From metacommunity dynamics to rapid biodiversity assessment: DNA-based approaches expand horizons in both fundamental and applied ecology

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Abstract

Molecular tools have long been recognised as having enormous potential to expand horizons in ecology, but the promise remains substantially unfulfilled. In this thesis, I apply genetic approaches to two ecological problems that have proved difficult to solve using traditional techniques.

Chapters 1 and 2 apply molecular tools to a community ecology problem to ask what mechanisms govern the persistence of an ant-plant metacommunity. I first use molecular data to clarify the number of coexisting ant species, and then employ population genetic techniques to investigate dispersal scale and other elements of life-history in the three most common species. Where hostplant density is high, a clear dispersal hierarchy is detected, which correlates positively with ant body size and negatively with fecundity, consistent with the hypothesis of a dispersal-fecundity trade-off. The hierarchy is less clear when hostplant density is low because one species shows dispersal plasticity, dispersing longer distances when hostplants are scarce. Results are discussed in the context of mechanisms that allow the coexistence of multiple symbionts with a single plant host.

Chapters 3 to 8 address the use of molecular tools for informing decision-making in environmental management and biodiversity conservation. COI metabarcoding data are used to analyse patterns of arthropod diversity in the contexts of sustainable forest management (Chapter 5), agricultural management (Chapter 6), and habitat restoration (Chapter 7). It is shown that this potentially revolutionary technique can detect even fine-scale environmental changes, accurately characterise the biodiversity response to management variables, and be used to test the usefulness of convenient indicator variables. COI data is shown to outperform 18S data in recovering alpha and beta diversity information, and reference-based OTU-picking is demonstrated to be a useful approach where there is interest in the responses of a particular set of species. Potential applications and current limitations are discussed in Chapter 8.

Table of Contents

List of tables

List of figures

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Preface

A thesis of two parts

This thesis comprises two quite different studies, one of which uses population genetics to solve problems of fundamental ecology, while the other addresses environmental management problems using bulk DNA barcoding of arthropod samples.

The population genetics study was originally intended to make up the entire thesis, going on from the point reached at the end of Chapter 2 to build a spatially explicit model of species coexistence using the dispersal information derived from indirect genetic techniques. Unfortunately, several obstacles were encountered, sadly including the death of Dr Gabriel Debout, who was advising on the population genetics analyses. Moreover, when the data were analysed, it became apparent that additional samples were required if high confidence quantitative estimates of dispersal scale were to be obtained for species in the genus *Azteca*, and this coincided with it becoming extremely difficult to obtain permission to export samples from Peru for use in molecular studies because of concerns about access and benefit sharing following the 2010 Nagoya Protocol. At about this time, I also came to the strong conclusion that my long-term interests lay in applied – rather than fundamental – ecology, and my PhD supervisor (Dr Douglas Yu) was becoming involved in the field of DNA metabarcoding, which seemed to offer the opportunity to influence policy and improve the way in which biodiversity is measured and managed in the UK.

A common theme

The two studies are linked by the overarching theme of using genetic approaches to address ecological problems that have been difficult or impossible to solve using traditional methods. Molecular ecology is not a new field (indeed, the journal bearing its name recently celebrated its twentieth anniversary), but it is yet to fulfil its promise. In a recent paper, Andrew *et al*. (2013) provided a comprehensive overview of the range of applications for molecular tools in ecology. They used the analogy of the drunk who searches for his keys beneath a street lamp (because that is the area that is illuminated) to convey how molecular techniques have expanded our capacity to explore and understand the natural world (*"we are now at the point where the whole street is illuminated, and many 'key' questions that we have wanted to ask all along can finally be addressed"*). Though there is undoubtedly truth in this, molecular approaches have still not yet become mainstream in ecology except in a phylogenetic and phylogeographic context, and there

has been particularly minimal uptake in the disciplines of community ecology and macroecology (Johnson *et al*., 2009).

In large part, the promise of molecular ecology is unfulfilled because (and here I generalise) most people drawn to the field of ecology (including those who now teach it, apply it, and set policy around it) are attracted by the fieldwork and natural history element, not by the prospect of working in a laboratory; those naturally drawn to molecular work are more likely to turn to fields such as biomedical science. Furthermore, most undergraduate ecology courses do little to give students confidence in evaluating and employing molecular methods, with the result that many ecologists consider genetics inaccessible, too complicated, or simply irrelevant to their work. On the other side of the disconnect, molecular scientists (and also bioinformaticians) often develop powerful tools but lack the inclination or ecological knowledge to apply them to real problems.

This thesis addresses both of the major gaps identified by Johnson *et al*. (2009).

Community ecology

Chapters 1 and 2 apply molecular tools to a community ecology problem to ask what mechanisms govern the persistence of an ant-plant metacommunity, with a particular focus on spatial processes and dispersal. The difficulty of measuring dispersal using traditional approaches has been a major obstacle to the development of the field of metacommunity ecology because it has limited the potential for testing theories in natural systems. Here, I use genetic techniques to resolve a morphologically intractable species complex, make relative and quantitative estimates of dispersal scale, investigate reproductive behaviour, and understand the effects of environmental heterogeneity on processes that govern local and regional distribution patterns within a metacommunity.

Macroecology

Chapters 3 to 8 apply molecular tools to a macroecology problem, describing the response of arthropod communities to environmental management variables. Invertebrate species make up the majority of animal biodiversity but are largely ignored when it comes to selecting and evaluating management and conservation actions. Instead, like searching for keys beneath a street lamp, policy is guided almost entirely by consideration of an unrepresentative subset of species that are large, charismatic, and easily identified. Molecular tools can illuminate the street by enabling measurement of a more representative subset of diversity, which can be treated as a true response variable (i.e. it is measurable repeatedly over time and space). This allows evidence-based decision making and the detection of large-scale trends. However, the ecological community is not used to approaching biodiversity in this way, and the challenge here is to

persuade applied scientists and environmental managers to look beyond what they are familiar with and to recognise that it is both possible and desirable to do better.

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Many people have helped me with parts of this thesis. Thanks to Gabriel Debout, who helped so much at the very beginning, and will always be remembered; to Brent Emerson, who was a much-needed academic presence and a great support during the first two years, before moving overseas; and to Cock van Oosterhout for providing population genetics wisdom. Thanks to Miguel and Alejo, Frank Azorsa, and Simon Leishman for your help collecting ants and escaping charging peccaries in the heat of Peru, and to Sam Wheatman, Sam Bocock, and Joe Middleton for your help measuring trees and escaping charging highland cows in the snow in Thetford Forest.

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Chapter 1: Species delimitation in a genus of neotropical plant-ants

1.1 Summary

In this brief chapter, a species complex within the ant genus *Azteca* (Hymenoptera: Dolichoderinae) is resolved using a combination of (1) phylogenetic analysis of a single mitochondrial gene (COI), (2) Bayesian assignment based on multilocus genotypes, and (3) queen morphology.

There was a high level of agreement between methods in terms of the grouping of individuals into separately evolving lineages, with strong genetic support for the existence of five *Azteca* species that are regular inhabitants of the myrmecophyte *Cordia nodosa*. These are designated *A.* 'depilis 1', *A.* 'depilis 2', *A. ulei* '1A', *A. ulei* '1B', and *A. ulei* '2'. Morphospecies were consistent with genetic species except that the most common morphospecies, *A. ulei*, was found to comprise three separate lineages (*A. ulei* '1A', *A. ulei* '1B', and *A. ulei* '2').

Finally, for the purposes of future analyses of this group of ants, it is demonstrated that the program STRUCTURE can be used to assign individuals reliably to species based on their multilocus genotypes. It is suggested that this should be carried out with the number of clusters set to be greater than five, so as to enable the detection of anomalous individuals.

1.2 Introduction

In the first part of this thesis, I use molecular approaches to investigate the metacommunity dynamics of an ant-plant symbiosis in the Peruvian Amazon. In this system, it has been hypothesised that the co-existence of multiple ant species living symbiotically with the ant-plant *Cordia nodosa* Lam. (Boraginaceae) can be explained by spatial niche partitioning (Yu *et al*., 2001; 2004). This hypothesis is tested in Chapter 2 (which also includes a detailed introduction to the study system and previous work), but it is first necessary to address the fact that it remains unclear exactly how many *C. nodosa*-associated ant species there are. Therefore, the first step is to use genetic data to delimit species boundaries.

1.2.1 Species identification problems in the genus Azteca

C. nodosa is commonly inhabited by two genera of ants (one colony per plant, with a few exceptions; Yu *et al*., 2001; 2004). One of these genera is represented by a single species, *Allomerus octoarticulatus* var *demererae* Wheeler (Hymenoptera: Myrmicinae); the other, *Azteca* Forel (Hymenoptera: Dolichoderinae), includes a group of species that are morphologically distinguishable only by their queens (Yu *et al*., 2004; Bruna *et al*., 2005). Since the queens are difficult to collect, it is usually impossible to make field identifications to species level for *Azteca* samples, and so empirical studies of this system have tended to pool the *Azteca* species (Yu *et al*., 2001; 2004; Bruna *et al*., 2005). However, a molecular approach to ecology requires accurate species delimitation, so that intraspecific genetic structure is not obscured by interspecific variation.

On the basis of differences in queen morphology, behaviour, and ecology, it is believed that *C. nodosa* is commonly colonised by at least three species of *Azteca*, only one of which, *A. ulei* var. *cordiae* Forel, has been formally described. The other morphospecies have been termed *A.* 'depilis 1' and *A. '*depilis 2' in previous studies (Yu *et al*., 2004; Edwards *et al*., 2006). A fourth putative morphospecies, *A.* 'depilis 3', has also been recorded, but is thought to be rare (Edwards *et al*., 2006).

1.2.2 An integrated approach to species delimitation

Here, I use evidence from both mitochondrial and nuclear genes, plus queen morphology, to separate the various *Azteca* species that occur at a single site in the Peruvian Amazon, and I develop a protocol for assigning individuals to species based exclusively on their multilocus genotypes.

Species concepts have been much debated over the decades (de Queiroz, 2007; Velasco, 2009, Frankham *et al*., 2012). I follow de Queiroz (2007) in considering species to be separately evolving lineages, for which monophyly constitutes important evidence. I test for the monophyly of *Azteca* morphospecies using Bayesian phylogenetic analysis of the mitochondrial Cytochrome c oxidase subunit I gene (COI), which mutates at a rate such that it is useful for detecting species-level divergence in animals (Hebert *et al*., 2003). However, it can be difficult to distinguish between species-level and population-level processes using this method, especially when species are closely related (Moritz and Cicero, 2004; Carstens *et al*., 2013), and it is generally not advised to rely on a single method for species delimitation (Carstens *et al*., 2013). Therefore, I also use the programs STRUCTURE (Pritchard *et al*., 2000) and STRUCTURAMA (Huelsenbeck and Andolfatto,

2007) to assign individuals to groups based on their multilocus genotypes. These programs were developed for inferring within-species population structure (Huelsenbeck *et al*., 2011) but they have been shown to be useful tools for the delimitation of closely-related species (Shaffer and Thompson, 2007; Hausdorf and Hennig, 2010; Rittmeyer and Austin, 2013), particularly when used in conjunction with phylogenetic analysis (Carstens *et al*., 2013).

Both STRUCTURE (Pritchard *et al*., 2000) and STRUCTURAMA (Huelsenbeck and Andolfatto, 2007) apply a Bayesian MCMC analysis to co-dominant microsatellite data in order to cluster individuals into groups such that Hardy-Weinberg equilibrium is maximised within groups. The programs differ from one another in that STRUCTURAMA allows number of clusters (*K*) to be treated as a random variable, following a Dirichlet process prior, and computes posterior probabilities for each value of *K*, while STRUCTURE requires *K* to be specified, and its output can be hard to interpret in terms of selecting the best value of *K* (Evanno *et al*., 2005). For this reason, I use STRUCTURAMA to find the number of clusters (i.e. species) that best fit the genotype data. The monophyly of individuals within each group is checked by reference to the COI phylogeny.

Finally, I assign individuals to species in STRUCTURE, using the information from STRUCTURAMA about the number of likely groups *K*. The graphical outputs from STRUCTURE are useful for assigning individuals to a known number of species, and for detecting anomalous genotypes (e.g. those that contain errors, represent additional rare species, or constitute misidentifications at the generic level). Here, I ask which values of *K* result in the correct assignment of individuals to species, while separating out anomalies, and I determine the parameters that should be used for species assignment in subsequent population genetic analyses.

1.3 Methods

The *Azteca* ants used in this analysis were collected at the Los Amigos Research Station (CICRA) in Madre de Dios, Peru, during September 2009, as part of the sampling of colonies for population genetics analysis (See Chapter 2 for full details). In addition, I used DNA from 23 *Azteca* queens that had been collected by D. Yu and G. Debout on previous field trips and identified to morphospecies.

1.3.1 Laboratory steps

DNA extraction

DNA was extracted from whole worker ants, or from two legs of a queen, using Zygem's (Hamilton, New Zealand) prepGEM Insect kit following manufacturer's instructions. This rapid method of DNA extraction omits any cleaning step, meaning that the DNA is likely to contain proteins that will cause degradation over time. Nonetheless, its quality is sufficient for studies requiring relatively short DNA sequences, or microsatellite markers, and my experience is that good quality data can be obtained following more than a year's storage of DNA at -20 ^{o}C.

Sequencing for COI

23 queens and 139 workers were sequenced for a 658 bp region of the mitochondrial Cytochrome c oxidase subunit I (COI) gene. PCR was performed using the standard invertebrate barcode primers described by Folmer *et al*. (1994) (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'; HCO2198: 5' –TAAACTTCAGGGTGAC-CAAAAAATCA-3'). For each sample, 1.5 µl DNA was amplified using 15.9 µl water, 2.5 µl QIAGEN PCR Buffer (Valencia, CA), 1.5 µl $MgCl_2$, 2 µl dNTP mix, 1 µl primer for each direction, and 0.1 µl *Taq* DNA polymerase. Thermal cycling consisted of an initial denaturation phase of 5 minutes at 95 ^{o}C, followed by 40 cycles of 1 minute at 95 ^{o}C, 45 seconds at 52 ^{o}C (annealing), and 1 minute at 72 ^{o}C (extension), with a final extension phase of 5 minutes at 72 ^{o}C.

For sequencing, 6.35 µl water, 1.5 µl sequencing buffer, 0.15 µl reverse primer, and 1 µl BigDye solution (v. 3.1) were added to 2 µl PCR product for each specimen. Samples were sequenced at The Genome Analysis Centre (TGAC) in Norwich on a Life Technologies 3730XL sequencer.

Multilocus genotyping

The workers and queens used in the phylogenetic analysis were genotyped at eleven polymorphic microsatellite loci (Table 1.1). Eight of the primers are described in Debout *et al*. (2007), and three were developed by ecogenics GmbH (Zürich, Switzerland) for this study.

Table 1.1: Details of the primers used for amplifying the eleven microsatellite loci in *Azteca*.

Locus	Forward primer	Reverse primer	Source
Az002	ACCCTAATTGTGAGTGGTC	AGTGTCCAATCATAGGCAG	Debout *et al*., 2007
Az014	ATTTCATCCTCTTTCGCCTC	CGTCCTAACCTCACCTAACG	Debout *et al*., 2007
Az016	CAAATAGATGAAAAATAATGCCG	GCAACATTGTAACGGTCAGC	Debout *et al*., 2007
Az022	CATTTCTTCACTCACTTTGC	GACCGTGCTGTTACTCTATC	Debout *et al*., 2007
Az064	TTCTCTCCTTCAACTTCCTG	CGAGGATTAGTAGATCGGTG	Debout *et al*., 2007
Az035	AGAAATGTCCTTTACCTGAG	ATTGTAATAGTGATATTGTAAAGC	Debout *et al*., 2007
Az048	TGATATTTTATCTTCATCCTG	GTTTGCTTTAGAATTTTCAC	Debout *et al*., 2007
Az171	CATTTGTTTCCTCTTATCTC	CGAATTTAGATTCTTGGC	Debout *et al*., 2007
Az04135	TTCGCCGTTTACACTCGTTG	CATATCACTGTGCGCTGCC	New
Az08028	CTTTCGATATCCCACGCGAC	TCCTGAGTGTTCCATCGTCC	New
Az10230	TCGAACACCCGCTATACAAATGC	CAAACCGTGGCGTGACTATC	New

PCR was carried out in three separate multiplex reactions, using a QIAGEN Multiplex PCR Kit (Valencia, CA) with small reaction volumes. For each sample, 1 µl of DNA was evaporated by heating at 55 ^{o}C for 10 minutes, and 1 µl of Multiplex PCR Master Mix and 1 µl of primer mix were then added to each sample. A drop of mineral oil was used to prevent evaporation during PCR. Thermal cycling consisted of an initial period of 15 minutes at 95^{o}C (denaturation) followed by 40 cycles of 30 seconds at 94^{o}C, 90 seconds at 56^{o}C (annealing) and 60 seconds at 72^{o}C (extension), with a final extension phase of 30 minutes at 72 ^{o}C. PCR product was diluted to 1% of its original concentration and genotyped with a ROX500 size standard on an Applied Biosystems 3730 sequencer at the NERC Biomolecular Analysis Facility in Sheffield. Resulting genotypes were checked and scored in GeneMapper v. 4.0 (Applied Biosystems, Paisley, UK).

1.3.2 Phylogenetic analysis of COI sequence data

COI sequences were checked by eye for reading errors and aligned using the free online software BioEdit Sequence Alignment Editor v. 7.0.5 (Ibis Biosciences, Carlsbad, CA). A Bayesian Markov chain Monte Carlo (MCMC) analysis was carried out in MrBayes v. 3.2 (Huelsenbeck and Ronquist, 2003), with *Dolichoderus* sp. used as outgroup (sequence accessed via BOLD Systems v. 3 (Ratnasingham and Hebert, 2007), sample ID: BIOUG00914-C07). A single run was conducted, consisting of four chains that were run for 500,000 generations under the General Time-Reversible model of sequence evolution, with substitution rates following a Gamma distribution and a proportion of invariant sites (GTR + Γ + I). A 50% majority-rule consensus tree was subsequently produced and visualised in FigTree v. 1.3.1. Finally, 75% consensus sequences (i.e. consensus includes all bases that are conserved in > 75% of sequences) were generated in

Geneious 6.0.5 for each clade that represented a potential species, and these were used to calculate the level of divergence between pairs of clades.

1.3.3 Bayesian assignment using multilocus genotype data

The purpose of the following analyses is two-fold: first, I use STRUCTURAMA v. 2 to derive an independent estimate of the number of *Azteca* species from multilocus genotype data, and I compare the groupings of individuals with those given by the COI phylogeny to check for monophyly; second, for the purposes of future analyses, I ask which parameters allow the accurate assignment of individuals to species in STRUCTURE.

Analysis in STRUCTURAMA

For this stage of the analysis, I excluded any individuals that fell outside the main species clades in the COI phylogeny. Such 'outliers' may be attributable to sequencing errors, or they may represent additional *Azteca* species that only nest opportunistically in *C. nodosa* plants, since *Azteca* ants are commonly plant cavity nesters (Yu & Davidson, 1997). Because I was interested in clustering at the species level, I selected the no-admixture model in STRUCTURAMA, which is appropriate for the consideration of fully discrete populations. Following Hausdorf and Hennig (2010), the number of populations was set to be a random variable, and a total of 1,000,000 cycles were run, with sampling conducted on every 100th cycle and the first 4000 cycles discarded as burn-in. The posterior probability of each number of groups was examined to choose the most likely number of species, and the grouping of individuals was compared manually with that suggested by the COI phylogeny to check for monophyly.

Analysis in STRUCTURE

Having identified the most likely number of species, a subsequent analysis was performed in STRUCTURE 2.3.4. Here, I included all workers from the phylogenetic analysis, including outliers.

K was initially set to the value found by the STRUCTURAMA analysis to correspond to the most likely number of species. It was subsequently increased in order to assess whether, by using a larger *K*, outliers could be separated from the major groups before subdivision of species clusters began to occur. I selected the no-admixture model and allowed allele frequencies to vary between 'populations' (i.e. species). Five runs were performed for each value of *K* from *K*=5 to *K*=10, each run consisting of 100,000 iterations, with a burn-in phase of 10,000 iterations. Results for each *K* were averaged across runs in CLUMPP 1.1.2 (Jakobsson and Rosenberg, 2007) using the *Greedy* algorithm with 1000 random permutations tested. Results were then visualised via bar plots produced in DISTRUCT 1.1 (Rosenberg, 2004).

1.4 Results

1.4.1 Phylogenetic analysis of COI

High quality sequences were obtained for all 23 queens and 136 workers. The placement of the morphologically identified queens in the Bayesian phylogeny was broadly in agreement with expectations: each morphospecies (*A.* 'depilis 1', *A.* 'depilis 2', and *A. ulei*) formed a distinct, highly supported, monophyletic group (figure 1.1). Most workers (96%) were closely related to at least one of the queens (< 2% sequence divergence), which enabled them to be assigned to a morphospecies. Six workers could not be assigned to a species because their sequences were highly divergent from those of all queens. These are considered outliers.

The deepest split within *Azteca* separated *A.* 'depilis 1' and *A.* 'depilis 2' from *A. ulei* (Panel A in Figure 1.1). *A.* 'depilis 1' and *A.* 'depilis 2' are also clearly differentiated from one another in well-supported clades, each with a Bayesian posterior probability of 1.0. Consensus sequences for these two clades were divergent at 5.4% of nucleotides, which is consistent with their representing separate species. There was no evidence of a third *A.* 'depilis' species.

Unexpectedly, a deep division also occurred within the *A. ulei* morphospecies, and a divergence level of 7.3% between the consensus sequences for the two main clades (*A. ulei* '1' and *A. ulei* '2' in Figure 1.1) is strong evidence for the presence of two separate species. Further sub-divisions occurred within each of the two *A. ulei* species, as well as within *A.* 'depilis 2'. These were shallow but strongly-supported (posterior probability > 0.99), and could represent either species-level or population-level divergences.

Of the six outliers (tips marked '?' in panel A of Figure 1.1), four shared the same haplotype but were not closely related to any queen. These likely represent an additional but rare symbiont or an opportunistic nester. A fifth outlier was as distantly related to the other *Azteca* specimens as was the outgroup, and probably represents a misidentification to genus, while the last was ambiguously positioned within *A. ulei*.

In summary, phylogenetic analysis of COI suggests that the three morphospecies in fact comprise at least four separately evolving lineages (*A.* 'depilis 1', *A.* 'depilis 2', *A. ulei* '1', and *A. ulei* '2'), with the additional diversity occurring within the *A. ulei* morphospecies.

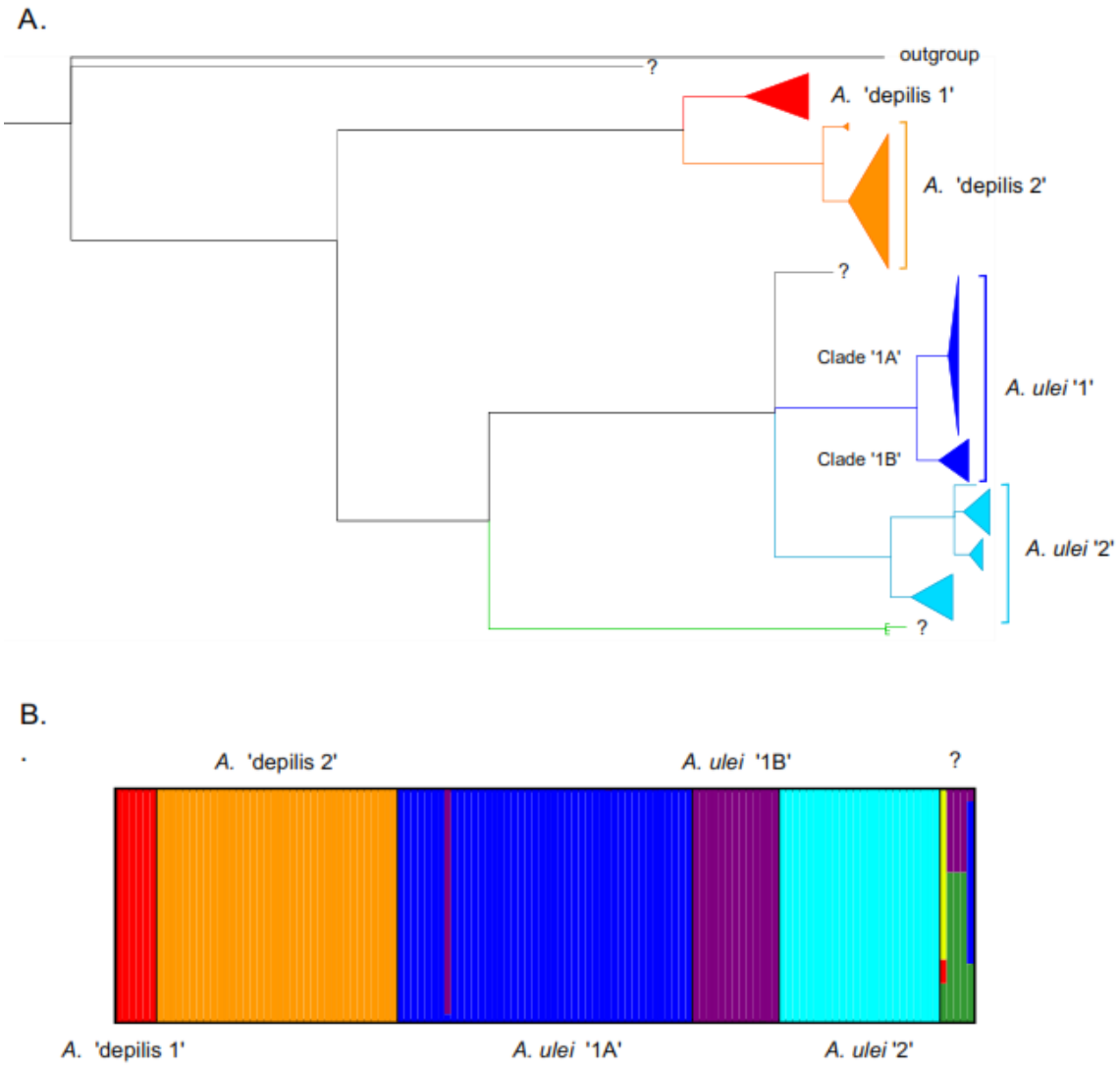

Figure 1.1: Assignment of individuals to species. (A) a 50% consensus tree from Bayesian phylogenetic analysis based on mtDNA sequences (COI) using the GTR+Γ+I model of evolution. Clades with <99% support have been collapsed, and species names are taken from morphological identifications of queens assigned to each clade. Outliers (not closely related to a queen sequence) are marked as '?'. (B) STRUCTURE bar plot averaged over 5 runs in which the same individuals have been assigned to 7 clusters based on their multilocus genotypes. Individuals are ordered according to their position in the phylogeny.

1.4.2 Bayesian assignment using multilocus genotype data

Analysis in STRUCTURAMA

The six phylogenetic outliers were excluded from the STRUCTURAMA analysis. For the remaining individuals, the number of groups with the highest posterior probability was five (P = 0.81), while

there was a low probability of there being four groups (P = 0.19), and no support for any other number of groups. The five groups corresponded with the following clades in the Bayesian phylogeny: (1) *A.* 'depilis 1', (2) *A.* 'depilis 2', (3) *A. ulei* '2', (4) clade A within *A. ulei* '1', and (5) clade B within *A. ulei* '1' (Figure 1.1A). The clades represented by these last two groups are monophyletic and robust, but shallow, with consensus sequences diverging by only 1.3%. Nevertheless, I accept the five species outcome on the basis that the split between *A. ulei* '1A' and *A. ulei* '1B' occurs when the number of clusters in STRUCTURAMA is specified to be as low as three (results not shown).

Overall, there was a high level of agreement with the mtDNA phylogeny in terms of the grouping of individuals. Only two individuals (1.6%) were assigned to clusters that did not match their positions in the phylogeny: one occurred in the *A.* 'depilis 1' clade in the phylogeny but was assigned to *A. ulei* '1B' by STRUCTURAMA, and the other occurred in the *A. ulei* '1A' clade in the phylogeny and was assigned by STRUCTURAMA to *A. ulei* '1B'.

Analysis in STRUCTURE

This analysis included all individuals from the phylogenetic analysis, including outliers. When the number of clusters (*K*) was set to five (the most likely number of groups according to the STRUCTURAMA analysis), the presence of the outliers meant that non-outliers were not assigned to the same clusters as in the STRUCTURAMA analysis. However, this was rectified by increasing *K*, which resulted in the separation of the outliers and gave assignments that were consistent with STRUCTURAMA for all other individuals (Figure 1.1). The only exception was that the *A.* 'depilis 1' individual that had been assigned by STRUCTURAMA to *A. ulei* '1B' was correctly assigned by STRUCTURE to *A.* 'depilis 1', leaving just one individual in disagreement with the phylogeny. Increasing the number of clusters to *K*=10 did not result in the subdivision of any of the five species.

With the exception of the outliers and the misassigned individual, all ants were assigned to species clusters in STRUCTURE with a posterior probability greater than 0.99 for all tested values of *K*. Outliers were either assigned to a sixth group, or failed to be assigned with high probability to any one group (Panel B in Figure 1.1).

1.5 Discussion

These results provide strong evidence for the presence of at least five co-occurring species of *C. nodosa*-associated *Azteca*, pointing to a greater level of cryptic diversity than has been assumed in previous studies. In particular, the *A. ulei* morphospecies is shown to comprise three separate species. This would seem to explain some previous, unpublished results that found a surprising lack of spatial genetic structure for *A. ulei* populations, even at very large spatial scales (G. Debout, unpublished data). Given the results of this study, it seems likely that species lumping masked any population-level variation within individual species.

STRUCTURAMA has been shown by other authors to be a reliable tool for species delimitation (Hausdorf and Hennig, 2010; Rittmeyer and Austin, 2013), and the high level of agreement between the mitochondrial DNA and the microsatellite data indicates that the results are likely to be robust, since the two data types constitute independent evidence. Interestingly, the same six individuals were identified as outliers in both analyses, which suggests that their ambiguous assignment is not attributable to sequencing or genotyping error. With the exception of the highly divergent individual that seems to be misidentified at the genus level, these probably represent additional species of *Azteca* that are rarely found on *C. nodosa*.

For subsequent analyses (Chapter 2), my results indicate that STRUCTURE can be used to assign individuals to the five main *Azteca* species based on their multilocus genotypes. The value of K should be set to $5 < K < 10$, and individuals that are not assigned to a group with a posterior probability greater than 0.99 should be excluded from subsequent analyses. The use of positive control specimens for each species allows clusters to be matched to named species. Where individuals have been sampled from multiple geographic locations, the analysis should be performed separately for each site in order to minimise the probability that species will be split as a result of population-level differences.

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Chapter 2: Species coexistence and foundress dispersal in a metacommunity of neo-tropical plant-ants

2.1 Summary

It has been hypothesised that the coexistence of multiple ant species belonging to the genera *Allomerus* (1 species) and *Azteca* (at least 5 species; Chapter 1) that are symbionts of the same species of hostplant (*Cordia nodosa*) can be explained by a dispersal-fecundity trade-off when hostplant density varies across the landscape (Yu *et al*., 2001; 2004). However, this has not been tested empirically because of the difficulty associated with measuring dispersal in the field. In this chapter, I use indirect genetic techniques based on multilocus genotypes to investigate dispersal scale in the two most common species of *Azteca* (*Az.* 'depilis 2' and *Az. ulei* '1A') and in *Allomerus octoarticulatus*. In addition, mating system and fecundity analyses are performed for the two *Azteca* species (previous data exist for *Allomerus*). The primary aim of the chapter is to confirm that *Allomerus* foundresses are more dispersal limited than are *Azteca* foundresses. Secondary aims include deriving quantitative estimates of dispersal parameters that could be used in the future to inform a spatially-explicit model of species coexistence, and determining whether a dispersal-fecundity trade-off can explain coexistence within *Azteca* as well as between *Azteca* and *Allomerus*.

Where *C. nodosa* density was high, a clear dispersal hierarchy was identified, which was positively correlated with body size. *Allomerus* uniquely displayed dispersal plasticity, dispersing longer distances when hostplant density was low, which is suggestive of interspecific differences in dispersal and host-selection behaviour. Quantitative estimates of average dispersal distance were obtained using fine-scale isolation by distance and a sibship analysis, but they were mostly associated with a high degree of uncertainty. The exception was for *Allomerus* at high hostplant density locations, where an estimate of around 100 m (median foundress dispersal distance) was obtained from both analyses and fitted well with results from a previous empirical study (Yu *et al*., 2004). Life-history analyses confirmed that female fecundity was low in *Azteca spp.* compared with *Allomerus*. The largest species, *Az. ulei* '1A', also had lower *male* fecundity than *Allomerus*, in addition to the highest rate of polyandry and a tendency to inbreed when hostplant density was low. Results are consistent with a dispersal-fecundity trade-off enabling coexistence between genera, but a different mechanism is needed to explain the coexistence of species within the genus *Azteca*.

2.2 Introduction

2.2.1 The species co-existence problem

The classic Lotka-Volterra models of competitive exclusion (Lotka, 1925; Volterra, 1926) predict that the maximum number of species in an ecosystem cannot exceed the number of distinct limiting resources. The apparent failure of such models to account for the high species richness encountered in many natural environments has led to an extensive body of literature that seeks to explain how species are able to coexist despite a large degree of resource overlap (e.g. Hutchinson, 1961; Janzen, 1970; Connell, 1970; 1978; Shmida and Ellner, 1984; Chesson, 2000; Leigh *et al*., 2004; Novotny *et al*, 2006; Nathan *et al*., 2013) and to understand how the loss of resource heterogeneity disrupts stable coexistence (Davies *et al*., 2000; Allan, 2003; Abrams and Wilson, 2004; Saxer *et al*., 2009). Empirical testing of species coexistence theory is greatly complicated by the vast complexity of the natural world and our incomplete knowledge of species' traits, requiring most models to make simplifying assumptions about the distribution, movement and interaction of species (Logue *et al*., 2011).

2.2.2 Species coexistence mechanisms

Chesson (2000) proposed that in a community where two or more species share a common limiting resource, coexistence may be obtained by a combination of 'equalising' and 'stabilising' mechanisms (Chesson, 2000). Equalising mechanisms act to reduce the fitness difference between competing species, and might include, for instance, increased levels of predation on the competitively dominant species (Chesson, 2005). Neutral coexistence (Bell, 2001; Hubbell, 2001) can be viewed within this framework as an extreme equalising case where species are of exactly equal fitness and can therefore coexist for very long periods of time in the absence of any stabilising mechanism (Adler *et al*., 2007; Chave, 2004; Wooton, 2005). Stabilising mechanisms increase intraspecific competition relative to interspecific competition and classically involve niche partitioning. This may take the form of (1) partial dependence on unshared resources, (2) limitation by species-specific natural enemies (Janzen-Connell hypothesis; Connell, 1970; Janzen, 1970), or a switching of fitness rankings over (3) time or (4) space (Amaresekare, 2003; 2004; Chesson, 2005). For stable coexistence between two species, meaning that either species is able to recover from low density, the stabilising effect must be greater than the net fitness difference of the two species (Chesson, 2000). In reality, natural communities tend to be shaped by the complex interaction of multiple processes and mechanisms: equalising and stabilising, neutral and

non-neutral, spatial and temporal, local and regional (Shmida and Wilson, 1985; Wilson, 1990; Snyder and Chesson, 2003; Amaresekare, 2004; Tilman, 2004; Mouquet *et al*., 2005; Leibold and McPeek, 2006; Adler *et al*., 2007).

2.2.3 Coexistence in the context of metacommunities

Where spatial processes are invoked to explain coexistence, the system is best considered within the conceptual framework of a metacommunity, which is defined as a set of local communities that are linked by dispersal of multiple, potentially interacting species (Wilson, 1992; Leibold *et al*., 2004). A metacommunity approach recognises the interaction of species at a variety of scales and emphasises that the interplay of local and regional processes can produce a more complex community than can processes operating at any one spatial scale (Hoopes *et al*., 2005). An idea central to both the 'species sorting' (Pulliam, 1988; Leibold, 1998) and 'mass effect' (Shmida and Wilson, 1985) metacommunity hypotheses is that regional coexistence can occur if (1) each species is competitively dominant under a different set of environmental conditions, and (2) environmental conditions vary across a landscape (Chesson and Huntly, 2001; Amaresekare and Nisbet, 2001; Yu and Wilson, 2001; Levine and Rees, 2002; Kneitel and Chase, 2004; Hoopes *et al*., 2005). Local mixing can then be achieved via source-sink dynamics; that is, a species can persist in locations where it is the inferior competitor and has negative population growth (sinks) via dispersal from nearby locations where it is the dominant competitor and has positive population growth (sources) (Warner and Chesson, 1985; Amaresekare and Nisbet, 2001; Mouquet *et al*., 2005). This represents a unified theory of coexistence within the metacommunity framework, and has been termed 'environmental niche partitioning' (Debout *et al*., 2009).

2.2.4 Obstacles to research on metacommunities

Despite an extensive body of theoretical literature, research on metacommunities has advanced little in recent years. In large part, this is because of difficulties associated with testing theories in natural systems (Logue *et al*., 2011). There are three main obstacles: first, the complexity of ecosystems and species traits makes it difficult to isolate specific mechanisms that are responsible for maintaining coexistence (Meynard *et al*., 2013); second, most models are based on patch occupancy (e.g. Levins and Culver, 1971; Yu and Wilson, 2001; Gravel *et al*., 2010), but discrete patches rarely occur in nature, meaning that it is usually not straightforward to quantify the amount of space available for colonisation; and third, it is extremely difficult to measure dispersal (Jacobson and Peres-Neto, 2010).

Obligate symbioses (e.g. ant-plant and host-disease systems) are useful for metacommunity studies because interactions are simplified and patches occur naturally and are clearly defined, with each host organism representing a patch (Yu and Wilson, 2001; Yu *et al*., 2001; Mihaljevic, 2012). This makes it easy to score the occupancy of patches and to quantify the amount of available space (i.e. un-occupied hosts). Moreover, plant hosts have the additional advantage of being sessile, which allows their spatial distribution to be easily mapped. Previous studies (Yu *et al*., 2001; 2004; Debout *et al*., 2009) have taken advantage of these features to generate hypotheses for the coexistence of multiple ant species in ant-plant systems. However, the problem of measuring dispersal remains and has limited the ability of these studies to fully parameterise theoretical models (Yu *et al*., 2004; Jacobson and Peres-Neto, 2010).

2.2.5 The importance of dispersal in metacommunity dynamics

Dispersal is integral to the concept of metacommunities (Snyder and Chesson, 2003; Jacobson and Peres-Neto, 2010; Salomon *et al*., 2010; Matias *et al*., 2012; Liao *et al*., 2013). On a local scale, it is a key factor in determining the ability of individuals to colonise available patches (Levins and Culver, 1971; Yu and Wilson, 2001); on a regional scale, it provides the means for interaction between communities and determines the extent to which rescue effects and source-sink dynamics can operate (Amaresekare, 2003; 2004; Matias *et al*., 2012). Furthermore, the scale of dispersal determines the spatial scale at which local and regional community dynamics operate (Davies *et al*., 2005).

Thus, an inability to measure dispersal severely limits empirical testing of metacommunity theory, thereby rendering the theory less useful for practical purposes (Cain, 2000; Bowler and Benton, 2005; Jacobson and Peres-Neto, 2010). With the effects of processes such as climate change and habitat fragmentation now issues of great concern in the context of biodiversity conservation, it is important that ecologists take advantage of modern tools and techniques that have the potential to inform robust ecological models.

Below, I consider the limitations of common empirical approaches for characterising dispersal and discuss the potential of direct and indirect genetic approaches.

2.2.6 Measuring dispersal

Field experiments

Dispersal has most often been measured using field experiments. However, this is difficult when (1) dispersal distances are long; (2) the organism of interest is sessile with a single dispersal event, rendering mark-recapture approaches useless (Suni and Gordon, 2010); or (3) the landscape is

difficult for researchers to move through, or to search thoroughly, an example being tropical rainforest, where analyses that rely on exhaustive sampling are often unrealistic (Nathan *et al*., 2003). For some organisms that are highly dispersal limited, recolonisation or isolation experiments (e.g. Yu *et al*., 2004) may yield some information about dispersal scale. However, to perform this kind of experiment on a large scale in a challenging landscape, or to repeat it in multiple sites, becomes extremely labour intensive. Alternatively, inverse modelling approaches can be used to estimate dispersal kernel parameters via a maximum-likelihood method based on the observed distribution of dispersed propagules and the spatial location of potential parents (Ribbens *et al*., 1994). However, this is reliant on both exhaustive sampling of potential parents, and knowledge of their individual fecundities, which is often unavailable, and studies tend to be conducted at a scale that is too small to capture the full distribution of dispersal distances (e.g. Bruna *et al*., 2011). Indeed, a common criticism of field studies in general is their failure to capture rare long-distance dispersal events, which are of critical importance in determining a species' ability to invade new territory or to disperse regionally in a metacommunity (Silvertown, 1991; Clark, 1998; Cain *et al*., 2000; Nathan *et al*., 2003).

Direct genetic approaches

Genetic techniques have long been recognised as having potential for revealing dispersal characteristics (Cain *et al.*, 2000; Wang and Smith, 2002). Direct genetic approaches, which involve the assignment of dispersed offspring to parents using polymorphic genetic markers, have been widely used to describe dispersal in trees (e.g. Dow and Ashley, 1996; Konuma *et al*., 2000; Godoy and Jordano, 2001; Robledo-Arnuncio and Garcia, 2007). Increasingly sophisticated statistical approaches have been implemented to fit dispersal kernels to this type of data (Pairon *et al*., 2006; Robledo-Arnuncio and Garcia, 2007), but a limiting factor is the requirement to map all potential parent plants in the study area (Nathan *et al*., 2003).

Indirect genetic approaches

Indirect genetic methods, including 'isolation by distance' (IBD), rely on inferring dispersal characteristics from observed patterns of spatial genetic structure (SGS) in adult populations. This has several advantages over direct methods. First, it does not require exhaustive or random sampling, meaning that individuals can be sampled by making use of existing infrastructure, such as trail systems, so long as an appropriate geographic area is covered (Cain *et al*., 2000; Vekemans and Hardy, 2004). Second, it captures effective dispersal over multiple generations (Wang and Smith, 2002), and therefore averages out the effects of stochastic events that can cause

temporarily atypical dispersal patterns (Matias *et al*., 2012). Third, it is more likely to capture the effects of rare, long-distance dispersal events (Cain *et al*., 2000).

IBD is based on the relationship between geographic distance and genetic distance (or similarity). At an appropriate geographic scale, and when a population is in drift-dispersal equilibrium, this relationship is expected to be positive, linear, and virtually independent of mutation rate and dispersal kernel (Rousset, 1997; 2000; Hardy and Vekemans, 1999; Vekemans and Hardy, 2004). When genetic distance is regressed against the logarithm of geographic distance, the inverse of the regression slope gives Wright's (1943) 'neighbourhood size', $4\pi\sigma^2 D_e$, where σ^2 is the mean squared axial parent-offspring distance, and D_e is effective population density (Rousset, 1997). Thus, σ^2 can be estimated from the regression slope if D_e is known. Due to the assumption of drift-dispersal equilibrium, and the fact that the analysis provides an average over multiple generations, studies implementing IBD should only be carried out where populations are considered to have been stable for at least tens of generations prior to sampling (Hardy and Vekemans, 1999; Vekemans and Hardy, 2004), if results are to be considered representative of current population dynamics.

IBD has been used to study dispersal in a wide range of animal and plant taxa including trees (Hardy *et al*., 2006), mammals (Sumner *et al*., 2001; Spong and Creel, 2001; Broquet *et al*., 2006; Frantz *et al*., 2010; Selon *et al*., 2010), birds (Coulon *et al*., 2010; Corrales and Höglund, 2012), reptiles (Hoehn *et al*., 2007), fish (Planes and Fauvelot, 2002; Bradbury and Bentzen, 2007; Planes *et al*., 2009), marine invertebrates (Maier *et al*., 2005; Pinsky *et al*., 2010), and insects (Watts *et al*., 2004; 2007; Debout *et al*., 2009; Charman *et al*., 2010; Suni and Gordon, 2010; Oleksa *et al*., 2013). Where dispersal estimates from IBD have been compared with estimates derived from other (usually field-based) methods, results have been found to vary by about a factor of two (Sumner *et al*.,2001; Spong and Creel, 2001; Broquet *et al*., 2006, Watts *et al*., 2007; Selon *et al*., 2010). Discrepancy may be attributable to (1) uncertainty inherent in empirical dispersal estimates that are based on capture-mark-recapture studies of highly mobile species (e.g. Sumner *et al*., 2001; Broquet *et al*., 2006; Selon *et al*., 2010), or (2) incorrect estimation of effective density (D_e) and interpretation of σ in the IBD analysis. Unfortunately, accurate estimation of D_e is not straightforward, requiring either sampling over multiple generations to find genetic coalescence rates (Wang and Whitlock, 2003; Robledo-Arnuncio and Rousset, 2009), or having a detailed understanding of the reproductive parameters of the study population (e.g. Serbezov *et al*., 2012; Selon *et al*., 2010). However, it is generally accepted that variation among adults in lifetime reproductive success reduces D_e below census density in natural populations (Hedgecock, 1994; Frankham, 1995; Watts *et al*., 2007; Oleksa *et al*., 2013), and so authors who use adult or

colony census density for D_e (e.g. Sumner *et al*., 2001; Debout *et al*., 2009; Suni and Gordon, 2010; Corrales and Höglund, 2012) will inevitably underestimate the scale of dispersal. Likewise, σ is often incorrectly interpreted as average dispersal distance (e.g. Sumner *et al*., 2001; Suni and Gordon, 2010). I provide a detailed discussion of the relationship between σ and average Euclidian parent-offspring distance in section 2.3.9, below.

2.2.7 Study system

My study system is a neotropical ant-plant community in the Peruvian Amazon. The ant-plant *Cordia nodosa* Lam. (Boraginaceae) provides housing in the form of stem swellings (domatia) to at least six ant species (Figure 2.1), with a single colony occupying each plant in almost all cases.

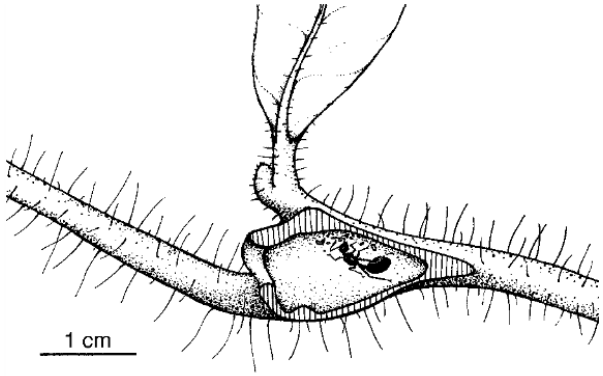

Figure 2.1: illustration of an *Allomerus* queen with brood inside a *C. nodosa* domatium (Bailey, 1924)

In return for housing, the ants defend the plant against herbivory by patrolling leaves. The most frequent ant symbiont is *Allomerus octoarticulatus* var. *demerarae* Wheeler (Myrmicinae, named *Allomerus demerarae* in some earlier papers and referred to hereafter in this study as *Allomerus*). *Allomerus* is a castration parasite of *C. nodosa* because it destroys the hostplant's flowers and prevents most reproduction. The system survives because the other five (at least) ant species (Chapter 1), which are all in the genus *Azteca* Forel (Dolichoderinae), are pure mutualists, allowing their hostplants to flower and fruit freely. Therefore, an explanation of how the two ant genera coexist on a single hostplant species is an explanation of how this ant-plant symbiosis persists in the face of parasitism. The *Azteca* species are currently undescribed, with the exception of *Azteca ulei* var. *cordiae* Forel. However, analysis of genetic data suggests that *Az. ulei* is itself three separate, closely related species (see previous chapter).

Approximately 80% of hostplants are occupied by an established colony of *Allomerus* or *Azteca* at any given time. Most of the remaining plants are saplings, which do not yet have established ant colonies, or plants that have lost their colony and are partially inhabited by a variety of

opportunistic ants (not considered further). Finally, some plants are inhabited by the rare ant mutualist *Myrmelachista schumanni* Roger (Formicinae, < 2% of plants, also not considered further) (Yu *et al*., 2001).

All six focal ant species are specialised and obligate symbionts of *C. nodosa*, in the sense that female alates (winged, dispersing ant queens) enter its domatia and attempt to found colonies, and none is known to found colonies on any other plant species. Therefore, all species are entirely dependent on *C. nodosa* for colony establishment and survival (Yu *et al*., 2001), and coexistence cannot be explained by simple resource partitioning. Mechanisms enabling coexistence in other well-studied ant-plant systems include habitat niche partitioning between riverside and forest interior environments (*Cecropia*–ant system; Yu and Davidson, 1997), and partitioning according to hostplant quality (*Acacia*–ant system; Palmer *et al*., 2000), but these are unlikely to play a role in the *C. nodosa* system because viable saplings are found only in the forest interior, and there is little evidence for *Allomerus* and *Azteca* inhabiting hostplants of different qualities (although Yu *et al*. (2004) did find a significant tendency for *Azteca* foundresses to be found in larger saplings). Therefore, previous work on this system has focused on environmental niche partitioning (based on spatial processes) as a mechanism for coexistence (Yu *et al*., 2001; 2004).

2.2.8 Previous work

Because of the difficulty in distinguishing among *Azteca* species based on worker morphology, previous work on this system has pooled *Azteca* species and considered coexistence primarily at the generic level.

A dispersal-fecundity trade-off

Measurement of multiple life-history traits has suggested that *Azteca* is both the better disperser (female *Azteca* alates fly longer distances than do those of *Allomerus*; Yu *et al*., 2004) and the stronger competitor (*Azteca* foundresses easily kill *Allomerus* foundresses; Edwards *et al*., 2006), which means that the classic competition-colonisation trade-off (Levins and Culver, 1971) cannot explain coexistence (Yu *et al*., 2001). However, *Allomerus* colonies are more fecund, which has led to the hypothesis that a dispersal-fecundity trade-off is central to maintaining coexistence between genera (Yu and Wilson, 2001; Yu *et al*., 2001; 2004). Since competition is preemptive (foundresses compete to colonise available hostplants but are unable to displace established colonies), the trade-off can explain coexistence only in a metacommunity where the competitive hierarchy varies across space (Yu and Wilson, 2001).

A metacommunity

Across the landscape of the Department of Madre de Dios, Peru, where studies have been carried out (Yu & Pierce 1998; Yu *et al*., 2001; 2004), hostplant (*C. nodosa*) density varies spatially by over an order of magnitude. This natural experiment can be used to measure how relative abundances of the different ant species change as patch density varies, in effect, measuring how habitat loss determines extinction trajectory. Such a landscape-level census has revealed that variance in the relative abundances of the two genera is almost completely explained by patch density (Figure 2.2).

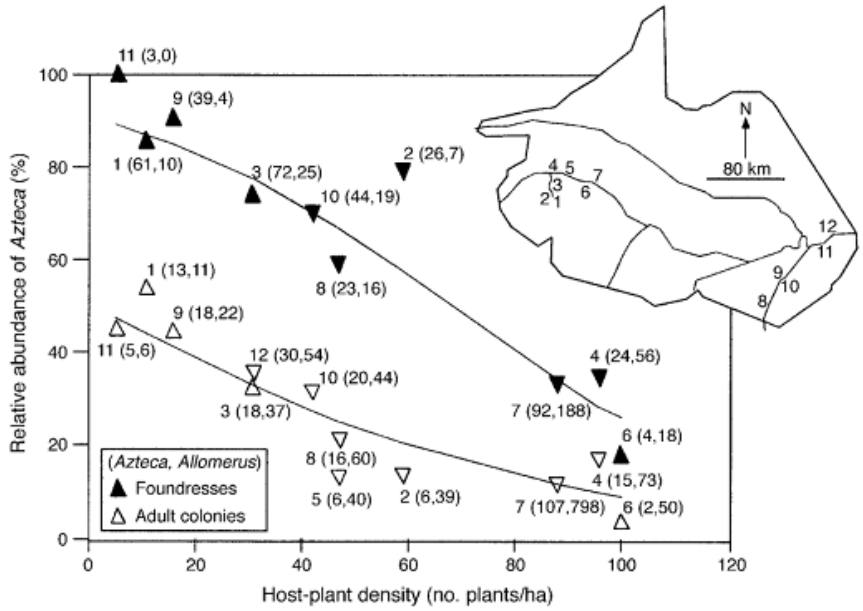

Figure 2.2: Figure from Yu *et al*., 2001 showing the relationship between hostplant density and the relative abundance of *Azteca* colonies and foundresses. Density explained 92.8% of variance in relative abundance for foundresses and 85.1% for colonies.

Hostplant density is thought to act as a resource niche axis for *Allomerus* and *Azteca* (Yu *et al*., 2001; 2004) because its variation across the landscape gives rise to a 'spatially heterogeneous competitive environment' (*sensu* Amarasekare 2003); in other words, the competitive hierarchy varies between sites according to hostplant density. The explanation for this is that *Allomerus*' superior fecundity makes it the more successful coloniser of *C. nodosa* saplings in high-density sites, while *Azteca*'s superior flying ability makes it the more successful coloniser of saplings in low-density sites, where saplings are more isolated. This results in regional coexistence, with *Allomerus* going to fixation at high density sites and *Azteca* at low density sites. Local mixing is then achieved via source-sink dynamics when dispersal occurs between high and low density sites (Yu *et al*., 2004; Amarasekare *et al*., 2004). The overall result is regional and local coexistence (Yu

& Wilson 2001; Yu *et al*., 2001; 2004), with the highest relative abundance of *Azteca* occurring in areas of low hostplant density, as seen in Yu *et al*. (2001).

A coexistence model

Yu and Wilson (2001) incorporated varying hostplant density with a dispersal-fecundity trade-off into an implicit data-driven model of species coexistence, which was adapted from the competition-colonisation model of Levins and Culver (1971). This model was parameterised with mortality and per capita colonisation rates for *Allomerus* and *Azteca* (species pooled) (Yu *et al*., 2001), but incorporation of detailed dispersal behaviour was not possible because of a lack of data.

Yu *et al*. (2004) conducted a sapling isolation experiment in a 16 ha plot to estimate the extent of dispersal limitation in *Allomerus* and *Azteca* (pooled). For *Allomerus*, a fall-off in the abundance of foundresses was observed at a distance of about 100-150 m from the plot edge, which suggests that *Allomerus* does not regularly disperse more than 150-200 m. However, *Azteca* foundresses did not show any such fall-off. This suggests that *Azteca* queens are better dispersers than are those of *Allomerus* but allows no quantitative estimate of dispersal scale. Two additional lines of evidence from Yu *et al*. (2004) support *Azteca* being the better disperser: first, *Allomerus'* distribution is significantly clumped at scales ≤ 100 m, while *Azteca* colonies do not show clumping at any spatial scale; second, *Azteca* queens are larger than *Allomerus* queens, with greater alitrunk depth, which corresponds to greater wing muscle mass (Zera and Denno, 1997).

Bruna *et al*. (2011) used inverse modelling to describe the dispersal kernels of a species of *Azteca* that inhabits the plant *Tococa bullifera* and concluded that the modal dispersal distance of *Azteca* was less than 10 m. However, I do not regard this dispersal kernel as being representative of the dispersal of the *C. nodosa*-associated *Azteca* species because it was conducted at a very small scale (9 ha) and failed to account for the low proportion of reproductively active *Azteca* colonies.

2.2.9 Aims of this study

Measuring dispersal

In this study, I use indirect population genetic techniques, including IBD, to derive relative and quantitative estimates of dispersal scale for *Allomerus* and *Azteca*. I consider that the study system is likely to have been stable over a sufficient time scale to satisfy the assumption of drift-dispersal equilibrium and that, since sites are located in the forest interior, isometric dispersal in two dimensions is a realistic assumption for all focal ant species.

The results from Yu *et al*. (2001; 2004) provide some strong a priori expectations of results – specifically, I expect to find that *Allomerus* foundresses do not regularly disperse more than about 200 m, but that *Azteca* foundresses do. The study is repeated at three separate locations in the Madre de Dios region of Peru, and I perform separate analyses in high and low density sites at each location in order to control for the effect of hostplant density on dispersal behaviour, which can be affected by landscape properties (Travis and Dytham, 1999; Clobert *et al*., 2009; Bonte *et al*., 2010; Coulon *et al*., 2010). This also helps to satisfy the requirement of constant population density in the IBD analysis.

Coexistence within Azteca

All previous work on the *C. nodosa* system has pooled *Azteca* species to some extent, either deliberately, to increase sample size and avoid issues associated with the difficulty of identification to species level (e.g. Yu *et al*., 2001), or unintentionally, because identification based on morphology has led to multiple separately evolving lineages being assigned to a single morphospecies (e.g. Fig. 8 in Yu *et al*., 2004). Since multilocus genotypes now allow species to be differentiated with greater certainty, a secondary aim of this study is to begin to differentiate the life-history traits of individual *Azteca* species, and I ask whether a dispersal-fecundity trade-off might also explain co-existence *within Azteca*. I focus on the two most abundant species, *Az. ulei* '1A' (hereafter, *Az. u1A*), and *Az.* 'depilis 2' (*Az. dp2*), since other species were insufficiently sampled for the majority of analyses. Under the hypothesis of a dispersal-fecundity trade-off, I would expect to find that (like for *Allomerus* and *Azteca*) the species differ in dispersal ability and fecundity (specifically foundress production), and that the better disperser is also the less fecund. Since dispersal ability is expected to correlate with body size, I predict that *Az. u1A*, the larger of the two species, will be the better disperser, while *Az. dp2* will be the more fecund.

Thus, overall, I expect to find that (1) *Allomerus* is the most fecund and the poorest disperser, (2) *Az. u1A* is the least fecund but the best disperser, and (3) *Az. dp2* is intermediate to the others in both traits.

Parameterising a spatially explicit model of species coexistence

The aim of this work is to provide information that can be used in the future to build a spatially explicit model of species co-existence. The majority of coexistence models have relied on artificially dividing a continuous landscape into sites, and specifying rates of dispersal within and between sites (e.g. Yu and Wilson, 2001). In contrast, a spatially explicit model treats the landscape as a continuum and uses detailed knowledge of species' traits and landscape properties to simulate community processes under different environmental scenarios (Dunning *et al*., 1995;

Bascompte and Solé, 1996; Smith and Lundholm, 2012). This is likely to provide more realistic predictions but is highly demanding of high quality data for parameterisation.

2.3 Methods

2.3.1 Outline of analyses

Life-history analyses

Mating system and fecundity influence effective density, which in turn affects estimates of dispersal scale that are based on spatial genetic structure. Such data are currently lacking for *Azteca* species, and so were gathered as part of this study. For mating system characterisation, the queen and 20 workers were genotyped for eight colonies of each species, and the number of patrilines per colony was estimated. To investigate fecundity, 68 entire hostplants were collected and scored for male and female alates and number of domatia. Worker genotypes were used to identify colonies that occupied multiple hostplants, which enabled detection of polydomy and examination of the relationship between colony size and fecundity for each species.

Dispersal analyses

For the dispersal analysis, georeferenced colonies were sampled for workers at three pairs of sites, and a single worker was genotyped from each colony. The resulting dataset was used to estimate spatial genetic structure at two levels: first at the grain of the six collection sites, and then at the grain of individual colonies within each site. Genetic structure was used to make inferences about the scale of dispersal, in both relative and quantitative terms.

Finally, a sibship analysis was conducted for *Allomerus* and *Az. dp2*. Newly-dispersed foundresses were collected from coppiced hostplants at site CIC-H (Table 2.1), and genotypes were used to identify full-sib pairs. The distribution of probability of sibship over geographic separation distance was used to provide an additional estimate of dispersal scale and to infer properties of the dispersal kernel.

2.3.2 Field collections

Collections for analysis of SGS

Sampling was carried out at three pairs of sites in Madre de Dios, Peru, between September and November 2009. Madre de Dios is characterized by extensive, mesic to seasonal lowland tropical rain forest (~2100 mm rain/year, Yu *et al*., 2001).

For each site pair, one site was situated in lowland, floodplain forest (typically with high *Cordia nodosa* hostplant densities) and the other in *terra firme* (upland) forest (typically low *C. nodosa* densities) (Yu *et al.,* 2001) (Figure 2.3). Although existing trail systems were used, spatial coverage

attempted to approximate a cross. This was in order to maximise the number of pairs of ant colonies that were separated by long distances (up to 5 km where possible).

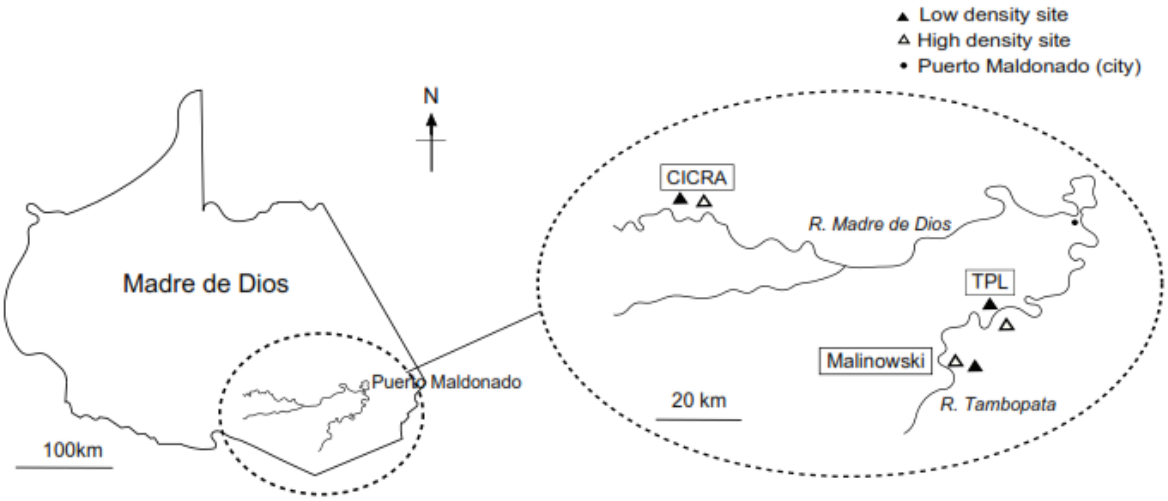

Figure 2.3: Map showing the location of the study area in the department of Madre de Dios and the location of the three pairs of sampling sites within the study area.

Hostplant density was calculated for each trail using a 4-m-wide strip census (2 m per trail side, with hostplants lying exactly 2 m from the trail edge counted as 0.5 plants). Each hostplant was scored for ant colony incidence, ant genus, and plant size (number of domatia) using the HanDBase Database Manager app. (DDH Software, Inc., Wellington, FL) on an iPod Touch (Apple Inc., Cupertino, CA) and georeferenced using a GPS60 (Garmin International, Inc., Olathe, KS) hand-held unit with external antenna.

Worker samples were collected from at least 30 *Allomerus* and 80 *Azteca* colonies at each site, the discrepancy in numbers arising from the knowledge that samples included multiple *Azteca* species that are indistinguishable in the field. The goal at each site was to collect a minimum of 30 colonies for at least one *Azteca* species. Collections were made from every hostplant encountered, except in two circumstances: (1) at the highest-density sites, every second or third *Allomerus* colony was sampled, so as to achieve the desired spatial coverage, and (2) where multiple *Azteca*-occupied hostplants were separated by less than 10 m, only one was sampled, since *Azteca* can be polydomous (Debout *et al.*, 2007b; *pers. obs.*). Approximately 20 workers were collected from each colony (either from the plant surface or from within domatia) into 100% ethanol.

Table 2.1: Details of the six sampling sites including location, total trail distance covered during sampling, whether hostplant density was considered 'high' or 'low', and the number of *Allomerus* and *Azteca* (species pooled) colonies from which workers were sampled. The goal was to collect a minimum of 30 *Allomerus* and 80 *Azteca* colonies (to account for multiple species of *Azteca*) at each site, while covering a spatial area of approximately 5 km x 5 km. Census densities were calculated using 4 m wide strip censuses by recording all hostplants lying within 2m of the trail edge, on either side, and their occupancy status. Plants rooted exactly 2m from the trail edge were counted as 0.5. Census density is given for hostplants (*C. nodosa*), and for colonies of *Allomerus* and *Azteca* (all species pooled), as well as for each of the two most common *Azteca* species, *Az. dp2* and *Az. u1A.*

Site	Location	Hostplant density	Watershed	River bank	Lat/Long	Total trail distance (m)	Colonies collected		Census density (ha^{-1})				
							Allomerus	*Azteca*	*C. nodosa*	*Allomerus*	*Azteca* (pooled)	*Az. dp2*	*Az. u1A*
CIC-H	Los Amigos Biological Station (CICRA)	High	Madre de Dios	North	S 12.3311 W 70.0414	13947	59	85	47.2	24.3	12.0	3.6	4.4
CIC-L	Los Amigos Biological Station (CICRA)	Low	Madre de Dios	North	S 12.3309 W 70.0614	6069	56	79	12.5	2.9	4.4	1.4	1.3
Mal-H	Malinowski guard station	High	Tambopata	South	S 12.5804 W 69.2859	13154	37	80	53.3	26.2	17.5	2.6	7.3
Mal-L	Malinowski guard station	Low	Tambopata	South	S 12.5921 W 69.2647	7410	51	80	10.3	2.7	5.3	1.4	1.1
TPL-H	Sachavacayoc Centre	High	Tambopata	South	S 12.5232 W 69.2154	14783	31	81	48.0	22.4	16.3	3.9	3.5
TPL-L	Tambopata Libertadores lodge	Low	Tambopata	North	S 12.4920 W 69.2420	4762	30	81	6.6	4.0	6.6	2.1	1.6

Collections for sibship analysis

In October 2009, 219 georeferenced *C. nodosa* trees were coppiced at site CIC-H in a figure-of-8 layout measuring approximately 3 km x 1 km (Figure 2.4). Coppicing destroyed the adult colonies, and the coppiced plants were then left to regrow domatia, which were colonised by dispersing queens and harvested four times at intervals of two to six months. All queens were collected into 100% ethanol.

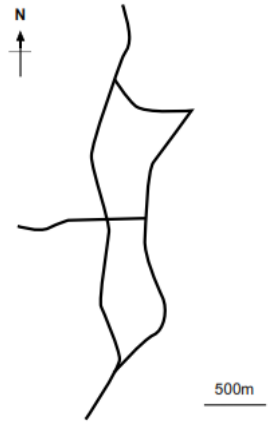

Figure 2.4: Map showing the trail system along which hostplants were coppiced for the sibship analysis at CIC-H.

Collections for mating system analysis

The mating system analysis (*Azteca* only) required colony queens to be collected along with a sample of their worker offspring. Occasionally, the colony queen, which is easily distinguished from reproductive offspring by the absence of wings, was encountered when making worker collections for the dispersal analysis. In this case, the queen was added to the collection, and these colony samples were used for both the dispersal and the mating system analyses. *C. nodosa* saplings provided another source of collections for the mating system analysis. Saplings lack established colonies but usually contain recently-dispersed foundress queens, often with an incipient colony consisting of a few tens of workers. When a sapling was encountered, its domatia were collected and later dissected in the field laboratory. Any foundress queen that had an incipient colony was collected into 100% ethanol along with 20 of her worker offspring.

Collections for fecundity analysis

Azteca-inhabited hostplants were collected from trails at the CICRA and Malinowski sites (Figure 2.3), ensuring that the full size range of *C. nodosa* (<10 – ~500 domatia) was represented. In order

to avoid collecting only part of a polydomous colony, the area surrounding any collected hostplant was searched, and any further *Azteca*-inhabited hostplants that were rooted within a 10 m radius of the collected plant, or made any physical contact with it, were also collected. In total, 68 hostplants were collected. These were dissected in the field laboratory and scored for number of domatia and reproductive offspring, including adult alates of both sexes and immature female alates (larvae and pupae), which are easily distinguished from workers by their larger size. All reproductives were collected into ethanol, along with a sample of workers from each hostplant.

2.3.3 Microsatellite amplification

DNA was extracted from whole worker ants, or from two legs of a queen, using Zygem's (Hamilton, New Zealand) prepGEM Insect kit following manufacturer's instructions. Individuals were genotyped at ten (*Allomerus*) or eleven (*Azteca*) polymorphic microsatellite loci (Table 2.2). For *Allomerus*, five of the primers are described in Debout *et al*. (2006), and five were developed for this study by ecogenics GmbH (Zürich, Switzerland). For *Azteca*, eight primer pairs were from Debout *et al.* (2007), and three were developed for the study. All *Azteca* markers amplified in all *Azteca* species, although allelic richness varied among species (Table 2.2).

PCR amplification was carried out in two (*Allomerus*) or three (*Azteca*) separate multiplex reactions, using a QIAGEN Multiplex PCR Kit (Valencia, CA) with small reaction volumes. DNA (1µl per sample) was evaporated by heating at 55 ^{o}C for 10 minutes, and 1 µl of Multiplex PCR Master Mix and 1 µl of primer mix were then added to each sample, with a drop of mineral oil to prevent evaporation. Thermal cycling was performed with an initial period of 15 minutes at 95 ^{o}C (denaturation) followed by 40 cycles of 30 seconds at 94 ^{o}C, 90 seconds at 52 ^{o}C (*Azteca*) or 53 ^{o}C (*Allomerus*), and 60 seconds at 72 ^{o}C, with a final extension phase of 30 minutes at 72 ^{o}C. PCR product was diluted to 1% of its original concentration and genotyped with a ROX500 size standard on an Applied Biosystems 3730 sequencer at the NERC Biomolecular Analysis Facility in Sheffield. Resulting genotypes were checked and scored in GeneMapper v. 4.0 (Applied Biosystems).

Table 2.2: Details of the microsatellite loci used for population genetics analysis of (A) *Allomerus* and (B) two species of *Azteca.*

A. *Allomerus*

Locus	Forward Primer	Reverse Primer	Marker length	No. of Alleles	Source	Notes
Ad040	GAAAGACAGATCGCTTCATC	GCCGATATTACTTCATTCAG	278-360	15	Debout *et al*., 2006	Heterozygote deficient
Ad045	TCTAACGGATTCTGCGAACG	ATCCTTAACGCCTGTGTTGG	205-255	19	Debout *et al*., 2006	
Ad109	TTACGAATAGTGCTCAAGAG	TAATGTAATGATTCTGAGCC	202-292	39	Debout *et al*., 2006	
Ad127	GGTAACGGCGTGTCAGCAGG	CCCTCCCACGCAGTAAATCC	184-236	12	Debout *et al*., 2006	
Ad166	GGTCCTTTGAGCAACTTAGC	CTGATCGCAATAGAGCAATG	146-230	31	Debout *et al*., 2006	
Ad01223	GTGAGCGACAGCTATTGGTAG	AATTGCACCGCGATCTGTG	72-88	9	New	Heterozygote deficient
Ad08302	TGCGAAAATCGAGAGGATGTG	TCTAGTGCGTGTGCTCTCTC	128-182	23	New	
Ad10399	TCTTGCGGAGATCCAACTCG	GAAACATCGCGGCGTACAG	86-120	16	New	
Ad12182	ATCGATAGCCGCGACTGG	CAAATTCGAGGAACACGGGG	96-136	12	New	Tri-nucleotide repeats
Ad12546	GTAATCGCCTTGGCTCCTTG	GCGTCATCGTGAGTTAGCG	92-154	15	New	

B. *Azteca*

			***Az. ulei* '1A'**		***Az.* 'depilis 2'**		
Locus	**Forward Primer**	**Reverse Primer**	**Marker length**	**No. of Alleles**	**Marker length**	**No. of Alleles**	**Source**
Az002	ACCCTAATTGTGAGTGGTC	AGTGTCCAATCATAGGCAG	123-129	3	123	1	Debout *et al*., 2007
Az014	ATTTCATCCTCTTTCGCCTC	CGTCCTAACCTCACCTAACG	218-266	17	207-257	8	Debout *et al*., 2007
Az016	CAAATAGATGAAAAATAATGCCG	GCAACATTGTAACGGTCAGC	318-418	33	324-334	5	Debout *et al*., 2007
Az022	CATTTCTTCACTCACTTTGC	GACCGTGCTGTTACTCTATC	114-210	34	145-201	25	Debout *et al*., 2007
Az064	TTCTCTCCTTCAACTTCCTG	CGAGGATTAGTAGATCGGTG	350-400	19	338-352	5	Debout *et al*., 2007
Az035	AGAAATGTCCTTTACCTGAG	ATTGTAATAGTGATATTGTAAAGC	134-210	29	134-150	6	Debout *et al*., 2007
Az048	TGATATTTTATCTTCATCCTG	GTTTGCTTTAGAATTTTCAC	288-312	12	290-306	9	Debout *et al*., 2007
Az171	CATTTGTTTCCTCTTATCTC	CGAATTTAGATTCTTGGC	168-200	10	171-189	13	Debout *et al*., 2007
Az04135	TTCGCCGTTTACACTCGTTG	CATATCACTGTGCGCTGCC	101-137	17	94-180	26	New
Az08028	CTTTCGATATCCCACGCGAC	TCCTGAGTGTTCCATCGTCC	226-272	18	242-254	7	New
Az10230	TCGAACACCCGCTATACAAATGC	CAAACCGTGGCGTGACTATC	206-240	15	209	1	New

2.3.4 Azteca species identification

Because of the difficulty of making morphological identifications, *Azteca* workers were identified to species level based on their multilocus genotypes (Chapter 1). This was carried out using the program STRUCTURE (Pritchard *et al*., 2000), with the number of clusters set to seven in order to allow detection of the five common species and separation of outliers (genotyping errors, misidentification to genus, and rare *Azteca* species; see Chapter 1). Three individuals of known identity were included for each species in every run, in order to match species names to clusters.

2.3.5 Relative abundance patterns

Following assignment to species, I used binomial regressions to test the relationship between hostplant density and the relative abundance of each species, given as the proportion of all *Allomerus* and *Azteca*-occupied hostplants inhabited by that species at the level of individual trails. I also tested whether the relationship between patch density and relative abundance varied between species. Finally, I performed a post-hoc analysis to ask whether the two most common *Azteca* species differed in their response to hostplant density. The resulting *p*-value was adjusted using Benjamini and Hochberg's (1995) correction for multiple tests ('*p.adjust(method="fdr")*' in *R*).

Assignment of *Azteca* colonies to species revealed that sufficient samples for the analysis of population genetic structure were collected only for the two most common species: *Az. u1A* and *Az. dp2*. Subsequent analyses are therefore limited to consideration of *Allomerus* and these two *Azteca* species.

2.3.6 Life-history analyses

Azteca mating system analysis

For each of eight colonies of *Az. u*1A and eight of *Az. dp2*, the queen and 20 workers were genotyped. For haplodiploid organisms in the absence of inbreeding, the effective number of male parents in a colony can be estimated as $nm = 2/(4r_{w-w} - 1)$ (Chevalet and Cornuet, 1982), where r_{w-w} is mean between-worker relatedness. The effective number of female parents can be estimated as $nf = 3/(4r_{w-w})$ (Ross, 1993). Between-worker relatedness, given by Wang's (2002) estimator, was calculated for each colony using the program COANCESTRY 1.0 (Wang, 2011), and the average within-colony relatedness was compared between species in *R* using a one-way analysis of variance (ANOVA) test, which can account for unequal variances. Male haploidy in the Hymenoptera means that paternal genotypes can be recreated based on those of the queen and

her female offspring (Debout *et al*., 2010). Based on this approach, the program MATESOFT 1.0 (Moilanen *et al.*, 2004) was used to derive an independent estimate of number of males by assigning offspring to patrilines. For colonies containing one or two patrilines only, standard errors and confidence intervals were generated by MATESOFT by jackknifing and bootstrapping over groups, respectively. However, for colonies containing more than two patrilines, MATESOFT is not able to derive these measures and returns only an estimate of average mate number.

Fecundity analysis

The reproductive characteristics and productivity of *Allomerus* are extensively documented elsewhere (Yu *et al*., 2004; Debout *et al*., 2010), and Yu *et al*. (2004) showed that the productivity of *Azteca* (pooled) increases with colony size, such that only the largest colonies produce large numbers of reproductive offspring (alate queens and males). Here, I improve on that analysis by accounting for polydomy in *Azteca*, and I extend it to ask whether the two focal species of *Azteca* vary in terms of fecundity.

One worker per hostplant was genotyped for species identification and to identify plants that shared a colony. This was carried out using COLONY 2.0 (Wang, 2004; Jones and Wang, 2010) to identify full-sib or half-sib pairs. Based on the results of the mating system analysis, *Az. dp2* workers were analysed under the assumption of male and female monogamy, but female polygamy (i.e. polyandry) was invoked for *Az. u1A*. Typing and scoring errors were each set to 0.01 for all species. Where workers were identified as siblings (including half-sibs for *Az. u1A*) and originated from neighbouring hostplants, polydomy was inferred and hostplants were pooled to give the total size of the colony in number of domatia. The effect of colony size on fecundity was investigated for the two focal *Azteca* species. I followed Yu and Pierce (1998) in using square-root scale linear regressions for this analysis.

Hostplant size

Hostplant size in number of domatia was recorded for all sampled colonies. These data were analysed using a quasi-Poisson GLM to test for differences among species. Differences in average hostplant size would indicate differential colony mortality rates.

2.3.7 Basic population genetics analyses

Tests for equilibrium were carried out in Micro-Checker (Van Oosterhout *et al*, 2004). This included testing for the presence of heterozygote deficit, null alleles, large-allele drop-out, and scoring error due to stuttering. Corrected allele frequencies were generated to account for any null alleles detected. GenePop 4.0 (Raymond and Rousset, 1995a) was used to test for linkage

disequilibrium among loci to ensure the independence of genetic markers, and to generate inbreeding coefficients (F_{IS}; Wright, 1951) for each species at each site.

2.3.8 Large-scale spatial genetic structure

GenePop was used to generate estimates of pairwise geographic and genetic distances (given by $F_{ST}/(1-F_{ST})$; Weir and Cockerham, 1984) between sites. Geographic distance was calculated based on the UTM coordinates of a sampled colony located approximately in the centre of the sampling area at each site. For each species (*Allomerus*, *Az. dp2*, and *Az. u1A*), I performed a linear regression to test for the positive effect of geographic distance on genetic distance that is expected under isolation by distance. I also tested for differences among species.

Next, I tested for genotypic differentiation between sites in GenePop. For each ant species, log-likelihood ratio (*G*) based exact tests (Raymond and Rousset, 1995b) were performed for all pairs of sites against the null hypothesis that genotypes are drawn from the same distribution in all populations. Because the distances separating site pairs ranged from less than 5 km to over 80 km (Figure 2.3), I hypothesised that *Allomerus*, the more dispersal-limited species, would show significant genetic differentiation even between neighbouring sites, while the better-dispersing *Azteca* species would only show differentiation between the most distant site pairs. All *p*-values were Bonferroni-corrected for the number of pairwise comparisons within species.

2.3.9 Fine-scale spatial genetic structure

The program SPAGeDi (Hardy and Vekemans, 2002) was used for analysis of isolation by distance at the grain of individual colonies within sites. SPAGeDi uses individual genotypes and geographic coordinates to calculate kinship coefficients and geographic separation distances for all pairs of sampled individuals and to estimate the slope of the regression, which is a product of σ^2 (half the mean squared axial parent-offspring distance, discussed below), and D_e (effective population density). Where an estimate of D_e is provided by the user, SPAGeDi returns a value of σ in addition to the slope of the regression line and associated statistics.

Following Vekemans and Hardy (2004), the kinship coefficient of Loiselle *et al*. (1995) was used as the measure of pairwise genetic similarity, and pairwise geographic distances were calculated as straight-line distances derived from UTM co-ordinates recorded in the field. Where possible, an estimate of the variance associated with σ was calculated by jackknifing over loci.

Estimating effective population size

Estimating D_e is not straightforward when comprehensive sampling of more than a single generation has not been undertaken (Wang and Whitlock, 2003; Robledo-Arnuncio and Rousset, 2009; Pinsky *et al*., 2010) and when reproductive behaviour is not fully understood. However, it is generally accepted that effective density is some fraction of census density, due to variation among adults in reproductive success (Hedgecock, 1994; Frankham, 1995; Watts *et al*., 2007). Effective population size, N_e, can be estimated as $N_e = \frac{4N}{2+V_k}$ where $V_k$is the variance in reproductive success (Kimura and Crow, 1963; Hedrick, 2005), but V_k is also difficult to estimate without carrying out parentage analysis (e.g. Serbezov *et al*., 2012). In the case of the *C. nodosa*-associated ant species, previous research has shown that a greater proportion of *Allomerus* colonies are producing reproductive queens and males (alates) at any given time than are *Azteca* colonies (Yu *et al*., 2001, 2004). Therefore, *Allomerus* would be expected to have a higher D_e/D ratio than would the *Azteca* species, unless *Azteca* mating systems allow multiple adults to contribute to their offspring through male or female polygamy.

In the absence of direct measures of effective density, and given that overestimating D_e results in underestimating σ, the conservative approach is to calculate σ for *Azteca* using the same D_e/D ratio that is used for *Allomerus*. Therefore, I expect to underestimate *Azteca*'s dispersal ability, which means that if the IBD analysis nonetheless finds σ to be higher for *Azteca* than for *Allomerus*, then it can be confidently inferred that *Azteca* species are indeed the better dispersers. For trees, N_e generally lies between 0.1*N* and 0.5*N* (Frankham, 1995). Hardy *et al*. (2006) derived values of σ for neotropical trees using a variety of D_e estimates and found that convergence of the iterative procedure for estimating σ was achieved more often when using values at the higher end of this range. Therefore, I use D_e = ½ × census density for all species.

Estimating and interpreting σ

For a population spread over two dimensions with isotropic dispersal, σ^2 is defined as 'half the mean squared axial parent-offspring distance' (Rousset 1997, 2000) and represents the variance of dispersal distance on a single axis. The relationship between σ and (the parameter of interest) the mean Euclidean dispersal distance (*d*), is given by the equation $\sigma^2 = \frac{1}{2}E[d^2]$, which (unfortunately) cannot be solved for *d*. However, σ can be used to parameterise a dispersal distribution (Broquet and Petit, 2009), which is helpful for visualising differences among species. Here, estimates of σ are used to parameterise a negative-exponential distribution $P(d)=\alpha e^{-\alpha d}$, where $\alpha=\frac{1}{\sigma}$ (see Broquet and Petit (2009), p.198). Estimating the shape of dispersal kernels is notoriously difficult and error-prone (Clark *et al*., 1999; Nathan *et al*., 2003, Kinlan and Gaines,

2003), and so this distribution was chosen because it is easy to parameterise. I plotted the cumulative distribution function (CDF) of the negative exponential and found the point on the x-axis at which CDF=0.5, which is the distance at which the area beneath the original curve is equal to 0.5. This distance or more is travelled by 50% of dispersing ant reproductives, and it can therefore be considered an estimate of the median dispersal distance.

2.3.10 Sibship analysis

To obtain information about the actual shape of the dispersal kernel for the focal ant species, I carried out a sibship analysis of ant queens colonising *C. nodosa* saplings. The idea here is that as sister female alates disperse from their natal colony, the distribution of pairwise distances should contain information on (1) dispersal scale, and (2) the shape of the dispersal kernel. This type of data has been used to effectively characterise pollen dispersal kernels in plant populations (Robledo-Arnuncio *et al*., 2007; de-Lucas *et al*., 2008).

All queens collected from the coppiced hostplants at CIC-H (Figure 2.4) were genotyped, and full-sib pairs were identified in COLONY (full-sib and half-sib for *Az. u1A*). "Male monogamy" (i.e. monogyny) was assumed for all species on the basis of (1) previous studies of *Allomerus* (Debout *et al*., 2010) and (2) mating system results presented here for *Azteca*, both of which suggest that monogyny is the norm. "Female polygamy" (i.e. polyandry) was invoked for *Az. u1A*, as per the fecundity analysis above, while monandry was assumed for *Allomerus* and *Az. dp2*. Although low levels of facultative polyandry have been recorded in *Allomerus*, the majority of queens tend to be singly mated (Debout *et al*., 2010), while mating system analysis in this study detected no instance of polyandry for *Az*. *dp2*. On the basis of population genetics results, inbreeding was assumed to be present only for *Az. u1A* and, since out-crossing is the most common strategy for all focal ant species, the "dioecious" option was chosen, following recommendations in the software user guide. Typing and scoring errors were set to 0.01 for all loci.

A run of the longest available length was conducted using the full-likelihood analysis method with no size prior and without updating allele frequencies during the run. Following the run, the distance separating each pair of full sisters was calculated using the geographic coordinates of the coppiced plants in which they had been found. The number of full-sib pairs in each distance class was normalised by the total number of possible pairs of queens in that class to account for the fact that distance classes were not evenly represented in the sampling design. A binomial regression was used to test for an effect of separation distance on the probability of sibship for each species, and a minimum estimate for median parent-offspring distance was calculated as half the median distance separating sibling pairs. These estimates were compared with those

derived from the IBD analysis, and the sibship results were used to make inferences about the relative shapes of the dispersal kernels for the focal ant species.

2.4 Results

2.4.1 Ant frequencies and hostplant density

In total, 264 *Allomerus* and 486 *Azteca* colonies were sampled across the six sites (Table 2.6). *Azteca* comprises five species in our collections (Chapter 1), of which *Az. dp2* and *Az. u1A* were found to be the most abundant (Figure 2.5).

All five species were present at all six collection sites, and there was no evidence of spatial partitioning within sites: colonies of all species were interspersed, even at very small spatial scales (Figure 2.5).

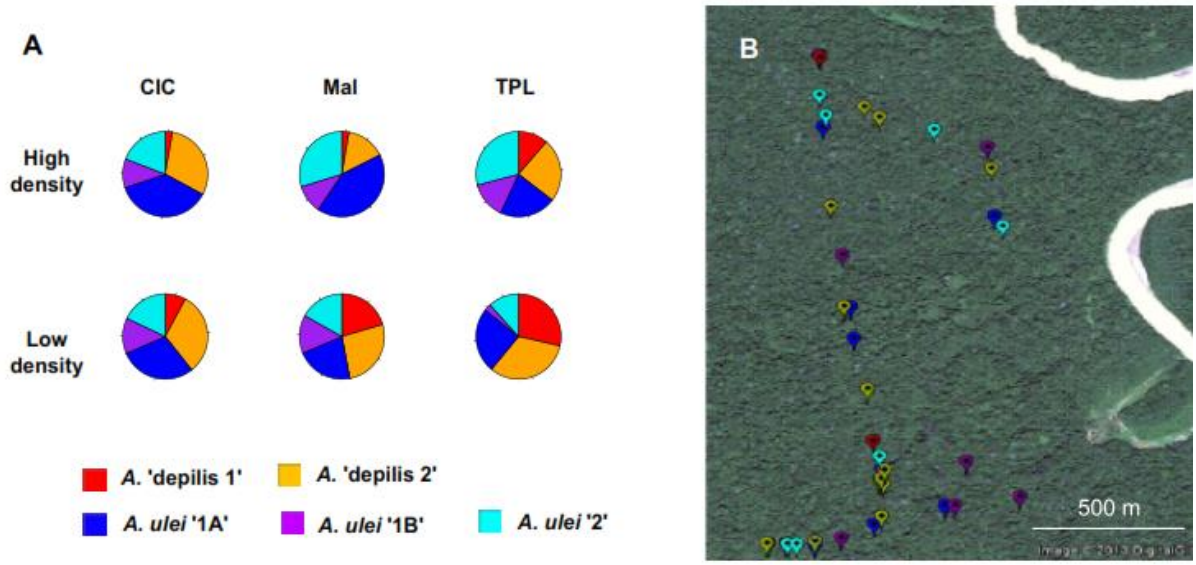

Figure 2.5: The distribution of *Azteca* species: (A) the proportion of collections identified to each species at each of the six collection sites; (B) map showing the locations of colonies of all five species along trails at site CIC-L.

I reconfirmed the key macroecological pattern that characterises this system; *Azteca* colonies are more frequent in sites with low hostplant density (binomial regression: variance explained = 79.1%, $\chi^2_{df=1}$=64.9, p<0.001; Panel A in Figure 2.6), which is consistent with *Azteca spp.* being better dispersers (Yu et al., 2001, 2004).

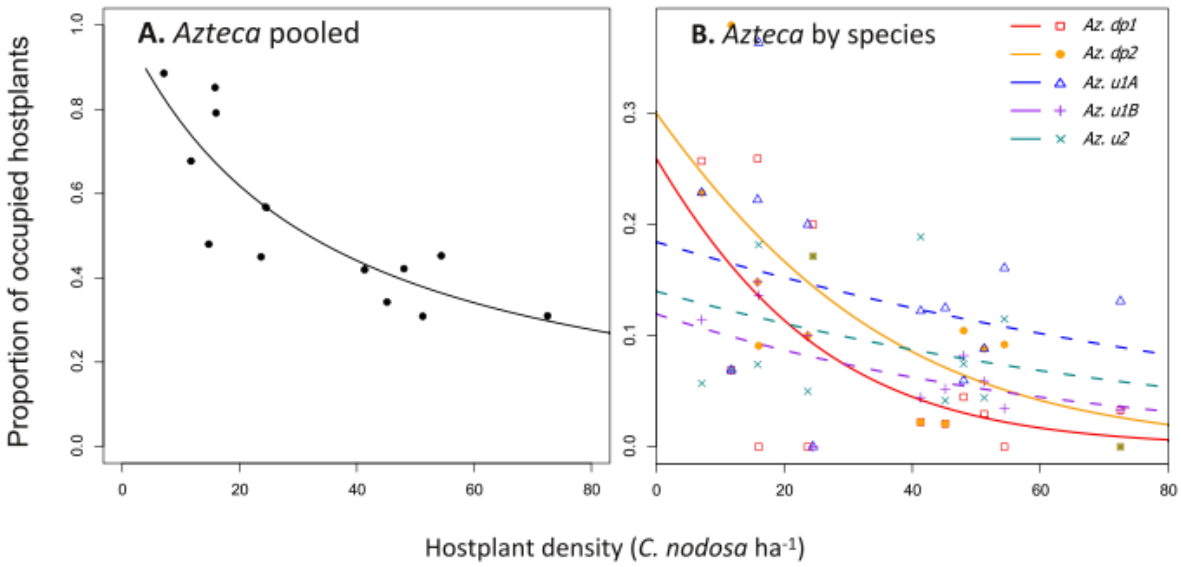

Figure 2.6: The effect of hostplant density on the relative abundance of *Azteca*, measured as proportion of all occupied hostplants, with species (A) pooled and (B) considered separately.

However, the relationship varied among species (binomial regression, interaction effect: $\chi^2_{(4,50)}$=124.59, p=0.001), with the two *Az.* 'depilis' species showing steeper hostplant density responses than the three *Az. ulei* species (Table 2.3; Figure 2.6). In fact, *Az. u1A* and *Az. u2* showed no significant decline in frequency with increasing hostplant density (although a weak negative relationship is suggested in both cases), and *Az. u1B* showed only a marginally significant decline (Table 2.3; Figure 2.6).

Table 2.3: For each *Azteca* species, results of binomial regressions for the effect of hostplant density on trail-level relative abundance, including the estimate and standard error of the regression slope, z-statistic, *p*-value, and the % variance in relative abundance that is explained by hostplant density.

	Slope	s.e. (slope)	z	p	% variance
Az. dp1	-0.05	0.0096	-5.24	**<0.001****	50
Az. dp2	-0.038	0.007	-5.132	**<0.001****	49
Az. u1A	-0.011	0.0066	-1.744	0.081	9
Az. u1B	-0.018	0.009	-2.021	**0.043***	28
Az. u2	-0.013	0.008	-1.731	0.083	9

A post-hoc test found that there was still a significant interaction between species and the effect of hostplant density on relative abundance when only the two most common species (*Az. u1A* and *Az. dp2*) were considered ($\chi^2_{df=20}$=7.29, p=0.007). However, this result became marginally non-significant when the *p*-value was adjusted for ten possible pairwise comparisons ('*p.adjust*

(method="fdr")' in *R;* adjusted *p*=0.069). Thus, there is a suggestion that *Az. u1A* and *Az. dp2* may respond differently to variation in hostplant density, but this lacks robust statistical support.

Hereafter, the scope of this study is limited to consideration of *Allomerus* and these two species of *Azteca*, since other *Azteca* species were insufficiently sampled for the purpose of conducting population genetic analyses.

2.4.2 Life-history analyses

Azteca mating system analysis

Variances were not homogeneous across species for Wang's (2002) within-colony relatedness coefficient (Bartlett's test: $K^2_{df=1}$=8.97, *p*=0.002), and so a one-way ANOVA test (*oneway.test()* in *R*), which includes a correction for non-homogeneity of variance, was used to test for an effect of species on within-colony worker relatedness. A significant difference between species was found (One-way ANOVA: $F_{df=1}$=13.14, *p*=0.007), with *Az. u1A* workers being less closely related to one another, on average, than those of *Az. dp2* (*Az. u1A*: mean = 0.52 ± 0.014 (s.e.); *Az. dp2*: mean = 0.71 ± 0.051). For all colonies, every worker shared at least one allele with the queen at all loci. Colonies are therefore taken to be monogynous, and variation in within-colony relatedness of workers is assumed to arise from different numbers of males. The number of males calculated from relatedness coefficients using the equation of Chevalet and Cornuet (1982) did not differ significantly from number of males detected using MATESOFT (Wilcoxon Signed Rank test: *V*=92, p=0.23). Where differences occurred for individual colonies, the calculated value usually underestimated the number of males because of an uneven distribution of offspring among patrilines. All *Az. dp2* colonies were monandrous, but multiple males were detected by MATESOFT at 6 of 8 *Az. u1A* colonies, with a maximum of three males per colony.

In summary, *Az. dp2* appears to be strictly monogynous and monandrous in its mating system, while *Az. u1A* is monogynous but queens often mate with two or three males.

Fecundity analysis

68 entire *Azteca*-inhabited hostplants were collected and scored for reproductive offspring. Analysis of worker genotypes in COLONY revealed 55 genetically distinct colonies, eight of which inhabited multiple hostplants. The maximum number of plants occupied by a single colony was five. Worker genotypes identified eighteen colonies as *Az. dp2* and seventeen as *Az. u1A*.

Az. dp2 was more fecund than *Az. u1A* in terms of male production (128.1.4 ± 69.8 vs. 7.94 ± 6.79 male offspring per colony; quasi-Poisson GLM: $\chi^2_{df=1}$=2217, *p*=0.019), which is largely attributable to the fact that only 12% of *Az. u1A* colonies contained male offspring, compared with 56% of *Az.*

dp2 colonies. There was no difference in female alate production (12.33 ± 9.24 vs. 4.41 ± 4.11 female alates per colony; $\chi^2_{df=1}$=69.91, *p*=0.40), and, for both species, the proportion of colonies containing female alates was low compared with available data for *Allomerus* (11% of *Az. dp2* colonies; 12% of *Az. u1A*; 47% of *Allomerus*).

Fecundity of *Az. dp2* (alate sexes pooled) increased with colony size (square-root-transformed linear regression: $F_{df=1}$=18.92, *p*<0.001). The slope of the relationship between fecundity and colony size did not differ from that of *Allomerus* (square-root-transformed linear regression, interaction effect: *F* =1.20, *p*=0.278), but *Az. dp2* produced fewer alates for any given number of domatia (effect of species: *F*=19.09, *p*<0.001; Figure 2.7). When the analysis was restricted to male alates, the effect of species also became non-significant (*F*=1.14, *p*=0.289), which indicates that the difference in overall fecundity is attributable to lower female alate production in *Az. dp2*. There were insufficient data to perform the same analysis for *Az. u1A* owing to the fact that very few colonies contained reproductive offspring of either sex.

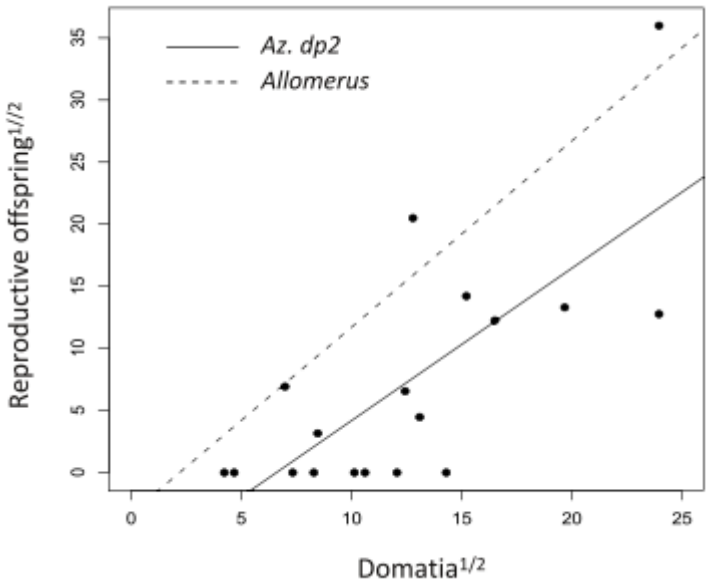

Figure 2.7: For *Az. dp2* (points and solid regression line), the square-root scale relationship between colony size (in domatia) and the number of reproductive offspring of both sexes (Reproductives$^{1/2}$ = -8.10 + 1.23 x Domatia$^{1/2}$). The dashed line shows the same relationship for *Allomerus* (Reproductives$^{1/2}$ = –3.70 + 1.55 x Domatia$^{1/2}$).

These results suggest that a fecundity hierarchy does exist within *Azteca*, with *Az. dp2* being more fecund than *Az. u1A*, but that this is driven by differential male production. In contrast, the difference in fecundity between *Allomerus* and *Az. dp2* is driven by lower female alate production in *Az. dp2*.

Hostplant size

The three ant species occupied hostplants of different sizes (quasi-Poisson GLM: $F_{df=2}$=30.97, p<0.001), with *Allomerus* inhabiting the smallest hostplants, and *Az. u1A* the largest (*Allomerus* = 30.04 ± 1.23 (mean ± s.e.) domatia; *Az. dp2* = 41.33 ± 2.86; *Az. u1A* = 56.83 ± 4.54; Figure 2.8). All pairwise comparisons were significant, even after correction for multiple tests (Table 2.4).

Table 2.4: Interspecific pairwise comparisons of hostplant size (in domatia) using a quasi-Poisson GLM with *p*-values corrected for multiple comparisons ('method="fdr"').

	χ2	*p*
Allomerus vs. *Az. dp2*	303.3	<0.001
Allomerus vs. *Az. u1A*	1542.1	<0.001
Az.u1A vs. *Az. dp2*	314.3	0.004

This is suggestive of a hierarchy in colony mortality rates, with *Allomerus* colonies having the highest mortality and *Az. u1A* the lowest. Differential mortality between genera is consistent with the previously reported tendency for a chrysomelid beetle, *Trachysomus sp.* to attack and kill *Allomerus*-inhabited hostplants but not those inhabited by *Azteca* (Yu and Pierce, 1998).

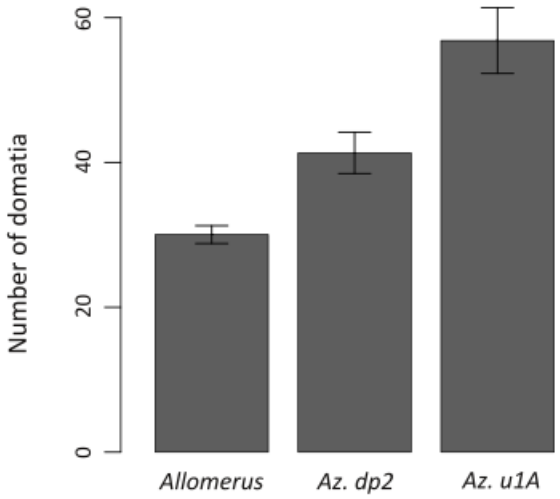

Figure 2.8: For each species, mean hostplant size measured by number of domatia. Error bars show ± 1 s.e.

2.4.3 Basic population genetics analyses

The microsatellite markers used for *Azteca* had been developed from *Azteca ulei* samples, and I found a substantially greater level of polymorphism for *Az. u1A* than for *Az. dp2* (Table 2.2). For *Az. dp2*, two loci (Az002 and Az10230) were monomorphic across all populations. Although this

meant that these markers were uninformative for detecting genetic variation in this species, they remained informative for the purposes of species identification.

Testing for equilibrium

Two *Allomerus* loci (Ad01223 and Ad040) showed a significant heterozygote deficit across multiple populations ($p < 0.05$) and so were excluded from subsequent analyses. For the remaining loci and for all *Azteca* loci, no evidence of allelic drop-out or scoring error due to stuttering was detected by Micro-Checker, and no more than one locus in any population showed signs of null alleles. Since this was a different locus in each population, it was likely caused by small sample size, as opposed to the actual presence of a null allele. Tests in GenePop found no evidence of linkage disequilibrium, suggesting that all loci vary independently.

Inbreeding

Inbreeding coefficients showed that outcrossing was the norm for *Az. dp2* (F_{IS} = -0.014 ± 0.013, mean across six sites ± s.e.), with no effect of colony density on inbreeding levels (linear regression, $F_{1,4}$ = 0.255, R^2 = 0.06, p=0.640). Very low levels of inbreeding were detected for *Allomerus* (F_{IS} = 0.023 ± 0.004), again with no significant density effect ($F_{1,4}$ = 3.00, R^2 = 0.43, p=0.158). *Az. u1A* had the highest overall level of inbreeding (F_{IS} = 0.074 ± 0.021), and a strong negative effect of colony density was detected ($F_{1,4}$ = 10.43, R^2 = 0.72, p=0.032), such that inbreeding was highest when colony density was low (Figure 2.9). This is consistent with male availability being low in *Az. u1A*.

Across all three species, the proportion of colonies that produce males (single value for each species, taken from the fecundity analysis for *Azteca*, and from G. Debout's raw data for *Allomerus*) also had a significant negative effect on both the level of inbreeding (linear regression: F=25.36, p<0.001) and the effect of colony density on inbreeding (interaction effect: F=11.44, p=0.004). In other words, lower male production is associated with higher levels of inbreeding and a stronger density effect. F_{IS} can be expressed as $F_{IS} = 0.17 – 0.356M + D(0.05M-0.024)$, where M is the proportion of colonies that produce males and D is the species-specific colony density (linear model, F_{IS} ~ Density * Males: $F_{3,14}$ = 12.29, $p < 0.001$).

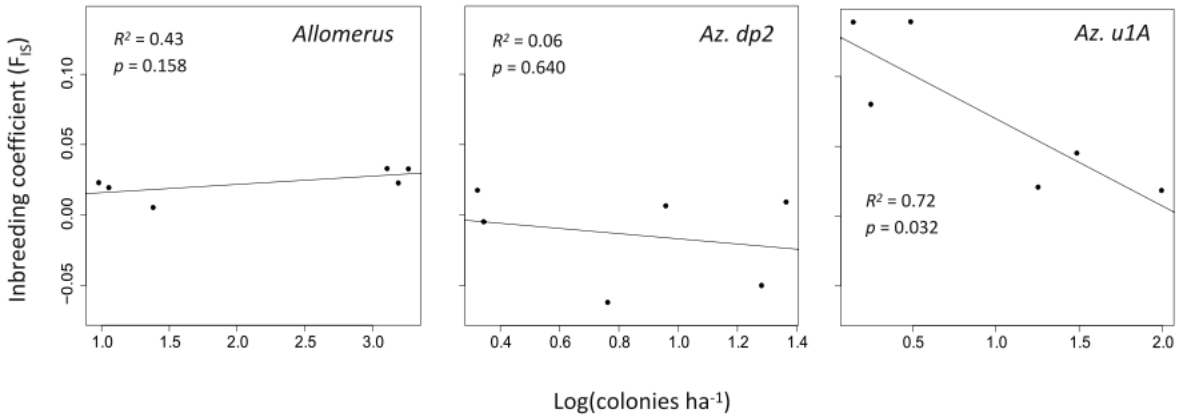

Figure 2.9: Effect of colony density on inbreeding for each of the three focal ant species.

2.4.4 Large-scale spatial genetic structure

Euclidean distances between sites ranged from 3.87 km separating CIC-H and CIC-L to 88.08 km separating CIC-L and TPL-H. Coarse-grained IBD was detected for all three species, with pairwise genetic distance between sites, given by $F_{ST}/(1-F_{ST})$, increasing with geographic distance (Table 2.5). Average genetic distance varied among species but the slope of the relationship between genetic and geographic distance did not (linear model, $F_{ST}/(1-F_{ST})$ ~ geographic distance*species; effect of Species: $F_{df=2}$=7.17, p=0.002; interaction effect: $F_{df=2}$=2.27, p=0.11). After correction for multiple tests, pairwise comparisons found that *Allomerus* and *Az. dp2* each showed significantly higher levels of genetic difference than *Az. u1A* but they did not differ from one another (*Allomerus* vs. *Az. dp2*: $F_{1,28}$=0.46, adjusted p=0.505; *Allomerus* vs. *Az. u1A*: $F_{1,28}$=14.07, p=0.024; *Az. dp2* vs. *Az. u1A*: $F_{1,28}$=6.15, p=0.029).

This indicates that, of the three species, *Az. u1A* has the highest level of gene flow between sites, which suggests that it is the least dispersal limited of the species (i.e. the best disperser).

Table 2.5: For each species, mean (and s.e.) pairwise genetic distance between sites ($F_{ST}/(1-F_{ST})$), and results of a linear regression of $F_{ST}/(1-F_{ST})$ against geographic distance across all pairs of sites. Df=1,13 for all regressions.

	Mean	s.e.	Slope	s.e. (slope)	*F*	R^2	*p*
Allomerus	0.014	0.0014	0.00011	0.00003	11.64	0.47	**0.005****
Az. dp2	0.017	0.0043	0.00028	0.0001	8.21	0.39	**0.013***
Az. u1A	0.0055	0.0017	0.00013	0.00003	18.82	0.59	**<0.001****

Exact tests conducted in GenePop provided further clarification of the dispersal hierarchy. Once again, *Az. u1A* was shown to have the highest level of gene flow between sites, being the only species for which no site differed genotypically from any other (Figure 2.10). In contrast, differentiation was detected between all pairs of sites for *Allomerus*, including those separated by very short distances (< 5 km). This is consistent with very low levels of gene flow as a result of dispersal limitation. Intermediate levels of gene flow were indicated for *Az. dp2*, for which only sites separated by a geographic distance greater than 77 km (i.e. comparing across watersheds; Table 2.1) exhibited significant genetic differentiation. Differences among species in the level of gene flow between sites can be clearly seen in the STRUCTURE plots in Figure 2.11.

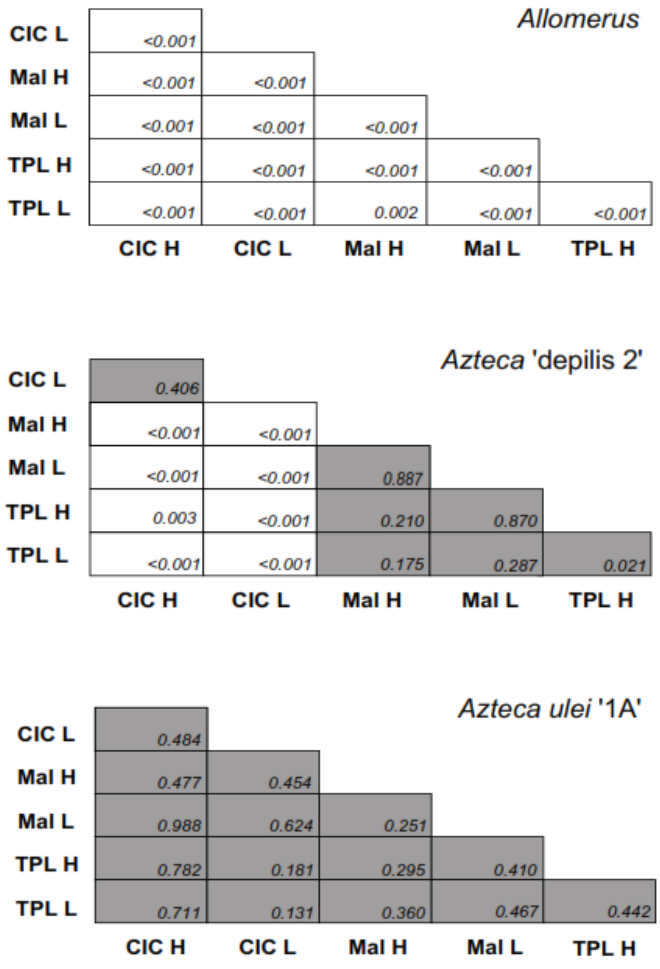

Figure 2.10: Matrices illustrating the presence (white cell) or absence (grey cell) of genotypic differentiation among populations of the three focal ant species. Raw *p*-values are shown but significance is assigned following Bonferroni correction for 15 tests.

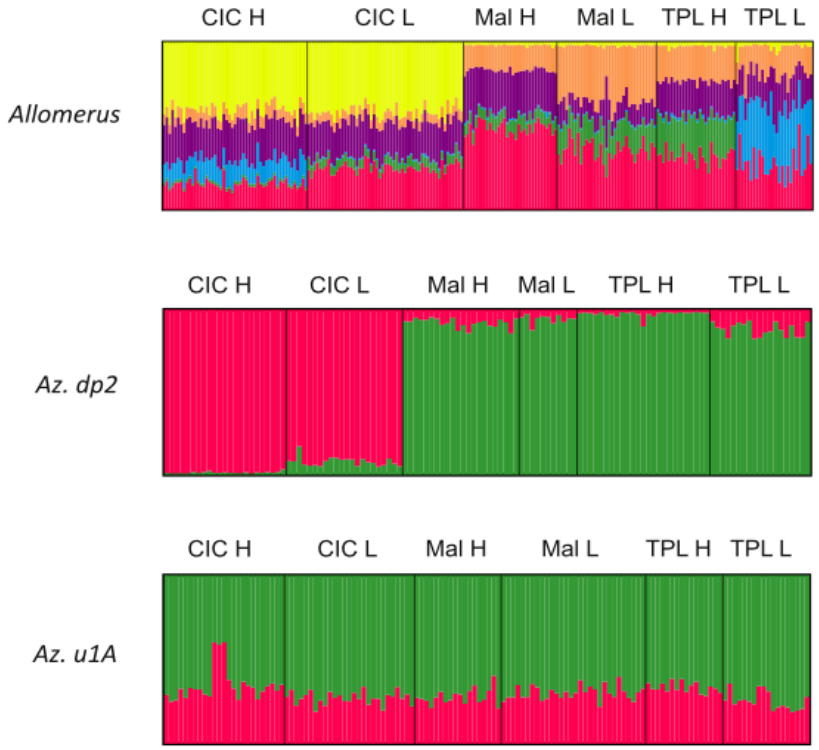

Figure 2.11: For each of the focal species, a plot showing the results of population assignment in STRUCTURE. Plots are created using DISTRUCT 1.1, and results are averaged over ten runs using CLUMPP 1.1.2. *K* was set to 6 for *Allomerus* and to 2 for *Az. dp2* and *Az. u1A*, and each run consisted of 100,000 iterations. Increasing *K* did not allow any further visual differentiation of populations for either of the *Azteca* species.

In summary, analysis of between-site genetic differentiation suggests that *Az. u1A* is the best disperser of the three species, and *Allomerus* the poorest.

2.4.5 Fine-scale spatial genetic structure

Fine-scale analysis of IBD performed in SPAGeDi successfully converged and yielded estimates of σ for all three focal ant species at CIC-H and CIC-L, and for *Allomerus* at all 6 sites (Table 2.6). An estimate for *Az. dp2* was also obtained at Mal-L, but otherwise the iterative procedure for estimating σ did not converge for the *Azteca* species at the Malinowski and TPL sites. Therefore, I focus on the two CICRA sites for making interspecific comparisons of dispersal distance.

Negative exponential distributions were fitted to all obtained σ values and used to estimate median dispersal distances (Table 2.6).

At CIC-H, *Allomerus* exhibited the lowest σ value and median dispersal distance (95 m), *Az. Adp2* exhibited an intermediate value (193 m), and *Az. u1A* exhibited the highest (319 m) (Figure 2.12).

This is consistent with the results from the large-scale spatial genetic structure analyses in identifying *Allomerus* as the poorest disperser and *Az. u1A* as the best.

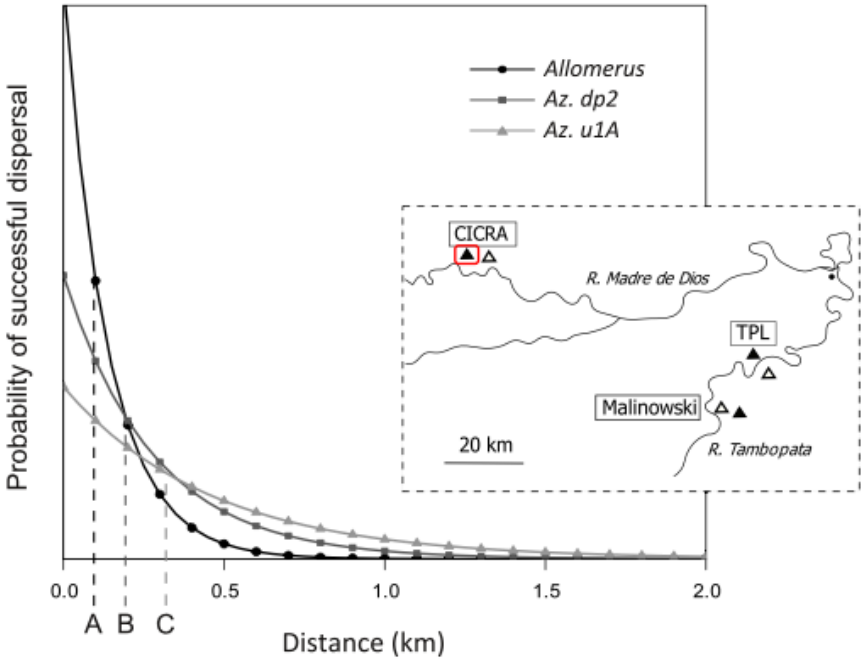

Figure 2.12: For site CIC-H, negative exponential distribution curves ($P(d)=\alpha e^{-\alpha d}$) parameterised with values of σ derived from analysis of fine-scale isolation by distance in SPAGeDi using $\alpha=1/\sigma$ (Broquet and Petit, 2009). Dashed lines show the point at which the area beneath the curve is equal to 0.5, which is an estimate of the median dispersal distance, for (A) Allomerus (0.095 km), (B) *Az. dp2* (0.193 km), and (C) *Az.u1A* (0.319 km).

At all three pairs of sites, *Allomerus* exhibited a substantially greater median dispersal distance at the low hostplant density site than at the high density site (Table 2.6; Figure 2.13, Table 2.6), with a more than two-fold difference at CICRA and Malinowski (CICRA - 161% increase; Malinowski – 122%; TPL – 53%). Overall, there was a significantly negative effect of hostplant density on the median dispersal distance of *Allomerus* (linear regression: d = 234 – 2.77 x hostplant density; F=17.4, R^2=0.81, p=0.014), which suggests that when hostplants are spaced farther apart, *Allomerus* responds by dispersing longer distances.

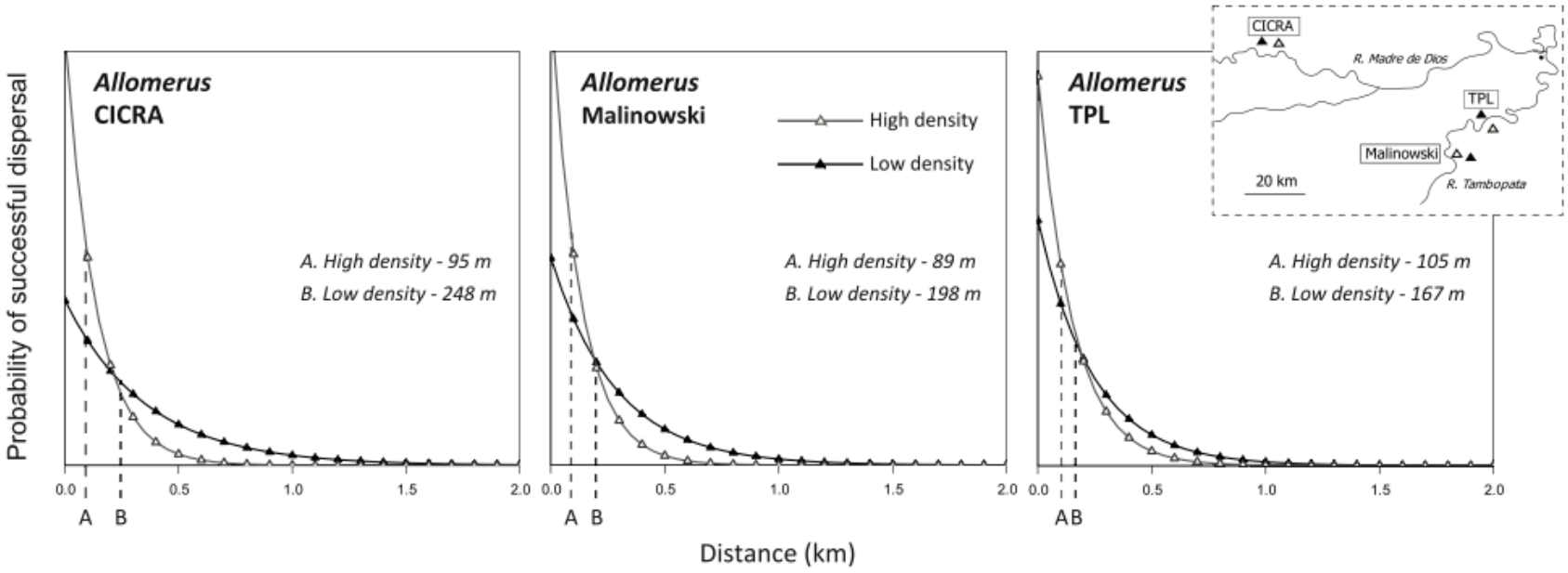

Figure 2.13: For *Allomerus*, negative binomial distribution curves parameterised with estimates of σ from the (A) high and (B) low hostplant density site at each sampling location (CICRA, Malinowski, TPL).

Table 2.6: Results of fine-scale IBD analysis. For each species at each site, the number of colonies genotyped (N); the value of D_e (0.5 x census density) used as input in SPAGeDi; the value of σ (mean squared axial parent-offspring distance) returned by SPAGeDi, with associated s.e. where available; and the estimated median Euclidean dispersal distance (*d*) derived from the cumulative distribution function of the σ-parameterised negative exponential distribution (CDF=0.5). 'no conv.' indicates that the iterative procedure for estimating σ in SPAGeDi did not converge, so no dispersal estimate is available. ## indicates that convergence was not achieved when some loci were removed, which prevented estimation of s.e. by jackknifing over loci.

		High hostplant density					Low hostplant density				
		N	D_e	σ	s.e. (σ)	*d* (km)	N	D_e	σ	s.e. (σ)	*d* (km)
CICRA	***Allomerus***	56	1200	0.126	0.078	0.095	56	140	0.358	##	0.248
	Az. dp2	22	180	0.279	##	0.193	28	70	0.332	##	0.230
	Az. u1A	27	200	0.46	##	0.319	26	70	0.423	0.282	0.293
Malinowski	***Allomerus***	42	1300	0.129	0.254	0.089	38	135	0.285	##	0.198
	Az. dp2	11	130	no conv.	N/A	N/A	22	70	0.613	##	0.425
	Az. u1A	31	365	no conv.	N/A	N/A	18	60	no conv.	N/A	N/A
TPL	***Allomerus***	29	1120	0.152	##	0.105	30	200	0.241	##	0.167
	Az. dp2	19	195	no conv.	N/A	N/A	25	195	no conv.	N/A	N/A
	Az. u1A	17	175	no conv.	N/A	N/A	19	80	no conv.	N/A	N/A

In contrast, *Az. dp2* responds weakly to hostplant density, showing only a 20% increase in estimated median dispersal distance at CIC-L compared with at CIC-H (Figure 2.14), such that estimates of *d* at CIC-L were very similar for *Allomerus* and *Az. dp2* (Table 2.6). For *Az. u1A*, there was no suggestion that hostplant density affected dispersal distance (Table 2.6; Figure 2.14).

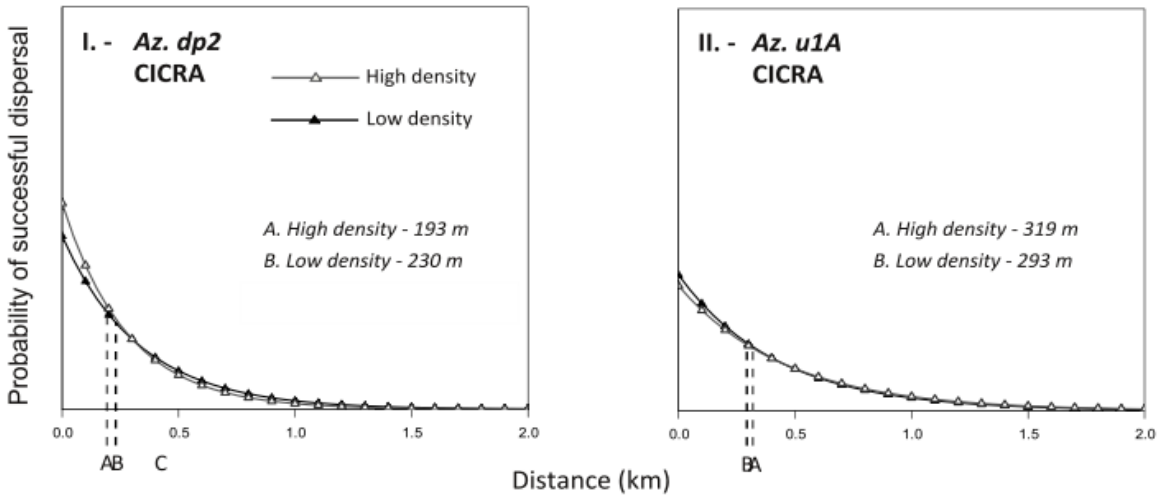

Figure 2.14: For (I) *Az. dp2* and (II) *Az. u1A*, negative binomial distribution curves parameterised with values of σ from the (A) high and (B) low hostplant density sites at CICRA.

In summary, fine-scale analysis of isolation-by-distance suggests that the average dispersal distance of *C. nodosa*'s ant symbionts is of the order of a few tens of metres to a few hundred metres. When hostplant density is high, results support the hypothesis that *Allomerus* is the shortest-distance disperser and *Az. u1A* is the longest-distance disperser (Table 2.6, Figure 2.12). However, when in low-density locations, *Allomerus* appears to increase its dispersal distance, making it similar to *Az. dp2* (Table 2.6, Figure 2.13). Nevertheless, recall that σ is expected to be underestimated for *Azteca*, since effective population size D_e has likely been overestimated. Taking this into account, *Allomerus* is probably the shortest-distance disperser in all locations.

2.4.6 Sibship analysis

Genotypes were obtained for a total of 320 *Allomerus*, 52 *Az. dp2*, and 20 *Az. u1A* foundresses harvested from coppiced hostplants at CIC-H.

For *Allomerus, s*ibship analysis in COLONY identified 92 full-sib pairs (posterior probability > 0.95). Probability of sibship decreased with increasing separation distance (binomial regression, probability ~ log(distance): χ^2=127.07, $p < 0.001$; Panel A in Figure 2.15), with 67% of full-sib pairs separated by distances smaller than 200 m (Figure 2.15), and 10% located in the same sapling (distance=0). The median separation distance was 125.95 m, from which the minimum value for median parent-offspring distance was inferred as ½ x 125.95 = 62.98 m, which fits well with the IBD estimate derived for this site (95 m). Mean separation distance was 199.08 ± 216.32 (mean ± s.e.), giving a minimum mean parent-offspring distance of 99.54 m. Panel A in Figure 2.15 illustrates that the frequency of long-distance dispersal events is low and that a thin-tailed dispersal kernel with a low modal distance is appropriate for *Allomerus*, at least where host-plant density is high (recall that this experiment was conducted at high density site, CIC-H). However, the maximum separation distance of 1110.42 m indicates a capacity for longer-distance dispersal where saplings occur in lower density.

For *Az. u1A*, no full-sib or half-sib pairs of sibling foundresses were identified with probability > 0.95, which is consistent with its inferred long dispersal distances (Table 2.6, Figure 2.12). Therefore, no analysis was possible.

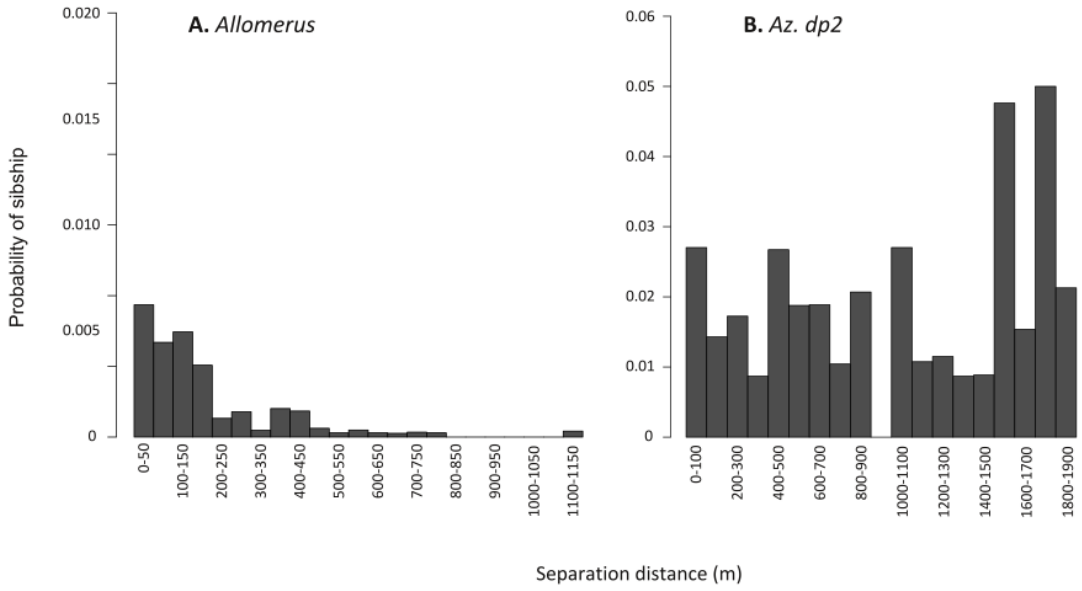

Figure 2.15: The relationship between Euclidean separation distance and the probability of sibship for (A) Allomerus and (B) Az. dp2 foundresses harvested from coppiced hostplants at CIC-H.

For *Az. dp2*, COLONY identified 41 pairs of full-sib foundresses. Overall, the probability of any pair of foundresses being full-sibs was higher than for *Allomerus* (*Az dp2: P* = 0.018, *Allomerus: P* = 0.0028; binomial GLM across all distance classes: $\chi^2_{df=1}$ =74.9, p<0.001), which reflects the fact that a greater proportion of *Allomerus* colonies produce female alates at any one time. Unlike for *Allomerus*, probability of sibship did not decline with increasing separation distance (χ^2=0.15, p=0.700; Panel B in Figure 2.15), and only 12% of sibling pairs were separated by less than 200 m. Median separation distance was 733.97 m, giving a minimum median parent-offspring distance of 367.0 m, which is considerably larger than the IBD-based estimate for the same site (193 m). Mean separation distance was 832.68 m, giving a minimum mean parent-offspring distance of 416.34 m. These results suggest that long distance dispersal is common for *Az. dp2*, with foundresses frequently travelling up to 2 km in search of a hostplant. Therefore, a fat-tailed dispersal kernel with a relatively high modal dispersal distance (different to *Allomerus*) is probably appropriate for *Azteca*, and low confidence should be attributed to estimates derived from a negative exponential distribution (Table 2.6).

2.5 Discussion

2.5.1 Determining relative dispersal abilities

This study provides three lines of evidence to support the conclusion from previous empirical studies (Yu *et al*., 2001; 2004) that *Allomerus* is more dispersal limited than *Azteca*. First, genetic structure among sites was strongest for *Allomerus* (Figure 2.10), which is indicative of limited gene flow as a result of dispersal limitation. Second, *Allomerus* had the lowest value of σ (i.e. strongest isolation by distance) in the fine-scale IBD analysis, at least at high hostplant density (Figure 2.12). Although $\sigma_{Allomerus}$ was slightly greater than $\sigma_{Az.dp2}$ at the low density site, recall that σ was expected to be underestimated for *Azteca* via overestimation of D_e (see below for detailed discussion). Third, dispersed sibling foundress queens of *Allomerus* were separated by shorter distances, on average, than were those of *Azteca* (Figure 2.15).

Results of the population genetics analyses also support my hypothesis that, within *Azteca*, dispersal ability correlates with the body size of dispersing queens. Genetic differentiation was detected between some pairs of sites for *Az. dp2* (smaller body size, Yu *et al*., 2004), but not for *Az. u1A* (larger queen body size) (Figure 2.3, Figure 2.10), and fine-scale analysis of IBD found that *Az. u1A* had the greater value of σ at both sites where estimates were obtained for both species (Table 2.7, Figure 2.14).

2.5.2 Estimating dispersal distance from analysis of IBD

Analysis of isolation by distance in populations at drift-dispersal equilibrium can yield quantitative estimates of dispersal scale. However, the accuracy of these estimates is affected by a range of factors that are difficult to account for. In this study, I have improved upon the approach of many other authors (e.g. Sumner *et al*, 2001; Debout *et al*., 2009; Suni and Gordon, 2010; Corrales and Höglund, 2012) by reducing D_e below census density and attempting to translate axial parent-offspring distances (σ) to estimates of Euclidean distances. Nonetheless, there remain several sources of uncertainty, which I discuss below. I subsequently present a more detailed interpretation of the dispersal characteristics of the three focal ant species in this study.

Spatial distribution of samples

Generating an accurate estimate of σ from the relationship between genetic and geographic distance relies on the assumption of constant within-site density of individuals (Rousset, 1997; 2000). Although I attempted to control for landscape-level variation in colony density by performing separate analyses for sites with high and low hostplant density, there were still

inconsistencies. In particular, hostplant density was less homogeneous at the Malinowski and TPL sites compared with at the CICRA sites, which may have contributed to the failure to detect isolation by distance in *Azteca* at most of these sites (Table 2.6). At Malinowski, the high and low density sites were directly adjacent to one another, meaning that they might have been influenced by dispersal from one to the other. The Mal-L trails that were closest to the Mal-H site had hostplant density more than twice that of those further away, and, although they accounted for only 31% of the total trail distance, 85% of *Allomerus* colonies were encountered on these trails. Thus, the assumption of constant density does not hold at this site. At TPL, the low density site was split over two separate trail systems (separated by approximately 4km) because there was an insufficient distance of trails at the original site, while the high density site consisted of a single transect and therefore does not truly represent a population in two dimensions.

Nevertheless, simulation studies (Leblois *et al*., 2003; 2004) have suggested that the analysis may be robust to deviations from the assumption of constant density, and despite the inconsistencies noted above, the results obtained for *Allomerus* are remarkably consistent across sites, with $\sigma <$ 1.6 at all low density sites and $\sigma >$ 2.4 at all high density sites. Therefore, the failures of the iterative procedure in SPAGeDi to converge on an estimate of σ are more likely to be attributable to small sample sizes and insufficient spatial coverage for the detection of IBD in *Azteca*. Ideally, sampling should cover an area of 10σ x 10σ (Rousset, 2000), which would equate to an area of approximately 5 x 5 km for *Au1A* based on estimates from CICRA. Although this was the target for spatial coverage in this study, it was not achieved at all sites due to limited trail networks.

In terms of sample sizes, it appears that 30 colonies per site are sufficient for detecting isolation by distance and obtaining an estimate of σ. Except in two cases (*Az. dp2* at TPL-L and *Az. u*1A at Mal-H), a value of σ was obtained wherever the number of colonies was 22 or higher; in no case was σ obtained where fewer than 22 colonies were included in the analysis (Table 2.6).

Uncertainty in estimating D_e

Accurate estimation of σ also depends on an accurate estimate of effective density (D_e). As mentioned above (see Methods section), D_e is difficult to calculate without sampling multiple generations or carrying out parentage analysis, which involves exhaustive sampling of potential parent colonies. In this study, the same ratio of effective density to census density was used to estimate D_e for all species, while acknowledging that this would affect the accuracy of dispersal estimates. Both mating system and fecundity influence the D_e/D ratio (Kimura and Crow, 1963), and data on these are now available for all three focal species, allowing specultion about how my

chosen approach to estimating D_e might have affected my conclusions about relative dispersal scales.

Analysis of the relationship between colony size and fecundity in *Azteca* improved on that conducted by Yu *et al*. (2004) in that I was able to detect polydomy using multilocus genotypes, which allowed me to pool domatia counts for hostplants that shared an ant colony. Like Yu *et al*. (2004), I found that fecundity is lower in *Azteca* than in *Allomerus*, and I showed that the difference is driven by lower female alate production in *Azteca* (Figure 2.7), with a smaller proportion of colonies producing female alates at any one time. This makes it highly likely that the ratio of effective density to census density is lower for *Azteca* than for *Allomerus*, meaning that the scale of dispersal (σ) is expected to have been underestimated for the two *Azteca* species. Therefore, if the correct D_e were used, the difference in dispersal scale between *Allomerus* and *Azteca* would likely be magnified compared with the estimates reported in this study, which lends yet more confidence to the conclusion that *Allomerus* is the most dispersal limited of the three species.

Determining relative D_e/D ratios for the *Azteca* species is more complex. *Az. u1A* is less fecund than *Az. dp2* as a result of producing fewer males, which will have the effect of lowering effective density relative to census density. However, the mating systems of the two species differ in that *Az. u1A* is polyandrous while *Az. dp2* is monandrous, which will *increase* effective density relative to census density for *Az. u1A* (Kimura and Crow, 1963). Moreover, there is the added complication that *Az. u1A* shows higher levels of inbreeding, especially when colony density is low (Figure 2.9). Therefore, it is not possible to say whether the observed difference in σ would be increased or reduced by the use of accurate D_e values in the IBD analysis, but the results from analysis of inter-site genetic differentiation (Figure 2.10) suggest that estimates would not be altered in such a way as to change the observed dispersal hierarchy.

Estimating Euclidean dispersal distances from σ

Even when axial parent-offspring distance (σ) is known, estimating Euclidean parent-offspring distance is not straightforward (Broquet and Petit, 2009; see Methods section). In this study, I used σ to parameterise negative exponential distributions, which were then used to derive estimates of median dispersal distance. The negative exponential distribution was chosen not because it accurately describes dispersal in the focal ant species, but because it is easy to parameterise since it requires only a single parameter (scale) to be input. In fact, the negative exponential distribution will never accurately describe dispersal in this system because all foundresses must travel to a new hostplant to found a colony, meaning that there is zero

probability of effectively dispersing 0 m. Other distributions, which are more likely to describe dispersal realistically (e.g. lognormal, gamma, inverse gamma, or Weibull distributions), are more complicated to use because they require both a scale and a shape parameter to be provided.

An additional complication is that the dispersal element captured by σ is the product of both male and female dispersal over many generations. In the *C. nodosa*-associated ant species, dispersal is considered to be heavily female-biased on the basis that females are many times larger than males (in *Azteca*, female dry weight = 12 x male dry weight; D. Yu, unpublished data), which means that IBD analysis is likely to underestimate foundress dispersal distance.

For all the reasons mentioned above, the distances obtained in this study from analysis of isolation by distance should be interpreted with caution.

2.5.3 Characterising dispersal in Allomerus

Despite the many uncertainties associated with the estimation of dispersal distance from IBD, the estimated median distances obtained for *Allomerus* using this method (89 – 319 m) are surprisingly consistent with both the empirical estimate from Yu *et al*. (2004) (150 – 200 m) and the estimate of minimum median dispersal distance derived from the sibship analysis (62.98 m). This may be because – at least areas of high hostplant density – *Allomerus* foundresses have a low modal dispersal distance (Figure 2.15), which means that (1) the negative exponential distribution provides a fairly good approximation of dispersal and (2) discrepancy between the scales of male and female dispersal is relatively small. In any case, the difference between male and female dispersal is likely to be lower in *Allomerus* than in *Azteca* because the difference in body size is smaller; *Allomerus* queens are smaller than *Azteca* queens (Yu *et al*., 2004), while males are approximately five times larger than those of *Azteca* by dry weight (D. Yu, unpublished data).

Because of the agreement among results obtained from different methods, it is possible to be fairly confident that, when hostplant density is high, the median dispersal distance for *Allomerus* foundresses is of the order of 100 – 200 m, with distances greater than 500 m rarely covered.

The IBD analysis revealed evidence of habitat-related dispersal plasticity, in that the effective dispersal distance of *Allomerus* increases when hostplant density becomes low (Figure 2.13). This makes sense if *Allomerus* foundresses colonise the first available sapling that they encounter, since the average distance to the nearest sapling on a given bearing should increase as density falls.

The disproportionate drop in the density of *Allomerus* colonies that was observed at sites with low *C. nodosa* density (*C. nodosa* density fell on average by a factor of about 4; *Allomerus* by a

factor of > 8) indicates an increased mortality rate of *Allomerus* foundresses in low density areas. This is likely to result from a combination of factors, including the simple inability to fly far enough to reach an available sapling, an increased risk of predation with increasing dispersal distance (Johnson *et al*., 2009), and the metabolic costs of longer-distance dispersal rendering foundresses more vulnerable to mortality from competitors, predators, and pathogens on arrival, thereby reducing the likelihood of successful colony establishment (Murrell *et al*., 2002; Clobert *et al*., 2009). Because the empirical and sibship analyses were carried out only in areas of high hostplant density, quantitative inferences about the scale of dispersal at low density sites should be treated with a greater degree of uncertainty than those at high density sites.

Dispersal characteristics for *Allomerus* have much in common with those reported for another obligate myrmecophyte of similar body size. Türke *et al*. (2010) studied the dispersal of *Crematogaster decamera* using a combination of empirical approaches and direct and indirect genetic techniques. Although they reported slightly higher estimates of average dispersal distance than were found for *Allomerus*, they detected a similarly strong level of genetic structure at small spatial scales (Figure 2.10) and interpreted their results as suggesting that foundresses settle on the first available sapling that they encounter. They also reported that unpublished data indicated shorter dispersal distances in areas of higher hostplant density.

2.5.4 Characterising dispersal in Azteca

For *Azteca*, interpretation of results is more tentative because (1) there is no existing empirical estimate of dispersal distance for comparison, (2) estimates of σ were obtained at fewer sites due to non-convergence in SPAGeDi, and, (3) for *Az. dp2*, estimates derived from the IBD and sibship analyses differed by about a factor of two (IBD = 197 m, sibship = 367 m for the same site).

The estimate from the sibship analysis is likely to be the more accurate due to the expected underestimation of σ in the IBD analysis. Furthermore, the distribution of sibling separation distances (Figure 2.15) suggests that the dispersal kernel of *Az. dp2* is poorly approximated by the negative exponential distribution, which will strongly affect the accuracy of the estimate from the IBD analysis. However, the sibship estimate is itself likely to be an underestimation of true dispersal distance because no drop-off in probability of sibship was observed at the scale of this experiment. Therefore, to accurately characterise the distribution of sibships for *Az. dp2*, more data are required, covering a larger area. Nonetheless, this analysis provides some useful information, pointing to a highly spread distribution of dispersal distances, with a high frequency of long-distance dispersal events and foundresses regularly dispersing distances greater than 500 m, even when hostplant density is high.

Interestingly, I found no evidence that the dispersal distance of either *Azteca* species increased with hostplant density (Figure 2.14, Table SIGMA). This suggests that *Azteca* queens disperse further than the distance to the nearest sapling when *C. nodosa* density is high, and points to a difference in dispersal behaviour between *Allomerus* and *Azteca*, which is not something that has been considered in previous attempts to model the dynamics of this system.

Why does Azteca disperse long distances when hostplant density is high?

There are several possible explanations for the long-distance dispersal of *Azteca* foundresses when hostplant density is high.

1. Local resource competition (LRC, Clark, 1978) may favour dispersing longer distances from the natal colony in order to avoid competing with siblings for colony establishment (Clobert *et al*., 2009). Moreover, since few *Azteca* colonies produce female alates at any one time – itself perhaps a consequence of LRC (Silk, 1983) – dispersing far from the natal colony has the added advantage of there being lower overall levels of competition with other *Azteca* foundresses, even when adult colony density is high. Competition with *Allomerus* is less of a concern, since *Azteca* foundresses of all species are able to invade domatia occupied by *Allomerus* foundresses (Yu *et al*., 2004).
2. The relatively low density of *Azteca* colonies may mean that foundresses have to travel further from their natal plant in order to mate, since males are highly dispersal limited. Little is known about the mating behaviour of *C. nodosa*-associated ants except that mating must occur before arrival at a sapling, since foundresses are able to produce diploid worker offspring upon arrival. In *Az. dp2*, my results suggest that out-crossing is the norm (Figure 2.9), which means that all foundresses must travel to a find a mate. In *Au1A*, the average distance to a mating aggregation will be even greater because only 12% of colonies produce males at any one time (compared with 56% of *Az. dp2* colonies). Thus, when colony density is low, the distances that must be travelled to mate with an unrelated male are likely to be very large, which perhaps explains why a higher level of inbreeding occurs in this species when colony density is low (Figure 2.9).
3. When saplings are plentiful, *Azteca* foundresses may take advantage of being relatively strong fliers to search for high quality saplings. This is supported by previous results that found the relative abundance of *Azteca* foundresses to be higher in larger saplings (Yu *et al*., 2004). Foundresses are known to use chemical cues to detect hostplants (Edwards *et al*., 2006), but the extent to which these cues convey information about hostplant availability and size is unknown, as is the extent to which visual cues may be used in sapling selection. I consider this hypothesis less likely to be correct because flight is

inherently dangerous for dispersing queens due to the threat of predation (Türke *et al*., 2010), and so choosiness that results in extended flight times is unlikely to be a successful strategy. It is more likely that the observed tendency for *Azteca* foundresses to occupy larger saplings is explained by their large body size (particularly in the *Az. ulei* group) making it impossible for them to colonise the smallest saplings.

4. Finally, *Azteca* foundresses would disperse longer distances than *Allomerus*, on average, if they were less efficient at locating saplings. However, if this were true then we would expect to see that *Azteca* also disperses further than *Allomerus* at low host-plant density sites, which is not the case. Moreover, Edwards *et al*. (2006) found that *Azteca* foundresses are actually *more* responsive to chemical cues from *C. nodosa* saplings than are *Allomerus* foundresses, which is consistent with their ability to locate and colonise isolated saplings.

In summary, the most likely explanations for the longer dispersal distances of *Azteca* are the avoidance of competition with sisters or conspecifics and/or the need to travel to find a mate.

2.5.5 What drives coexistence within Azteca?

The holy grail of coexistence theory is to explain the mechanisms that enable coexistence of multiple closely-related species. Five *C. nodosa*-associated *Azteca* species co-occurred across the study area, and this is the first time that it has been possible to separate the species and to start to consider how they might be able to coexist. Unfortunately, it was not realised at the time of collecting that samples comprised so many species, with the result that each species was under-sampled, and sufficient data for population genetics analysis were obtained only for the two most common species, *Az. dp2* and *Az. u1A*. Therefore, I focus my discussion on these two species.

I began by hypothesising that the same dispersal-fecundity trade-off that maintains coexistence between *Azteca* and *Allomerus* also maintains coexistence between *Az. dp2* and *Az. u1A*. Therefore, I expected to find *Az. u1A*, the larger species, to be both the better disperser and the less fecund of the two species, and to respond more strongly than *Az. dp2* to variation in hostplant density.

As discussed above, population genetics analyses found evidence that *Az. u1A* is indeed the better disperser (Figure 2.12), and *Az. u1A* was also found to be less fecund than *Az. dp2*. However, this difference was driven by differential male production, while female alate production – the key variable in terms of a dispersal-fecundity trade-off (Yu and Wilson, 2001) – did not differ between species. This lack of difference may well be attributable to very small sample sizes, with only two

colonies of each species containing female alates, and so more data are required if firm conclusions are to be drawn regarding the relative female alate production rates of the two *Azteca* species.

However, the relationship between hostplant density and relative abundance was not as expected under the hypothesis of a dispersal-fecundity trade-off. Rather than showing the strongest response to variation in hostplant density, *Az. u1A* showed no significant response at all, while *Az. dp2* followed the pattern previously reported for *Azteca* in showing a strong negative response (Figure 2.6). Taken together with the apparent lack of difference in female alate production, this strongly suggests that coexistence within *Azteca* is maintained by something other than a dispersal-fecundity trade-off and may not be dependent upon variation in hostplant density.

An alternative hypothesis is that *Az. u1A* is limited by being unable to enter domatia of the smallest saplings as a result of its large body size. Thus, the smaller ants (*Allomerus* and the *Az. dp2*) have a colonisation advantage in that they are able to colonise saplings before *Az. u1A*, and if they succeed in colony establishment before the sapling is large enough for *Az. u1A* to enter it, they cannot then be displaced. However, if a smaller species fails to establish a colony before the sapling grows large, then superior fighting ability may give *Az. u1A* an advantage. Yu *et al*. (2004) found that *Az. ulei* foundresses (species pooled) were more successful than those of *Az. dp2* in invading domatia occupied by *Allomerus* foundresses, and that the hierarchy in invasion success correlated with relative head size. It is probable that this hierarchy extends to *Az. u1A* being able to invade domatia occupied by *Az. dp2* foundresses, but this has yet to be tested.

If this hypothesis is correct, then *Az. u1A*'s ability to fly long distances may help it to arrive at large saplings that lack established colonies, which will be relatively rare even where hostplant density is high. It would also be expected that *Az. u1A* foundresses are able to detect large, uncolonised saplings via either chemical or visual cues. The ability to distinguish between large vs. small and colonised vs. uncolonised saplings on the basis of chemical cues could easily be tested using Y-tube experiments, as in Edwards *et al*. (2006).

Considering all five *Azteca* species, my data suggest that there is an ecological split between the *Az.* 'depilis' and *Az. ulei* species groups, with *Az. dp1* showing the same strong response to hostplant density as *Az. dp2*, and *Az. u1B* and *Az. u2* following *Az. u1A* in showing little or no response. There is currently little species-specific life-history data to allow the generation of hypotheses concerning mechanisms for the coexistence of species within each of these very closely related groups, but now that the number of species is known, appropriate sampling can be conducted that would allow such data to be gathered.

2.5.6 Conclusions and further work

Although the ultimate goal of this work – to develop a spatially explicit model of species coexistence – is still some way off, this study represents a key step forward in confirming the dispersal hierarchy and generating some initial quantitative estimates of dispersal. Perhaps more importantly, it demonstrates that indirect genetic techniques can be used to characterise dispersal in hard-to-observe species, thereby enabling the generation and testing of metacommunity hypotheses in natural systems.

While analysis based on isolation by distance is useful for gaining information about relative dispersal scales with minimal sampling effort, it carries a high degree of uncertainty and is difficult to interpret quantitatively in the absence of additional sources of information. For the purposes of building a spatially explicit model (i.e. without artificially dividing the landscape into discrete blocks as in Yu and Wilson, 2001), more accurate and detailed information is required concerning the dispersal kernels of the different species. Robledo-Arnuncio *et al*. (2007) and Charman *et al*. (2010) have shown that it is possible to derive dispersal kernels from the observed distribution of dispersed siblings, and this should be the focus of the next stage of this project. Sibship data needs to be augmented and extended to cover a larger area, particularly for *Azteca*, and the study should be repeated in an area of low hostplant density, in order to detect any landscape-related plasticity. The natural starting point would be to consider *Allomerus* and *Az. dp2*, since these are the species that seem most likely to coexist via the originally-hypothesised dispersal-fecundity trade-off, and for which the most data are available. This study has highlighted possible behavioural differences in dispersal and sapling selection strategy among ant species, and this is something that should be investigated further and considered in any ecological model of coexistence.

Finally, there remain open questions concerning (1) the ability of *Azteca* foundresses to differentiate between large vs. small and colonised vs. uncolonised saplings, (2) the role of mating behaviour in determining dispersal characteristics, (3) the relative contribution of male and female dispersal to observed spatial genetic structure, and (4) how *Az. dp1*, *Az. u1B*, and *Az. u2* compare ecologically with the more common *Azteca* species considered in this study.

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Chapter 3: Introduction to DNA metabarcoding

3.1 Biodiversity in the 21st Century

Since the RIO Convention for Biological Diversity in 1992, and as recognition of our own dependence on functioning ecosystems has grown (Cardinale *et al*., 2012), biodiversity research has ceased to be exclusively the realm of hobbiests and naturalists. Globally, billions of dollars are now spent annually on biodiversity conservation (Waldron *et al*., 2013), and targets for slowing or reversing biodiversity declines are set at political levels ranging from local to international (Mace *et al*., 2010). Our capacity to prioritise and make effective use of available funding to meet these targets is reliant on our ability to detect changes in biodiversity in terms of both the negative impacts of environmental change or ecosystem degradation and the positive impacts of conservation and management interventions (Niemelä, 2000; Kim and Byrne, 2006; Pereira and Cooper, 2006; Magurran *et al*., 2010).

3.2 The taxonomic impediment

Two recent reports illustrate the extent of current limitations in our ability to detect biodiversity changes. Released earlier this year, the State of Nature Report (Burns *et al*., 2013) was able to publish quantitative trends for only 5% of terrestrial and freshwater animal and plant species in England, even though British flora and fauna are generally considered to be among the best-known worldwide (Shaw and Hochberg, 2001; Price *et al*., 2011; Tyler *et al*., 2012). Invertebrate fauna represented the greatest knowledge gap, and the report states that one of its strongest messages is "*the need to know more about how nature is faring in England*" (Burns *et al*., 2013). A similar sentiment was expressed by authors of the Terrestrial Biodiversity Climate Change Impacts Report Card, also published this year, which included as one of its main evidence gaps the lack of monitoring of invertebrate groups, and emphasised "*the importance of maintaining good surveillance and monitoring to allow the detection and quantification of change*" (Biodiversity Report Card, 2013).

The knowledge gaps highlighted by these two reports are largely the result of the 'taxonomic impediment'. That is, identification of specimens based on morphology is too time-consuming and demanding of taxonomic expertise – which is in notoriously short supply (Kim and Byrne, 2006) – for the measurement of total animal and plant biodiversity to be practical at large spatial and

temporal scales, and this limits the potential for large-scale biodiversity assessment and the detection of trends (Weeks and Gaston, 1997; Kim and Byrne, 2006; Valentini *et al*., 2009). Invertebrate groups tend to be among the most difficult to identify morphologically due to their usually small body size, high diversity, and the fact that they often lack readily-observed distinguishing morphological characters (Samways, 1993). Furthermore, many invertebrates spend the majority of their lifecycles in immature life stages, and it is often impossible even for taxonomic experts to identify pre-adult specimens to species level (Smith, 1989; Packer *et al*., 2009).

In the absence of direct biodiversity data, conservation science has historically relied on either intuition (Sutherland *et al*., 2004; Ferraro and Pattanayak, 2006; Maron *et al*., 2012) or easily-measured but usually poorly-validated biodiversity surrogates (Lindenmayer and Likens, 2011) to guide conservation policy and investment. However, we are now in a situation where funding is finite and, to date, our efforts have been unsuccessful in slowing the rate of biodiversity loss (Rands *et al*., 2010; CBD, 2011). There is a pressing need to provide strong evidence bases for decision making (Ferraro and Pattanayak, 2006; Pullin and Knight, 2011), which requires data on a scale that cannot be met using morphological identification of taxa (Kim and Byrnes, 2006). Therefore, new approaches to measuring biodiversity need urgently to be developed, validated, and adopted for use. In particular, these must to be able to overcome the taxonomic impediment for invertebrates, which represent the majority of animal diversity. Moreover, invertebrates include groups on which we rely heavily for ecosystem services such as pollination, pest control, and soil and water quality, as well as taxa that have substantial economic impact as agricultural pests and disease vectors (Kremen *et al*., 1993; Samways, 1993; Shaw and Hochberg, 2001; Cardoso *et al*., 2011).

3.3 DNA-based taxonomy

3.3.1 DNA barcoding

DNA barcoding, first proposed a decade ago by Hebert *et al*. (2003), was an important step in developing new methods for rapid biodiversity assessment. The barcoding approach identifies species by using a standardised region of DNA in conjunction with digital reference databases. For animals, the barcode region is a 658 bp region of the mitochondrial COI gene, while two loci – MatK and TrnL – have been adopted as standard barcodes for plants (Hollingsworth *et al*., 2009). Markers are selected on the basis of having low variation within species but high variation

between species, which allows for unambiguous identification (Hebert *et al*., 2003). Crucially, this method overcomes the taxonomic impediment by moving away from morphology-based identification, meaning that specimens can be identified at all life stages and regardless of convergent morphology.

Despite facing initial scepticism (e.g. Will and Rubinoff, 2004; Ebach and Holdrege, 2005; Prendini, 2005; Will *et al*., 2005), DNA barcoding has gained traction and proved to be useful for a wide variety of applications from resolving cryptic species complexes (e.g. Hebert *et al*., 2004) to identifying agricultural pests (Lefort *et al*., 2012) and detecting labelling fraud in commercially available food (Di Pinto *et al*., 2013) and herbal medicine (Newmaster *et al*., 2013) products. However, traditional barcoding relies on Sanger sequencing, whereby each specimen is sequenced in a separate reaction, and this means that it remains prohibitively time consuming and expensive for the purposes of generating large-scale biodiversity data.

3.3.2 DNA metabarcoding

In recent years, this issue has been solved by the widespread availability and decreasing cost of high-throughput or 'next generation' sequencing (NGS), which allows barcoding to be scaled up by sequencing many specimens/species in a single reaction (Valentini *et al*., 2009). Originally developed for the study of diversity and function in microorganisms (e.g. Venter *et al*., 2004), which frequently defy morphological examination, this approach has become known as DNA 'metabarcoding' when applied to multicellular organisms (Pompanon *et al*., 2011; Taberlet *et al*., 2012a). Denoising NGS data, clustering sequences into groups that represent 'species' (molecular operational taxonomic units, or OTUs), and assigning taxonomy to those OTUs is bioinformatically challenging, but many methods have been developed and analysis pipelines compiled that make it possible for non-bioinformatics-experts to process NGS data for ecological research (Valentini *et al*., 2009; Coissac *et al*., 2012; Yu *et al*., 2012).

The most linear extension of traditional barcoding to metabarcoding is to progress from extraction and sequencing of DNA from individual organisms to bulk extraction and parallel sequencing of DNA from unsorted mixtures of organisms (e.g. a Malaise trap sample). Several studies have demonstrated that the majority of species in a sample can be recovered using metabarcoding, although usually with some drop-out and less than 100% of OTUs identified to species level (Hajibabaei *et al*., 2011; Yu *et al*., 2012; Carew *et al*., 2013). Nevertheless, using artificial arthropod communities of known composition, Yu *et al*. (2012) demonstrated that patterns of species richness (alpha diversity) and species composition (beta diversity) are recovered accurately, and Ji *et al*. (2013) found the same result for natural communities when

comparing metabarcode data with standard (i.e. based on traditional morphology) biodiversity data derived from the same samples. The latter study also demonstrated that metabarcode and traditional datasets would yield the same management and conservation decisions.

A second strand of metabarcoding identifies species based on environmental DNA, or *e*DNA (Ficetola *et al*., 2008; Taberlet *et al*., 2012b; Yoccoz, 2012; Epp *et al*., 2012). This uses short (< 300 bp) barcode regions to sequence degraded DNA that has been deposited in the environment via processes such as decomposition, defecation, urination, mucous secretion, salivation, and digestion. The use of *e*DNA in water samples has been well-validated as a method for describing communities of aquatic fauna (Ficetola *et al*., 2008; Thompsen *et al*., 2012a; 2012b; Minamoto *et al*., 2012; Pilliod *et al*., 2013), including for early detection of invasive species (Jerde *et al*., 2011; Dejean *et al*., 2012). Likewise, Yoccoz *et al*. (2012) showed that communities of plants can be reliably described from *e*DNA in soil (Yoccoz *et al*., 2012).

Finally, a third strand of metabarcoding identifies vertebrates from blood meals of invertebrates that feed on them, including leeches (Schnell *et al*., 2012), mosquitoes (Kent and Norris, 2005; Barrera *et al*., 2012), carrion flies (Calvignac-Spencer *et al*., 2013), horseflies (Rovie-Ryan *et al*., 2013), and ticks (Gariepy *et al*., 2012). This approach is currently being used by the World Wildlife Fund (WWF) to detect the rare saola in the rainforests of Vietnam and Laos (Saola Working Group, 2013).

Thus, methods that enable biodiversity data to be gathered at large spatial and temporal scales across a wide range of taxonomic groups have been developed and validated, and the next step is to encourage their adoption for informing management and policy decisions.

3.4 Scope of the present study

The principal aim of the next chapters in this thesis is to demonstrate the type of data that can be obtained – and the types of management questions that can be addressed – using a meta-barcoding approach. The three studies were designed to address three of the most pressing environmental management issues in the UK. The first considers how metabarcoding could be used for informing the sustainable management of plantation forests, the second focusses on environmental management in agricultural ecosystems, and the third demonstrates how metabarcoding can be used to track the success of habitat restoration, which is set to become an increasingly important issue as biodiversity offsetting becomes incorporated into government policy (DEFRA, 2013).

A common theme is the potential for large scale biodiversity data to enable adaptive management, which represents the interface between science and policy, integrating "*design, management and monitoring to systematically test assumptions in order to adapt and learn*" (Salafsky *et al*., 2002). Adaptive management accepts that the optimal management strategy is uncertain at the outset, but seeks to reduce uncertainty by treating the strategy as a scientific hypothesis and testing replicated implementations against controls (no management) to judge effectiveness in meeting objectives (Lee, 1999; Keith *et al*., 2011). Based on what is learned, the strategy can then be adapted to improve the likelihood of a successful outcome. This is in contrast with the more pervasive 'trial and error' approach to management (Duncan and Wintle, 2008), whereby a strategy is persisted with until the point when it is found conclusively to have failed. Despite widespread acceptance that adaptive management is the most logical approach to biodiversity conservation, there are few examples of it having been applied in practice, and one of the main obstacles is the difficulty of directly measuring biodiversity responses to management interventions (Keith *et al*., 2011). Metabarcoding should prove to be a useful tool in overcoming this obstacle.

A second theme is the testing of structural and compositional indicators. Biodiversity indicators are appealing because they can be measured quickly and inexpensively, yet the relationships between indicators and other elements of biodiversity tend to be based on intuition rather than on quantitative data (Simberloff, 1999; Lindenmayer and Likens, 2011). Since monitoring indicators is liable to misinform management if the assumed relationship between surrogate and target does not hold, (Saetersdal and Gjerde, 2011; Keith *et al*., 2011), there have been repeated calls for the usefulness of indicators to be validated (e.g. Simberloff, 1999; Bockstaller and Girardin, 2003; Duelli and Obrist, 2003; Lindenmayer and Likens, 2009; Cushman *et al*., 2009; Nicholson *et al*., 2013). However, these calls have so far gone largely unanswered because of the considerable data requirements of demonstrating robust surrogacy relationships (Duelli and Obrist, 2003; Lindenmayer and Likens, 2011). Metabarcoding has the potential to provide the necessary data for testing indicators so that those that are meaningful can be employed with greater confidence, and those that are not meaningful can be avoided.

These three studies should be considered pilot studies; their aim is not to make recommendations about how best to manage ecosystems for biodiversity, but rather to demonstrate the potential of the metabarcoding approach for informing such management decisions in the future. Thus, sampling effort is minimal, particularly in the agricultural ecosystem (Chapter 6), and sequencing coverage is generally lower than would be used for a full study on which management decisions would be based.

3.5 Choice of sampling and sequencing methodologies

3.5.1 Sampling

I focus on trap-sampled arthropods for several reasons: first, the Arthropoda represents a major portion of biodiversity, much of which is habitually overlooked in biodiversity assessments (Kremen, 1993; Shaw and Hochberg, 2001; Cardoso *et al*., 2011); second, it contains taxa that occupy a wide variety of functional roles and that are of significance in terms of both ecosystem services and economic impacts (Cardoso *et al*., 2011); and third, methodologies for metabarcoding arthropod samples have been well-validated, including in natural systems (Hajibabaei *et al*, 2011; Yu *et al*., 2012; Ji *et al*., 2013).

3.5.2 Sequencing

I focus primarily on the COI barcode region because an extensive body of research supports its ability to discriminate among arthropod species (e.g. Hebert *et al*., 2003; 2010; Hogg and Hebert, 2004; Ball *et al*., 2005; Barrett and Hebert, 2005; Packer *et al*., 2009), and it is the marker for which there exists the most comprehensive reference database, with sequences for 138,698 formally-described animal species currently held in the Barcode of Life Database (http://www.boldsystems.org, Ratnasingham and Hebert, 2007). However, I use the 18S region of the small subunit rRNA for metabarcoding soil arthropods in Chapter 6 because COI data from soil samples has been shown to be heavily dominated by bacterial OTUs (Yang *et al*., 2013). In Chapter 7, I compare the performance of COI and 18S in recovering patterns of diversity in Malaise-trap-sampled arthropods.

Several different NGS platforms are available, each of which carries its own advantages and disadvantages (summarised in Shokralla *et al*., 2012). I chose to use the Roche GS FLX+ platform (Roche Diagnostics Corp. Branford, CT), commonly known as '454' sequencing, because at the time of processing it was the only platform that could sequence the entire COI barcode region (658 bp). The main disadvantage of '454' sequencing is that its pyrosequencing technology is liable to generate homopolymer errors (for instance, AAA can be read as AA or AAAA). However, since COI is a protein coding gene, the programme MACSE (Ranwez *et al*., 2011) can be used to detect and remove such errors based on the presence of frameshift mutations, which limits the impact on data quality. Full details of laboratory and bioinformatics methods are given in Chapter 4.

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Chapter 4: Laboratory and bioinformatics protocols for COI and 18S metabarcoding

4.1 Using the mitochondrial COI barcode region

4.1.1 Laboratory steps

Samples were stored in 100% ethanol from the time of collection until DNA extraction. In all cases, laboratory work (DNA extraction to sequencing) was carried out by collaborators Y. Ji and C. Yang at the core laboratory of the State Key Laboratory of Genetic Resources and Evolution at the Kunming Institute of Zoology (KIZ), Kunming, China.

DNA extraction

Prior to DNA extraction, the bodies of all insects larger than a honey bee were removed from the sample with just a leg retained for each specimen. The purpose of this was to limit the cost of reagents during the DNA extraction step. Each trap sample was homogenised using the FastPrep-24 sample preparation system (MP Biomedicals, Santa Ana, CA, USA). Samples were processed in 50 ml lysing tubes with sterile 0.25-inch spherical ceramic beads, and homogenisation was carried out at 5 m/s for 1 minute at room temperature.

Genomic DNA was extracted from each homogenised sample using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, and DNA quality and quantity was checked using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). PCR amplification of a 658 bp region of the CO1 barcode gene was carried out using the degenerate primers *Fol-degen-for* 5'-TCNACNAAYCAYAARRAYATYGG-3' and *Fol-degen-rev* 5'-TANACYTCNGGRTGNCCRAARAAYCA-3' (Yu *et al*., 2012). These are based on Folmer *et al*.'s (1994) 'universal' primers and were created from an alignment of all 215 complete mitochondrial DNA sequences for Insecta that could be accessed via GenBank (see supplementary information in Yu *et al*., 2012 for further details). They have been demonstrated to successfully amplify a wide range of arthropod taxa, although amplification of Hymenoptera can be problematic (Yu *et al*., 2012). For each sample, a unique 10 bp multiplex identifier (MID) tag was added to the forward primer with the standard Roche Adaptor A. MIDs consisted of 10 bp nucleotide sequences and differed from one another by a minimum of 4 bp. Their use plays an important role in reducing cost by allowing multiple samples to be combined for sequencing with retrospective assignment of sequences to samples based on the MID sequence.

PCR amplification and 454 sequencing

PCR was carried out in 20 µl reaction volumes containing 2 µl of 10 x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.4 µM of each primer, 0.6 U HotStart Taq DNA polymerase (non-proofreading; TaKaRa Biosystems), and approximately 60 ng of genomic DNA. Each sample was amplified in three independent reactions, which were subsequently pooled. A touchdown thermocycling profile was used, which consisted of 2 minutes at 95 ^{o}C, 11 cycles of 15 seconds at 95 ^{o}C, 30 seconds at 51 ^{o}C (annealing), and 3 minutes at 72 ^{o}C (extension), decreasing the annealing temperature by 1 degree every cycle; followed by 17 cycles of 15 seconds at 95 ^{o}C, 30 seconds at 41 ^{o}C, and 3 minutes at 72 ^{o}C, with a final extension phase of 10 minutes at 72 ^{o}C. The use of non-proofreading Taq with fewer, longer cycles has the effect of reducing the frequency of chimeras (Lenz and Becker, 2008). PCR products were gel purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and quantified using the Quant-iT PicoGreen dsDNA Assay kit (Invitrogen), before being pooled in preparation for sequencing. Pooled samples were sequenced unidirectionally from the A amplicon end on a Roche GS FLX pyrosequencer at the KIZ.

In most cases, several separate pools were created, each comprising a subset of the MIDs, and each sequenced on a separate region of the sequencing plate. Using multiple pools has the advantage of allowing individual MID sequences to be re-used, which reduces overall cost. However, this is balanced against the disadvantage of losing space on the sequencing plate by subdividing it. For each dataset, the number of regions used was determined in part by the requirements of other datasets using space on the plate, and partly as a function of the number of MIDs.

4.1.2 Bioinformatic processing

Quality control

I used an updated version of the experimentally validated pipeline described in Yu *et al*. (2012) to denoise and cluster the reads into Operational Taxonomic Units (OTUs). Quality control was performed using QIIME version 1.6.0 (Caporaso *et al*., 2010b). First, the command *split_libraries.py* was used to strip the primer and MID sequences from the raw sequence output, and replace them with an appropriate sample descriptor added to the information line for each sequence. Simultaneously, the lowest quality reads were removed from the dataset. These included sequences that were less than 100 bp (below which sequences become taxonomically uninformative; Meusnier *et al*., 2008) or greater than 700 bp in length, contained a run of more than 9 homopolymers, or featured more than 2 mismatches in the primer sequence. Where multiple 454 regions had been used, the output files for all regions were concatenated to form a

single fasta file. Next, sequences were aligned against a dataset of high-quality Arthropoda sequences, using the QIIME command *align_seqs.py* to implement the alignment tool PyNAST (Caporaso *et al*., 2010a). The minimum requirement for percent sequence identity was set to 60%, and sequences that failed to meet this requirement were removed from the dataset.

The program MACSE (Ranwez *et al*., 2011) was used to detect and remove homopolymer errors. This program takes advantage of the fact that CO1 is a coding gene, and uses the presence of stop codons to infer frameshift mutations caused by homopolymer errors. This is achieved by aligning candidate sequences at the amino acid level against a high-quality reference dataset, which here consisted of a subset of the larger Arthropoda reference dataset mentioned above (66 70%-similarity sequences). Since this step is computationally intensive, sequences were divided into 40 subsets, which were run in parallel on the High Performance Computing Cluster (HPCC) at the University of East Anglia. Following the MACSE run, a Perl script (written for the purpose by X. Wang at the KIZ) was used to remove the homopolymer insertion errors identified by MACSE, strip the 66 reference sequences from each of the 40 output files, and combine sequences back into a single fasta file.

The final step in quality control was to use algorithms implemented in USEARCH (Edgar, 2010) and UCHIME (Edgar *et al*., 2011), via QIIME command *pick_otus.py*, to detect and remove chimeras and cluster highly similar sequences. Both reference-based and *de novo* chimera detection was performed in UCHIME, using the full high-quality Arthropoda dataset described above as reference for the former. Non-chimeric sequences were subsequently clustered at 99% similarity using the USEARCH algorithm, and a new fasta file was created, comprising the longest sequence from each cluster (QIIME command *pick_rep_set.py*). This step could be performed prior to the MACSE step to increase the speed of homopolymer detection in very large datasets, since clustering reduces the number of sequences to be processed.

OTU-picking

The next step was to cluster sequences into OTUs that as far as possible represented true species. For this, I used the Bayesian clustering program CROP (v.1.33; Hao *et al*., 2011). The method implemented in CROP improves on hierarchical clustering methods by moving away from reliance on a strict dissimilarity threshold. Instead, a probabilistic model is used to identify clusters in which > 95% of sequences differ by less than a user-specified percentage (here 2%) from a central 'seed' sequence. Finding the seed sequences that minimize cluster number is computationally intensive, requiring the use of 12 parallel CPU cores on the HPCC. Parameters were chosen following the authors' recommendations: number of blocks to be used in the first round of

clustering was set to 1/50 of the number of input sequences, and block size was set to 150,000/average sequence length.

Following the CROP run, the OTU map output, which recorded all CROP input sequences belonging to each cluster, was merged with the OTU map from the initial USEARCH clustering step (QIIME command *merge_otu_maps.py*) so that all post-quality-control sequences were linked to OTU clusters. An OTU table was then created (QIIME command *make_otu_table.py*), which gave the number of sequences assigned to each OTU in each MID sample

Taxonomic assignment

The final bioinformatics step was to add taxonomic information to OTUs by comparing sequences against a reference database. For this, the Barcoder method in the program SAP (Munch *et al*., 2008) was used, which uses a Bayesian Markov chain Monte Carlo (MCMC) analysis of phylogeny to calculate the posterior probability that a sequence belongs to a specific taxonomic group. This is achieved by sampling a large number of phylogenetic trees based on a set of relevant homologues compiled using NetBlast searches against GenBank. The posterior probability that an environmental sequence belongs to a given taxon is calculated as the fraction of trees in which the sequence forms a monophyletic group with that taxon. I used a minimum identity of 70%, which allows sequences to be assigned to higher taxonomic levels if there is no match to a species in the database. This step is also computationally demanding, with the average sequence taking around 30 minutes to process, and so sequences were split into 30 groups which were run in parallel on the HPCC. Some sequences caused SAP to crash. When this happened, the problem sequence was removed from the input file and a manual BLAST search was performed instead to assign the OTU to a taxonomic group.

Taxonomic information for each OTU was added to the OTU table, which could then be filtered by taxonomic group in preparation for analysis (QIIME command *filter_otus_from_otu_table.py*). As a check, I first filtered for chordates, which were usually represented in each dataset by a small number of OTUs, probably as a result of having sampled insects that had fed on them. Since the distribution of vertebrates is well known, it was possible to check that the species identified in the samples would be expected to occur in the sampling region. Detection of unexpected vertebrate species could indicate that sample contamination had occurred. Next, I filtered for Arthropoda to create the main dataset that would be used for diversity analyses, and finally for lower groups, including insect orders and Arachnida. For the arthropod and order-level datasets, all OTUs that were represented by just one sequence (single-read OTUs) were excluded.

4.2 Using the 18S rRNA barcode region

4.2.1 Laboratory steps

DNA extraction was carried out as described above for the COI barcode region.

PCR amplification and 454 sequencing

An approximately 830 bp region of the small subunit (SSU) 18S rRNA gene was amplified using the forward primer 18S1 1b (5'-GTCAGAGGTTCGAAGGCG-3'), which is specific to the Metazoa, and the 18S2a reverse primer (5'-GATCCTTCCGCAGGTTCACC-3'), which amplifies universally within the Eukaryota (Hamilton *et al*., 2009; Wu *et al*., 2011; Yang *et al*., 2013). Like for COI, a unique 10 bp MID tag for each sample was added to the forward primer with the standard Roche Adaptor A, which allowed samples to be multiplexed for sequencing. PCR amplifications were carried out in 10 µl reaction volumes containing 0.8 µl dNTP mixture (1.25 mM L^{-1} each base), 6.05 µl distilled water, 0.05 µl Taq DNA polymerase (non-proofreading; TaKaRa Biosystems, Dalian, China), 1.0 µl 10x PCR buffer (100 mmol L^{-1} Tris-HCl (pH 8.3), 500mM L^{-1} KCl, 15 mM L^{-1} $MgCl_2$), 0.2 µl each primer (20 µM L^{-1}), 0.5 µl DMSO, 0.2 µl BSA and 1.0 µl DNA template. Thermal cycling consisted of an initial denaturation phase of 2 minutes at 95 ^{o}C, followed by 30 cycles of 15 seconds at 95 ^{o}C (denaturation), 30 seconds at 57 ^{o}C (annealing) and 3 minutes at 72 ^{o}C (extension), with a final extension phase of 10 minutes at 72 ^{o}C. As for COI, each sample was amplified in three independent reactions that were subsequently pooled and sequenced unidirectionally from the A amplicon end on a Roche GS FLX at the KIZ.

4.2.2 Bioinformatic processing

Quality control

Sequences were processed largely in the QIIME environment, following the 'USEARCH/CROP' pipeline described in Yang *et al*. (2013). Primer and MID sequences were stripped from the raw sequences using the *split_libraries.py* command, replacing MID sequences with the appropriate sample descriptor in the information line. Simultaneously, as in the COI pipeline, reads that were less than 100 bp or greater than 700 bp in length were removed from the dataset, as were those with more than two mismatches in the primer sequence. However, whereas runs of up to nine homopolymers were retained in the COI pipeline, the cut-off for 18S was lower, at six. This was because the program MACSE, which detects homopolymer errors in coding genes based on the presence of frameshift mutations, could not be used for 18S because it is not a coding gene. The next step was to use USEARCH and UCHIME, via the QIIME command *pick_otus.py,* to cluster

sequences at 99% similarity, and to perform reference-based and *de novo* chimera detection. The QIIME-compatible Silva SSU rDNA alignment release 108 was used for reference-based chimera detection. This was downloaded from http://www.arbsilva.de/download/archive/qiime/ and is referred to hereafter as the 'Silva 108' reference database. OTU-picking was carried out using Bayesian clustering in CROP 1.33, as in the COI pipeline. After clustering in CROP, the longest read from each OTU was chosen as the representative sequence to be used for taxonomic assignment.

Taxonomy was assigned by BLASTing at a stringency of 1×10^{-3} against the Silva 108 database, via QIIME command *assign_taxonomy.py*. This method is much less computationally intensive than is SAP, which was used for assigning taxonomy to COI OTUs, but, unlike SAP, it does not return posterior probabilities of identity at each taxonomic level. Therefore, Yang *et al*. (2013) suggest that low confidence should be attributed to taxonomic assignment below the ordinal level. At ordinal level and above, high confidence can be given to assignments on the basis that posterior probabilities are generally high in SAP, and a high level of agreement between the two methods has been documented (Yang *et al*., 2013).

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Chapter 5: Metabarcoding as a tool for informing sustainable forest management

5.1 Summary

European forests are required to be managed sustainably, which includes consideration of biodiversity. However, management measures aimed at enhancing biodiversity are based almost entirely on intuition and best guesses, rather than on a solid evidence base in the form of comprehensive biodiversity data. In this chapter, I use COI metabarcoding of Malaise trap arthropod samples from fifteen plantation stands in Thetford forest, UK, to test some common assumptions about the management of temperate plantation forests for biodiversity.

Ordinations showed fine-scale separation of samples by both crop type and sampling week, emphasising the importance of controlling for sampling date when designing this type of experiment. Surprisingly, no evidence was found to support the assumption that a two-species forest crop (oak and Scots pine) supports more species than does a pure oak crop. Pure pine sites had lower species richness than either pure oak or mixed crop sites, but they contained many specialists. This suggests that replacing all pure pine and oak stands with a uniform mixture of pine and oak would result in decreased regional biodiversity through the loss of pine specialists. No commonly-used structural indicator of biodiversity was found to have a robust relationship with species richness at the scale of this study, but species *composition* could be predicted from a combination of three indicators – broadleaf/conifer ratio, tree species diversity, and plantation crop density. Species composition patterns were strongly correlated with those derived from a standard dataset (i.e. morphological identifications) based on pitfall trap collections of carabid beetles and spiders from the same fifteen sites. This both validates the metabarcode dataset and suggests that certain broad compositional indicators may be useful, at least at some spatial scales.

This study supports the hypothesis that "*management for diversity requires diversity of management*" (Lindenmayer *et al*., 2006). That is, to cater for the varied and complex requirements of species, the key is to maximise the *variation* in habitat variables at the landscape level, as opposed to uniformly attempting to maximise the *value* of variables that may be associated with high species richness.

5.2 Introduction

5.2.1 Sustainable forest management

UK plantation forests

Although deforestation is recognised as posing a major threat to biodiversity and ecosystem services worldwide (Millennium Ecosystem Assessment, 2005), the area of forest cover in Europe is actually increasing (Forest Europe *et al.*, 2011). In the UK, following millennia of deforestation, the 20th century saw intensive reforestation and afforestation, motivated principally by a need to increase timber production (Malcolm *et al*., 2001). Although this substantially increased the amount of forest cover (Rollinson, 2003), it has led to a situation where three quarters of the UK's forested area now consists of plantations. This is unusual in Europe, where plantations account for only 4% of overall forest cover (Forest Europe *et al*., 2011). Thus, the UK faces unique forest management issues, and a history of management for timber production has resulted in dominant forest characteristics that are considered to be poor for native biodiversity (Malcolm *et al*., 2001; Forestry Commission, 2011). Key characteristics include:

1. Over 50% of plantations consist of monocultures (Forest Europe *et al*., 2011), which tend to be homogeneous in age and size structure over large areas.
2. Management practices have traditionally included the clearance of lying and standing deadwood, veteran trees, and naturally regenerating saplings (Malcolm *et al*., 2001).
3. Many plantations are dominated by non-native tree species such as Sitka spruce (*Picea sitchensis*), which alone accounts for 29% of UK forest cover (Forest Europe *et al*., 2011).

In recent decades, it has become recognised that forest management needs to balance the profitability of forest products against negative impacts on biodiversity (Noss, 1990; Kangas and Kuusipalo, 1993; Kerr, 1999; Malcolm *et al*., 2001). It is also now widely believed that, with appropriate management, even plantations can play an important role in protecting and enhancing native biodiversity, so long as they do not completely replace natural ecosystems (Hartley, 2002; Quine and Humphrey, 2003; Brockerhoff *et al*., 2008; Gardner, 2012). A major international mandate was provided by the Convention on Biological Diversity (CBD) in 1992, which was the first treaty to provide a legal framework for biodiversity conservation. Key objectives of the CBD include (1) maintenance of natural ecological processes in managed forest, (2) mitigation of the impacts of threatening processes – such as climate change, invasive species, and pollution – on forest biodiversity, and (3) protection and enhancement of forest biodiversity through the conservation of habitats and priority species (Forestry Commission, 2011).

In response to the requirements of the CBD, European guidelines produced by the Ministerial Conference on the Protection of Forests in Europe (MCPFE) defined sustainable forest management as:

> "*the stewardship and use of forests and forest lands in a way, and at a rate, that maintains their biodiversity, productivity, regeneration capacity, vitality and their potential to fulfil, now and in the future, relevant ecological, economic and social functions, at local, national, and global levels, and that does not cause damage to other ecosystems*." (MCPFE, 1993)

5.2.2 Current methods of forest biodiversity monitoring in the UK

With biodiversity included as a key component of sustainable forest management, there is a clear need for large scale biodiversity monitoring if sustainable management methods are to be designed, tested, and tracked (McElhinny *et al*., 2005). However, to fully census animal and plant biodiversity at any but the smallest scales has been a prohibitively difficult task because of the taxonomic impediment: the majority of animal diversity in forests exists within poorly known invertebrate groups, taxonomic capacity in Europe is in short supply, and the process of identifying very large numbers of specimens is therefore slow. Thus, monitoring has tended to focus on (1) individual species that are considered at risk of extinction, and (2) surrogate measures of biodiversity, or indicators. Indicators are forest attributes that can be measured quickly and cheaply by forest managers and that convey information about the wider state of the biological community (Noss, 1999; Marchetti, 2004; Coote *et al*., 2013). 'Compositional indicators' refer to key taxa that are considered representative of a broader segment of biodiversity; 'structural indicators' comprise aspects of the forest habitat that are thought to affect – and therefore predict – biodiversity (Smith *et al*., 2008).

Threatened species

The EU Habitats Directive requires any species or habitat that is rare or endangered at the European level (European Protected Species (EPS)) to be maintained at – or restored to – a favourable level wherever it occurs. EPSs are therefore subject to individual conservation measures and population monitoring and include woodland species such as the common dormouse (*Muscardinus avellinarius*), the smooth snake (*Coronella austriaca*), and all species of bat (Chiroptera) (Forestry Commission, 2011). In addition, many woodland species receive protection under the framework of the UK Biodiversity Action Plan (BAP), produced first in 1994 and updated in 2007, which was the UK government's response to the requirements of the CBD. Under the BAP, priority species and habitats in the UK were identified for conservation based on their national threat status and rate of decline. Around 450 priority species and nine priority

habitats occur in UK forests and woodlands and are subject to targeted conservation measures (Forestry Commission, 2011). There is a widely held assumption that conservation measures implemented for the protection of one threatened species will confer general biodiversity benefits; that is, threatened species are considered to act as umbrella species (Simberloff, 1998).

Compositional indicators

As in other ecosystems, birds are the most widely used compositional indicator of forest quality, largely due to ease of monitoring and the wealth of available population data. The Forestry Commission (2002) follows multiple authors (Furness and Greenwood, 1993; Custance, 2002; Gregory *et al*., 2005; 2008) in describing birds as 'good indicators of the broad state of wildlife and the countryside' because they use a wide range of habitats and occupy key positions in the food chain. Butterflies and bats are also considered useful indicators of forest condition in young and mature forest respectively (Forestry Commission, 2002). Indices of abundance exist for birds (Gregory *et al*., 2008) and butterflies (Liley *et al*., 2004), while bats are monitored as EPSs. Carabid beetles (Coleoptera: Carabidae) and hoverflies (Diptera: Syrphidae) are commonly cited as being potentially informative indicators of invertebrate diversity (Ferris and Humphrey, 1999; Humphrey *et al*., 1999; Pawson *et al*., 2011; Koivula, 2011), but large-scale monitoring of these groups does not currently take place.

Structural indicators

Certain forest characteristics are generally considered beneficial for biodiversity and are measured as indicators of sustainable forest management (Ferris and Humphrey, 1999; Winter *et al*., 2008; Forest Europe *et al*., 2011). Structural indicators are closely linked to management strategies, and they generally include characteristics that broadly promote the operation of natural ecological processes in managed forests and align them more closely with natural forests (Humphrey, 2005). Many studies have proposed lists of structural biodiversity indicators for temperate forests (e.g. Noss, 1999; Ferris and Humphrey, 1999; Hartley, 2002; Angelstam and Dönz-Breuss, 2004; Lindenmayer *et al*., 2000, 2006; Smith *et al.,* 2008), and common themes include horizontal and vertical structural complexity, tree age structure, the quantity and quality of lying and standing deadwood, levels of natural regeneration, the prevalence of native tree species, and tree species diversity. Other proposed indicators track the heterogeneity and connectivity of forest stands at a landscape level. Winter *et al*. (2008) considered the potential for standardising the use of structural indicators at a European level and identified sixteen key variables based on the indicators mentioned above that are currently measured to some degree by the majority of European countries in their National Forest Inventory (NFI) surveys. However,

to date, there is no formal European standardisation beyond the very general guidelines published by the MCPFE (Forest Europe, 2002; Table 5.1). The UK NFI currently records data pertaining to the majority of proposed indicator variables, but not always in great detail. For instance, stumps are assigned to broad diameter size classes, and lying deadwood is recorded only by its diameter at the point where it intersects a transect line (45 m of transect in each 1 ha plot). These data do not allow precise quantification of deadwood volume.

Table 5.1: Structural indicators featured in the Forest Europe (2002) guidelines.

Structural indicator	Description as given in Forest Europe (2002)
4.1 - Tree species composition	Area of forest and other wooded land, classified by number of tree species occurring and by forest type
4.2 – Regeneration	Area of regeneration within even-aged stands and uneven-aged stands, classified by regeneration type
4.3 – Naturalness	Area of forest and other wooded land, classified by "undisturbed by man", by "semi-natural" or by "plantation", each by forest type
4.4 - Introduced tree species	Area of forest and other wooded land dominated by introduced tree species
4.5 – Deadwood	Volume of standing deadwood and of lying deadwood on forest and other wooded land, classified by forest type
4.7 - Landscape pattern	Landscape-level spatial pattern of forest cover

5.2.3 How well do current indicators represent general forest biodiversity?

The usefulness of all current approaches is limited by lack of data.

Threatened species

While there is little doubt that it is desirable to protect species that are at risk of extinction, the absence of biodiversity data across all taxonomic groups means that the threat status of many species, particularly invertebrates, is simply not known (Kapos and Iremonger, 1998; Mossman *et al*., 2013). Therefore, there exists substantial taxonomic bias in conservation priorities, with efforts concentrated on vertebrate diversity, while large numbers of invertebrate extinctions almost certainly occur unnoticed (Possingham *et al*., 2002; Dunn, 2005; Cardoso *et al*., 2011a; Cardoso, 2012). A striking illustration of this is observed in the IUCN Red List data from 2010: of an estimated > 60,000 species of arachnid, only 33 have been evaluated for IUCN Red List criteria, and 18 of these are listed as Critically Endangered, Endangered, or Vulnerable. In contrast, 100% of mammals and birds have been evaluated (IUCN, 2010).

Because data are so sparse, changes on threatened species lists often occur as the result of changes in knowledge, rather than changes in actual threat status (Burgman, 2002; Quayle and Ramsay, 2005), and this limits the usefulness of such lists for monitoring progress towards conservation goals. Moreover, the list of species covered by the EU Habitats Directive is criticised by Cardoso (2012) for showing strong biases in terms of the geography, body size, appearance, and range size of species, in addition to taxonomic bias. More generally, the threatened-species approach to biodiversity conservation results in a somewhat tunnel-vision mentality, with judgements being based on the status of a few rare species rather than that of whole biological communities (Possingham *et al*., 2002). Furthermore, the working assumption that threatened species can act as umbrella species is often unfounded (Seddon and Leech, 2008; Cardoso, 2012), and measures designed to support individual threatened species can even have negative effects on other components of biodiversity (Rubinoff, 2001). In sum, there are reasons to believe that threatened species lists do not provide an effective framework for conserving overall forest biodiversity.

Compositional indicators

Little data exist to support the assumption that patterns observed in any one taxonomic group can predict patterns in other groups (Cushman *et al*., 2010; Koivula, 2011; Lindenmayer and Likens, 2011). In fact, many studies have found that different taxonomic groups differ substantially in their response to changing forest characteristics (e.g. Humphrey *et al.*, 1999; Smith *et al*., 2008; Baini *et al*., 2012; Coote *et al*., 2013). For instance, carabid beetles and hoverflies show opposite reactions to field layer cover (Humphrey *et al*., 1999; Jukes and Pearce, 2003), and spider diversity increases as forests mature (Smith *et al*., 2008; Coote *et al*., 2013) while bird diversity is higher in young forest (Moss, 1979; Fuller and Browne, 2003). To make matters more complex, Gaspar *et al*. (2010) have found that correlations between diversity measures of different arthropod groups varied according to geographic scale, with different groups found to be useful surrogates for other groups at transect, forest fragment, and landscape scales. Gaspar *et al*. (2010) suggest that the best overall surrogates of total arthropod diversity are the Hemiptera and Araneae, but the mega-diverse insect orders Diptera and Hymenoptera were not considered in their study, either as indicator or target groups, and the study only encompassed a single habitat type.

For all these reasons, several studies have concluded that compositional indicators can only provide meaningful information about sustainable management and overall biodiversity if multiple such indicators are measured (e.g. Dudley *et al*., 2005; Maleque *et al*., 2009; Koivula, 2011), which essentially defeats the point of using compositional indicators in the first place.

Apart from the lack of data linking them to other components of biodiversity, there are additional problems associated with the use of birds, bats and butterflies as indicators of overall forest biodiversity. Being charismatic and much-studied, all these groups are the subject of targeted conservation efforts. Interventions such as the provision of artificial nesting sites, supplemental feeding, and habitat alteration based on the specific requirements of these groups serve to decouple them from the underlying components of biodiversity that they are supposed to represent (Landres *et al*., 1988; Lindenmayer *et al*., 2000). Such interventions are common, and some of them, such as measures for the conservation of bat populations, are mandated under the EU Habitats Directive (Forestry Commission, 2011), but they undermine the ability of these groups to act as reliable indicators of overall biodiversity.

Structural indicators

It makes intuitive sense that measures seeking to promote natural ecological processes in plantation forest should support the persistence of native biological communities. Likewise, it seems a reasonable assumption that a more physically complex habitat should provide a greater number of ecological niches and, by extension, higher species richness (Simberloff, 1999; Noss, 1999; McElhinny *et al*., 2005; Lindenmayer et al., 2006). However, there is little baseline scientific research to provide a solid link between forest structural indicators and biodiversity (Larsson and Danell, 2001; Failing and Gregory, 2003; Lindenmayer *et al*., 2006; Coote *et al*., 2013). Simberloff (1999) set the agenda for scientists, stating that "*a major scientific thrust will be needed to transform ideas on managing forests for biodiversity into practical, effective tools. The key components of this thrust will be careful natural history, controlled and replicated field experiment, and intensive monitoring*". This is essentially a call for adaptive management, but it has remained largely unanswered because of the so-far-insuperable requirement for biodiversity data that encompass multiple taxonomic groups and forest types (Lindenmayer *et al*., 2006).

The most comprehensive study to have addressed the relationship between structural indicators and a wide range of biodiversity components is the 'Biodiversity Assessment Project', which was carried out by the UK Forestry Commission between 1995 and 1999. However, even this study did not go so far as to use an experimental approach to test the effects of different management approaches, and many potentially important invertebrate taxa were not considered. Although invertebrates were sampled thoroughly from different forest strata (Jukes and Pearce, 2003), the taxonomic impediment unsurprisingly forced the authors to limit their study of invertebrates to a few well-known and easily-identified groups: beetles (Coleoptera), Hemiptera (Cicadomorpha only) and hoverflies (Diptera; Syrphidae). More recently, Smith *et al.* (2008) and Coote *et al*. (2013) have tested the usefulness of various structural and compositional indicators in a range of

plantation forests in the UK, but the only invertebrate taxa considered were spiders (both studies) and hoverflies (only Smith *et al*., 2008).

The consistent lack of attention to the vast majority of invertebrate taxa severely limits the scope of sustainable management efforts, since invertebrates represent the majority of animal species, both worldwide and in the UK, and many small, uncharismatic species perform biological functions that are critical for maintaining ecosystem health (Cardoso *et al*., 2011b). All studies that have tested the effects of structural indicator variables on a range of taxonomic groups have found that responses vary substantially among groups (Jukes and Pearce, 2003; Smith *et al*., 2008; Cootes *et al*., 2013). Therefore, it is not valid to assume that untested invertebrate groups will respond like tested groups to any given forest structural variable.

The importance of testing the usefulness of coarse indicators is highlighted by the fact that where detailed studies of their relationship with elements of biodiversity have been carried out, the results have usually been complex, and sometimes even counterintuitive. For instance, both Jukes and Pearce (2003) and Lassauce *et al.* (2011) found a surprising lack of relationship in the UK/ temperate biome between deadwood volume, one of the most commonly cited coarse biodiversity indicators, and the species richness of saproxylic beetles, even though the guild is by definition dependent on deadwood at some point in the life-cycle. Importantly, Lassauce *et al*. (2011) found a much stronger relationship between deadwood volume and saproxylic beetle diversity in boreal forest. This suggests that structural indicators may vary in their applicability between biomes, in which case standardising the use of indicators at a European level may not be a useful approach. Smith *et al*. (2008) and Coote *et al*. (2013) also failed to find evidence to support the usefulness of several common structural indicators among the taxonomic groups considered in their studies. Moreover, of the indicators that were found to be useful in Ireland (Smith *et al*., 2008), several were not supported in the rest of the UK (Coote *et al*., 2013). Indicators supported by both studies included conifer canopy cover, which had a negative relationship with bird diversity but a positive relationship with bryophyte diversity, and stand age, which had a positive relationship with spider diversity (Coote *et al*., 2013). The complexity of the relationship between biodiversity and environment is illustrated by the fact that, in addition to the variation in response between taxonomic groups, there was also found to be a significant interaction between forest type (broadleaf/conifer forest) and the strength of biodiversity responses to indicator variables (see also Oxbrough *et al*., 2005).

In the absence of data to fully validate the effectiveness of different management strategies, Lindenmayer (2000) advocates a risk-spreading approach, in which multiple management strategies are employed at a variety of scales. However, this can be costly for management and is

difficult to justify, since at least some strategies are likely to have negative impacts on the commercial productivity of managed forest, and there is no direct evidence that any given measure enhances biodiversity. Indeed, some evidence suggests that even intensively managed monoculture plantations of even-aged, non-native conifers are already important reservoirs for biodiversity (e.g. Humphrey *et al*., 1999; Jukes and Pearce, 2003). Thus, there is still a need for adaptive management, as called for by Simberloff (1999), to quantify and characterise the biodiversity benefits of different management approaches so that they can be objectively balanced against the economic costs of diminished timber yield, and can contribute meaningfully to decision-making. This will never happen while measurement of biodiversity is so time consuming and demanding of expertise as is currently the case.

Even when coarse indicators correlate with species richness, they are limited in what they can convey about actual changes in biological communities, since they only provide information about the *potential* for species richness (Redford, 1992; Dudley *et al*., 2005). In fact, assessing progress in achieving biodiversity goals by monitoring structural indicator variables is liable to confuse means with ends (Failing and Gregory, 2003). Management strategies that promote certain forest characteristics are the means of increasing biodiversity, but the fundamental objective is not the forest characteristics but the biological communities themselves.

5.2.4 Detecting fine-scale temporal and spatial trends in invertebrate biodiversity

An element of forest biodiversity that has serious economic implications is the spread of forest pests. Forest Europe *et al*. (2011) states that "*Heavy attacks of insects and phytopathogens… may cause major impacts on forests, resulting in a weakening of forest ecosystem health and vitality, and economic losses. Insect populations are also likely to react to long-term change processes such as climate change*." Pest species that have extended their range northwards from central Europe to pose an economic threat to forestry in the UK include the oak processionary moth (*Thaumatopoea processionea*), which is already established in parts of the UK (Morecroft and Speakman, 2013), and the pine processionary moth (*T. pityocampa*), which has not yet arrived but is thought likely to pose a threat in the near future (Netherer and Schopf, 2010). Structural indicators are not useful for monitoring the spread of pest species, nor are they likely to be able to detect rapid temporal changes in insect communities that could arise as a result of factors such as climate change, chemical toxicity, invasive species, or disease. Only repeated, direct monitoring is likely to detect these kinds of temporal changes; otherwise detection will only occur only once the effect size is very large or when charismatic, endangered, or economically important species are affected.

5.2.5 Metabarcoding as a tool for monitoring forest biodiversity

There is a clear need for a more effective approach to gathering biodiversity data. This is required for (1) the implementation of adaptive management programmes to test the effectiveness of different management strategies in enhancing the various components of biodiversity, (2) the selection of meaningful indicators for specific regions and forest types, and (3) the detection of temporal and spatial changes in community composition. The aspect of data gathering that requires the most urgent attention is the ability to characterise invertebrate communities, in order to enable groups that are currently overlooked to make a contribution to decision-making.

Metabarcoding has the potential to answer this need, since previous studies have found that it can produce accurate and reliable alpha and beta diversity information at a fraction of the time and cost of traditional survey methods (Yu *et al*., 2012; Ji *et al*., 2013), and without requiring taxonomic expertise.

In this chapter, I describe a pilot project carried out in collaboration with Forest Research, which aims to demonstrate the potential of the metabarcoding approach for conducting biodiversity monitoring in UK forests. I explore the ability of metabarcoding to provide data that can be used for (1) detecting fine-scale spatial and temporal variation in arthropod community composition, (2) evaluating the biodiversity effects of management strategies such as planting mixed-species forest crops, (3) testing the usefulness of structural indicators of biodiversity, and (4) identifying which species are responsible for driving trends or are associated with particular forest characteristics. I use a sampling method that primarily traps insects from the order Diptera. Despite being the most species-rich group of arthropods, Diptera is almost always overlooked in biodiversity studies because of the difficulty associated with sorting and identifying the inevitably large numbers of specimens, which tend to be characterised by small body size (Jukes and Pearce, 2003). I aim to show that the metabarcoding approach allows hyper-diverse groups like Diptera to contribute to biodiversity analyses.

5.2.6 Study site

Thetford Forest, located on the Norfolk-Suffolk border in East Anglia, covers an area of approximately 185 km^2 and is the largest lowland conifer forest in the UK (Pedley *et al*., 2013). Planted in the early twentieth century, it is intensively managed by the UK Forestry Commission for both timber production and recreational purposes. The forest is composed of a matrix of even-aged stands (2-16 ha), which are typically harvested by clear-felling at an age of 60-80 years (Pedley *et al*., 2013). The majority of stands are planted with conifers, specifically Corsican pine

(*Pinus contorta* var. *maritime*; 54% of forest area) and Scots pine (*Pinus sylvestris*; 31%), but some comprise broadleaf species such as beech (*Fagus sylvatica*; 4%) and oak (*Quercus robur*; 3%) (Balzter *et al*., 2007). There are also mixed-species stands, which feature both conifer and broadleaf species, and are widely considered to have ecological, social (aesthetic), and silvicultural benefits over single-species plantations (Kerr, 1999; Hartley, 2002; Felton *et al*., 2010; Norman *et al*., 2010; Griess and Knoke, 2011; Gamfeldt *et al*., 2013).

5.3 Methods

5.3.1 Field methods

Sampling arthropods for the Metabarcode dataset

Malaise traps were used to sample flying arthropods at fifteen sites in Thetford forest. Each site was located in a separate forest management unit, and was characterised by one of three categories of mature forest crop: "Oak" (100% *Quercus robur*; 5 sites), "Pine" (100% Scots pine, *Pinus sylvestris*; 4 sites), or a "Mixed" crop of oak and Scots pine (6 sites). All forest stands were planted between the years 1930 and 1941, with the exception of one Oak stand (O2377) and one Pine stand (P4751), which were planted in 1954 and 1967, respectively (Table 5.2). The management strategy for all sites allows the natural regeneration of understorey (non-crop) trees (R. Brooke (FC), *pers. comm.*).

Table 5.2: Table showing the size, location, and planting year of each sampled forest stand, as well as plantation crop type and the percentage of the crop made up of oak and Scots pine.

Site name	Management sub-unit	Stand Area (ha)	Location	Planting year	Crop type	Crop ratio (Oak/Pine)
M3009	3009b	3.38	0°40'42.4" E; 52°30'54.6" N	1932	Mixed	26/74
M3021	3021a	4.85	0°40'2.3" E; 52°30'23.3" N	1941	Mixed	80/20
M3324	3324	5.21	0°51'32.8" E; 52°30'4.4" N	1935	Mixed	76/24
M3345	3345a	5.17	0°51'15.0" E; 52°29'29.0" N	1932	Mixed	55/45
M3548	3548a	4.46	0°51'26.2" E; 52°26'4.8" N	1934	Mixed	91/9
M4716	4716	5.15	0°53'54.8" E; 52°25'35.5" N	1934	Mixed	97/3
O2377	2377a	4.73	0°36'56.5" E; 52°32'19.3" N	1954	Oak	100/0
O3335	3335	6.75	0°51'17.4" E; 52°29'47.6" N	1932	Oak	100/0
O3547	3547	2.41	0°51'20.4" E; 52°26'3.9" N	1934	Oak	100/0
O4714	4714	4.87	0°53'26.9" E; 52°25'30.2" N	1934	Oak	100/0
O4722	4722b	2.91	0°52'20.7" E; 52°25'5.0" N	1933	Oak	100/0
P3037	3037c	1.73	0°40'15.4" E; 52°29'45.2" N	1930	Scots pine	0/100
P3506	3506d	1.61	0°49'46.9" E; 52°27'14.6" N	1941	Scots pine	0/100
P3522	3522a	7.13	0°51'6.2" E; 52°27'14.9" N	1937	Scots pine	0/100
P4751	4751c	3.61	0°52'35.5" E; 52°24'50.8" N	1967	Scots pine	0/100

Sampling was carried out by Forestry Commission staff between 8th August and 4th October 2011. A single Malaise trap was erected in each chosen management unit, making use of previously-placed survey quadrats that had been marked out with stakes. The trap was placed as close as possible to the quadrat centre, in a gap between trees, and the collecting bottle was filled 2/3 full with 100% ethanol to kill trapped specimens and preserve their DNA. Samples were

collected weekly for eight consecutive weeks, resulting in a total of 120 (8 x 15) malaise trap samples.

Sampling arthropods for the Standard dataset

During May-August 2011, pitfall trapping was carried out at the same fifteen forest sites. Eight traps were installed around the perimeter of the 50 x 50 m quadrat at each site, four of which were sited on open ground, and four at tree bases. The contents of the pitfall traps were collected by Forestry Commission or University College Cork staff every 2-3 weeks, resulting in a total of 84-90 trapping days.

Surveying for coarse biodiversity indicators

In February 2013, fourteen of the fifteen sites were surveyed for structural biodiversity indicators. The fifteenth site (P3506) had already been harvested and so could not be fully assessed for structural characteristics. The methodology for surveying structural indicators was based on that of the National Forest Inventory (NFI) but did not follow it exactly because the custom mapping software for the NFI protocol was not available for use. Sampling sites were located using GPS coordinates and Forestry Commission maps. At each site, a 30 x 30 m grid was marked out, orientated on a North-South axis, with grid lines marked at 5 m intervals. Following the NFI protocol, measurable stems were defined by a diameter at breast height (DBH, measured 130 cm above the ground) ≥ 4 cm. Position in the grid was recorded to the nearest 0.5 m for each measurable stem, along with species, DBH, and whether it was alive or dead. For multi-stemmed trees (e.g. hawthorn), each stem ≥ 4 cm DBH was measured and recorded independently. Various indices of tree species diversity and forest structural complexity were derived from these data, as were measures of crop and non-crop tree density (Table 5.3).

A 10 m x 10 m subquadrat in each corner of the grid was censused for dead branches, trunks, and stumps. Adapting the NFI survey protocol, we counted all pieces of lying deadwood with a diameter ≥ 7 cm that intersected 15 m of transect line (3 x 5 m) in each corner, and all stumps lying within a 5 m radius of the subquadrat centres. Following the NFI protocol, the stump closest to the centre was scored for height and for cross-section widths along its North-South and East-West axes. Any other stumps were assigned to a diameter class: 4 cm ≤ d < 6 cm, 6 cm ≤ d < 14 cm, 14 cm ≤ d < 40 cm, or d ≥ 40 cm. The cross-sectional area of stumps was calculated based on the smaller of the two diameter measurements for the central stumps, and the lower bound of the diameter class for others.

Table 5.3: Names and descriptions of coarse biodiversity indicator variables considered in this study.

Variable	Description
Stem_density	*Number of measurable stems in 900 m^2 block*
Tree_species	*Number of tree species with at least one measurable stem*
%Pine	*Percentage of measurable stems (crop and non-crop) that are Scots pine. A measure of the broadleaf/conifer ratio*
Crop_density	*Number of crop stems in 900 m^2 block*
Non-crop_density	*Number of non-crop stems in 900 m^2 block*
Deadwood_area	*Total cross-sectional area of lying deadwood stems intersecting transect line*
Deadwood_count	*Number of lying deadwood pieces intersecting transect lines*
Stump_area	*Total cross-sectional area of stumps in circular plots*
Stump_count	*Total number of stumps in circular plots*
DS_area	*Deadwood_area + Stump_area*
DS_count	*Deadwood_count + Stump_count*
Simpson_count	*Simpson's diversity index for trees, based on count of measurable stems*
Simpson_area	*Simpson's diversity index for trees, based on cross-sectional area of measurable stems*
SCI	*Structural complexity index (Zenner and Hibbs, 2000)*
ESCI_1	*Enhanced SCI, modification step 1 (ESCI'). Incorporates triangle orientations (Beckschäfer et al., 2013)*
ESCI_2	*Enhanced SCI, modification step 2 (ESCI). Incorporates triangle orientations and stem density (Beckschäfer et al., 2013)*

5.3.2 Laboratory and bioinformatics steps

Metabarcode data

Bulk DNA extraction and high-throughput sequencing of the Malaise trap samples was carried out by collaborators at the KIZ, as described in Chapter 4. Each of the 120 samples was processed as a separate MID, and six 1/16 regions of a Roche GS FLX '454' sequencing plate were used. Subsequently, I performed quality control, sequence clustering, and taxonomic assignment of OTUs using the bioinformatics pipeline described in Chapter 4. The key output of the metabarcode pipeline is an "OTU table", which gives the number of sequence reads per sample for each OTU, together with associated taxonomic information. The OTU table was filtered to exclude single-read and non-arthropod OTUs, and this formed the basis for the majority of analyses. Taxonomic subsets of the main arthropod OTU table were created for the most prevalent lower-level taxa: Diptera, Hemiptera, Lepidoptera, Hymenoptera, Coleoptera, and Arachnida.

Standard data

Carabid beetles and spiders from the pitfall traps were identified to species level using morphological characteristics by Nadia Barsoum (beetles, using the key of Luff (2007)) and Lauren Fuller (spiders, using the key of Roberts (1993)), and species were scored for site-level abundance. Other taxa caught in the traps were discarded. I combined the beetle and spider datasets to form a 'standard' dataset, which I used for comparison with the Malaise trap metabarcode data.

5.3.3 Two metabarcode datasets

Data analysis was carried out in *R* v. 2.15.2 (R Core Team, 2012). Two main datasets were created for metabarcoded arthropod OTUs. These are referred to henceforth as the 'binary' and 'pooled' datasets.

Binary dataset

The binary dataset was an OTU by sample dataset in which every OTU was scored for presence-absence in each of the 120 malaise trap samples (15 sites, 8 samples per site). It was generated from the raw arthropod OTU table, using function *decostand()* in *R* package *vegan* v. 2.0-7 (Oksanen *et al*., 2013) to translate sequence read counts into simple presence-absence for each OTU. This dataset was used for visualising compositional differences among samples (beta diversity) and for explaining variation in arthropod species richness (alpha diversity). I also created a subset of this dataset that included only Diptera-assigned OTUs, which accounted for 58% of taxa.

'Pooled' dataset

The pooled dataset was an OTU by *site* dataset, in which the eight weekly samples were combined within site. The abundance of each OTU was scored on a scale of 1 to 8, representing the number of weeks in which that OTU was detected in that site. This pooling is not a direct measure of OTU abundance, as a long-lived species could certainly persist in a site for a long time even if at low numbers. However, the index is probably partly correlated with abundance and directly correlated with each species' contribution, over time, to a forest compartment's biodiversity. This dataset contained the same number of data points as the standard (beetle and spider) dataset (N=15) and was therefore used for testing whether metabarcode data returned the same community information as standard data collected from the same sites. In addition, it was used for (1) carrying out order-level alpha-diversity analyses for orders with low overall species richness, and (2) testing whether coarse habitat variables can be used to explain patterns of beta diversity.

For each sample (binary dataset) and each site (pooled dataset), species richness values were derived for (1) all arthropods, and (2) each of the most prevalent lower-level taxonomic groups, using function *specpool()* in *vegan*.

5.3.4 Temporal and spatial variation in community composition

I used ordination to visualise compositional differences between samples and sites. For all ordinations, OTUs that occurred in only one sample (binary dataset) or site (pooled dataset) were excluded. These 'singleton' OTUs can introduce analytical instability and are generally uninformative for beta diversity analyses.

Binary dataset

To check the robustness of the ordination results, I used two different ordination methods to visualise differences in species composition between Malaise trap samples (120 sample points). The first method was correspondence analysis (CA; function *cca()* in *vegan*), which is based on eigenanalysis; the second was nonmetric multidimensional scaling (NMDS; function *metaMDS()* in *vegan*), which ordinates based on rank-order of sample dissimilarities. For this, I used the Jaccard dissimilarity index, since it is appropriate for binomial data and gives no weight to shared absences. To aid visualisation, I used the *ordispider()* function in *vegan* to group ordination points by site and week. The average Jaccard dissimilarity of samples from Oak versus Pine sites (within sampling week) was compared with that of samples from Week 1 versus Week 7 (within site) by extracting and averaging the relevant dissimilarity values from the distance matrix.

The results of the two ordination methods were compared using a Procrustes test with 999 permutations, and linear regressions were used to test for correlation between sampling week and scores on the vertical ordination axes (CA2 and NMDS2).

Multivariate likelihood ratio (LR) tests were conducted in *R* package *mvabund* v. 3.6.11 (Wang *et al*., 2012) to test whether the species composition of samples varied significantly between sites and sampling weeks. This community analysis method fits a generalised linear model (GLM) to each OTU to test its response to predictor variables, and returns individual statistics for each OTU, in addition to statistics relating to the community-wide significance of the effect. This makes it possible to pick out the OTUs that respond to the test variable. The method improves on traditional community analysis approaches in that (1) it allows the user to specify the appropriate mean-variance relationship for the data, which minimises the chance of confounding location effects with dispersion effects, and (2) it is less disproportionately influenced by the OTUs with

the highest variance in abundance (not relevant when presence-absence data are used) (Warton *et al*., 2012; Wang *et al*., 2012).

The binomial error distribution was used for *mvabund* tests on the binary dataset, with pit.trap resampling (an as yet unpublished method developed by the authors of *mvabund*) and 999 bootstrap iterations. Any OTU with an unadjusted *p*-value < 0.05 was considered to show a response to the test variable, and the direction of its response was discerned based on the fitted coefficients. I used this analysis to pick out the species that were specialists of each crop type and those that showed a significant temporal response in (1) Oak and Mixed sites (pooled because they overlapped in the ordinations), and (2) Pine sites. It should be noted that table-wide adjustment for per-OTU significance was not applied because the high number of OTUs rendered all OTUs as non-significant responders. This means that it is not possible to assign high confidence to identifications of responding OTUs.

Pooled dataset

Because the CA and NMDS ordinations returned very similar results for the binary dataset, I used only NMDS for the pooled dataset (15 points, weeks pooled within site). This was based on a quantitative version of the Jaccard dissimilarity index so as to preserve OTU frequency information. Ordination points were coloured and grouped by crop type, and the continuous environmental variable %Pine was displayed as surface contours (function *ordisurf()* in *vegan*). I also clustered sites using UPGMA cluster analysis (function *hclust()* in *vegan*) on the Jaccard distance matrix. A heatmap was then created, showing the frequency of each OTU in every site, with sites arranged to match their positions in the cluster dendrogram. This allowed the between-site variation in species composition to be visualised in the raw data.

5.3.5 Comparing metabarcoding and standard datasets

Here, I compare the metabarcoded Malaise trap community data with the standard dataset comprising abundance data for the pitfall-trap-sampled carabid beetles and spiders. Perfect correlation is not necessarily expected because the two datasets are made up of very different sets of species.

A quantitative Jaccard distance matrix and an NMDS ordination were created from the standard dataset, and compared with those of the pooled metabarcode dataset using Procrustes and Mantel tests, each with 999 permutations. For both datasets, I then used multivariate LR tests in *mvabund* to test for an effect of crop type (Oak/Mixed/Pine) on community composition. Inspection of diagnostic plots (residual vs. fit plot; function *plot.manyglm* in *mvabund*) indicated

that different mean-variance relationships should be specified for the two datasets: the abundance data in the standard dataset was best suited to negative binomial error distribution, while the Poisson distribution was more appropriate for the frequency data in the metabarcode dataset. In addition to testing for an overall effect of crop type, I also performed post hoc tests making pairwise comparisons among crop types. *p*-values were adjusted for three pairwise comparisons using Benjamini and Hochberg's (1995) correction method (*p.adjust(method=fdr)* in *R*).

5.3.6 Does mixed-crop forest have biodiversity benefits?

Mixed sites are expected to efficiently support a greater number of species (both Pine and Oak specialists) within individual sites. Therefore, I tested whether Mixed sites had higher species richness than Oak or Pine sites. The total species richness per crop type was estimated using the Chao2 incidence coverage method (Chao, 1987; Colwell and Coddington, 1994), using *vegan* function *specpool()*, and compared between pairs of crop types using Welch's *t*-tests. Resulting *p*-values were adjusted for three pairwise tests.

5.3.7 Are monocultures bad for biodiversity?

A monoculture was defined as a site in which all measurable stems (≥ 4 cm DBH) in the 30 x 30 m survey quadrat were assigned to a single tree species. I used the tree survey data from the study sites to determine which of the 9 sites that were characterised by a single crop species (Pine or Oak) represented true monocultures. The distinctiveness of the arthropod communities at these sites was explored via examination of their position in the ordinations, and additional NMDS ordinations were created from order-specific subsets of the pooled dataset to assess whether patterns observed in the main arthropod ordination were driven by a particular group. Multivariate Poisson LR tests in *mvabund* were used to pick out OTUs that were associated with the monoculture sites.

Finally, I tested whether the average observed species richness of Malaise trap samples from monoculture sites differed significantly from that of samples from non-monoculture sites, using a Poisson-distributed generalised estimating equation (GEE; function *geeglm()* in *R* package *geepack* (Højsgaard *et al*., 2006)) to account for the non-independence and temporal correlation of samples within a site. For this test, and for subsequent GEE tests, correlation structure was set to "ar-1" to reflect the fact that the correlation was expected to be greatest between subsequent samples (Zuur *et al*., 2009). I did not use estimated total species richness in this case because there were only a small number of monoculture sites, and the non-monoculture sites contained a

much wider variety of forest types, which meant that it was not appropriate to combine them for comparison with monocultures.

5.3.8 How well do structural indicators predict biodiversity?

For determining the predictive ability of structural biodiversity indicators, I mostly omitted site P3506 from the analyses, since this site had been harvested before tree species composition and forest structure could be scored. However, inspection of the harvested stumps and the surrounding forest suggested that it had been a pine monoculture, very similar in structure to site P3522. This enabled me to assign three coarse indicator values: %Pine = 100%, Crop_density = 45, and Non-crop_density = 0, and I included this site when testing the individual effects of these variables on beta-diversity.

Alpha diversity

I first tested for an effect of the enhanced structural complexity indicator (ESCI_2; Table 5.2) on species richness, since this has previously been found to be a good predictor of diversity in tropical forests (Beckschäfer et al., 2013). To account for repeated measures, I used a GEE model, with correlation set to "ar-1" and samples grouped by site.

Next, I performed a stepwise regression, on a multiple-term GEE model to test whether any combination of indicator variables could be used to predict species richness.

There were a total of 16 candidate coarse indicator variables (Table 5.3), many of which were closely related to one another. In order to reduce this to a more manageable number, I used linear models to assess the level of correlation between all possible pairs of variables. All variables that were not strongly correlated (adjusted $R^2 < 0.5$) with any others were included in the initial GEE model, along with one chosen from each subset of inter-correlated variables. From each subset, I chose the variable that was least related to any variable outside the subset. For instance, Stem_density, Non-crop_density and ESCI_2 formed a correlated subset linked by the theme of tree density. However, Stem_density includes an element of Crop_density, which is uncorrelated with any other variable and so qualifies independently for inclusion in model selection, and ESCI_2 is weakly correlated with other diversity and complexity measures, which form their own subset. Therefore, the variable chosen to represent this subset in the initial model was Non-crop_density. In subsets where multiple variables were equally valid for inclusion, the stepwise regression was carried out with each variable in turn, and the one that resulted in the most significant term in the reduced model was ultimately chosen to represent the subset.

The stepwise regression was carried out manually. All chosen variables were initially included as terms in the model, and the term with the highest *p*-value was eliminated at each step until all remaining terms were significant ($p < 0.05$). The Pearson residuals of the reduced model were plotted to check visually for normality and homoscedasticity, and a linear regression was used to check that the residuals did not vary in size with predicted species richness. I also plotted each of the variables from the reduced model individually against observed species richness, to illustrate the direction of each effect.

Next, I asked whether the environmental variables that predict species richness vary among taxonomic groups within the Arthropoda. I performed a stepwise regression for each of the most prevalent insect orders: Diptera, Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera. The same variables were included in each initial model as had been included for the main arthropod analysis. For Diptera (the most species-rich group), I used a GEE model applied to sample-level species richness data, as above. However, for other orders, *site*-level species richness data (i.e. from the pooled dataset) were used because the number of species per individual Malaise trap sample tended to be very low. Since these datasets did not contain repeated measures, Poisson GLMs were used instead of GEE models, and I was able to make use of *R*'s automatic function for stepwise regression, which operates based on AIC values. As a check, I also performed the stepwise regression manually based on *p*-values, as for the GEE models above.

Beta-diversity

The same variables that were included in the initial GEE models for species richness were also tested for their effect on beta-diversity. The most robust method of significance testing for community data is multivariate analysis in *mvabund*. Therefore, I first tested the individual significance of each variable as a single term with *mvabund*, using Poisson multivariate LR tests on the pooled arthropod community dataset.

I next tested the significance of variables in multiple-term *mvabund* models. One variable, %Pine, clearly had a greater individual effect on community composition than did any other, and so I conducted the model selection process in such a way as to ensure that %Pine was retained in the model. I began by using a forward selection approach, testing the significance of each variable as a second term in a model that included %Pine as its first term. I subsequently tested every possible combination of the terms that were significant in the two-term models, with %Pine retained as the first term in all tests.

For each variable that was significant in any of the *mvabund* models, I returned to the single-term *mvabund* analysis for that variable, and used OTU-specific *p*-values and LR coefficients to pick out

the OTUs that showed the strongest response in either direction. Effects were visualised in the raw data via heatmaps with sites were arranged according to their score for a given variable.

For comparison, I also used two types of permutation tests, each with 999 permutations. The first was PERMANOVA (function *adonis()* in *vegan*), which was used to test the effect of individual variables on a quantitative Jaccard distance matrix; the second used canonical correspondence analysis (function *anova.cca()* in *vegan*).

Finally, I produced a quantitative Jaccard distance matrix of sites based on values of the indicators that were significant in the multiple term *mvabund* model, and used a Mantel test to compare this with the one based on the pooled community dataset of Malaise trap arthropods.

5.4 Results

5.4.1 Detection of OTUs and assignment of taxonomy

Sequencing returned 120,433 raw sequence reads across the 120 samples, which represents relatively low coverage. Quality-control filtering reduced this to 51,262 reads, and Bayesian clustering in CROP at 98% similarity produced a total of 2884 molecular OTUs. Taxonomic identification to ordinal level was achieved for 92% of OTUs using SAP, and 19% were identified to species. A total of 613 OTUs were assigned to Arthropoda and contained > 1 read (non-'single-read' OTUs). These were retained for downstream analysis, following Yu *et al.* (2012). Over half of the arthropod OTUs (N=353) were identified as Diptera, with lower prevalence of Hemiptera (44), Hymenoptera (31), Lepidoptera (33), Coleoptera (38), Arachnida (34), and others. Removal of singleton OTUs reduced the full dataset to 496 OTUs across the 120 samples, and the pooled dataset to 448 OTUs across the 15 sites.

5.4.2 Data exploration

Correspondence analysis (CA) of the full dataset extracted only 5.2% of the total inertia in the first two axes (2.8% in CA1 and 2.4% in CA2), and stress was high in the two-dimensional NMDS analysis (0.311), with no convergent solution reached. However, increasing dimensionality to four dimensions achieved a convergent solution after 31 iterations, together with an improved stress value (0.186). Although these results suggest that neither method was able to produce a very robust projection of the data onto two dimensions, the two methods nonetheless produced very similar ordinations (Figures 5.1, 5.3; Procrustes rotation of the first two CA axes against a 2-dimensional NMDS ordination: R^2=0.86, p=0.001). Multivariate binomial LR tests in *mvabund* provided support for the observation that the arthropod community composition of samples varied both by site ($LR_{df=105}$=6685, p<0.001) and by sampling week ($LR_{df=119}$=1410, p<0.001).

Variation across forest types

The first ordination axis (CA1 and NMDS1) sorts samples by forest crop type (Pine/Mixed/Oak). There is substantial overlap between Mixed and Oak sites, while Pine sites are more distinct – two of them (P3522 and P3506) particularly so (Figures 5.1). Figure 5.2 allows visualisation of the taxa driving these community differences: species at the top of the chart are common in Oak and Mixed sites, but not in Pine sites, while species at the bottom of the chart are highly prevalent in Pine sites and rare or absent in Oak and Mixed sites. The distinctiveness of sites P3522 and P3506 can also be observed in the heatmap.

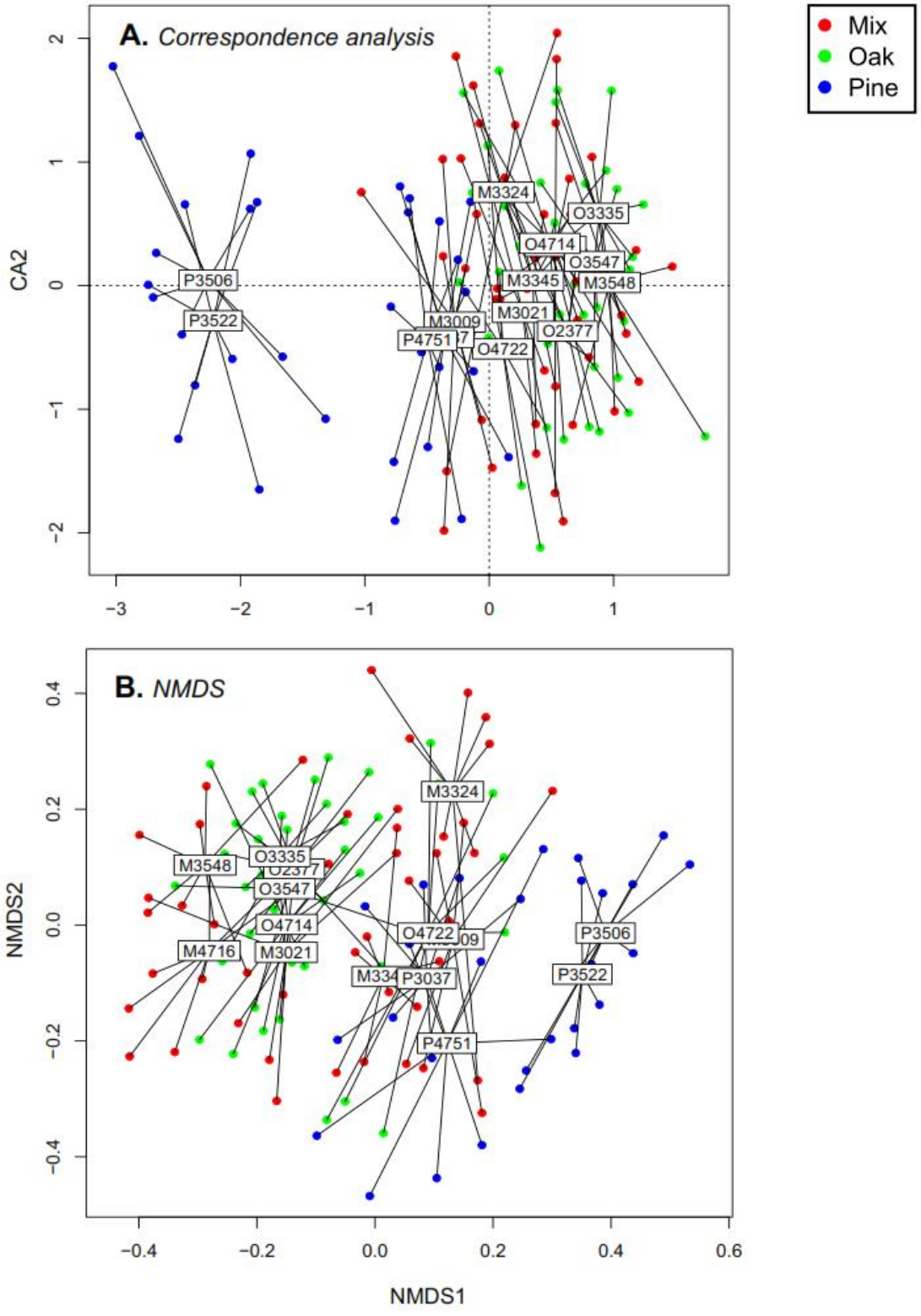

Figure 5.1: CA and NMDS ordinations showing all Malaise-trap samples. Samples are grouped by compartment, and coloured according to crop type.

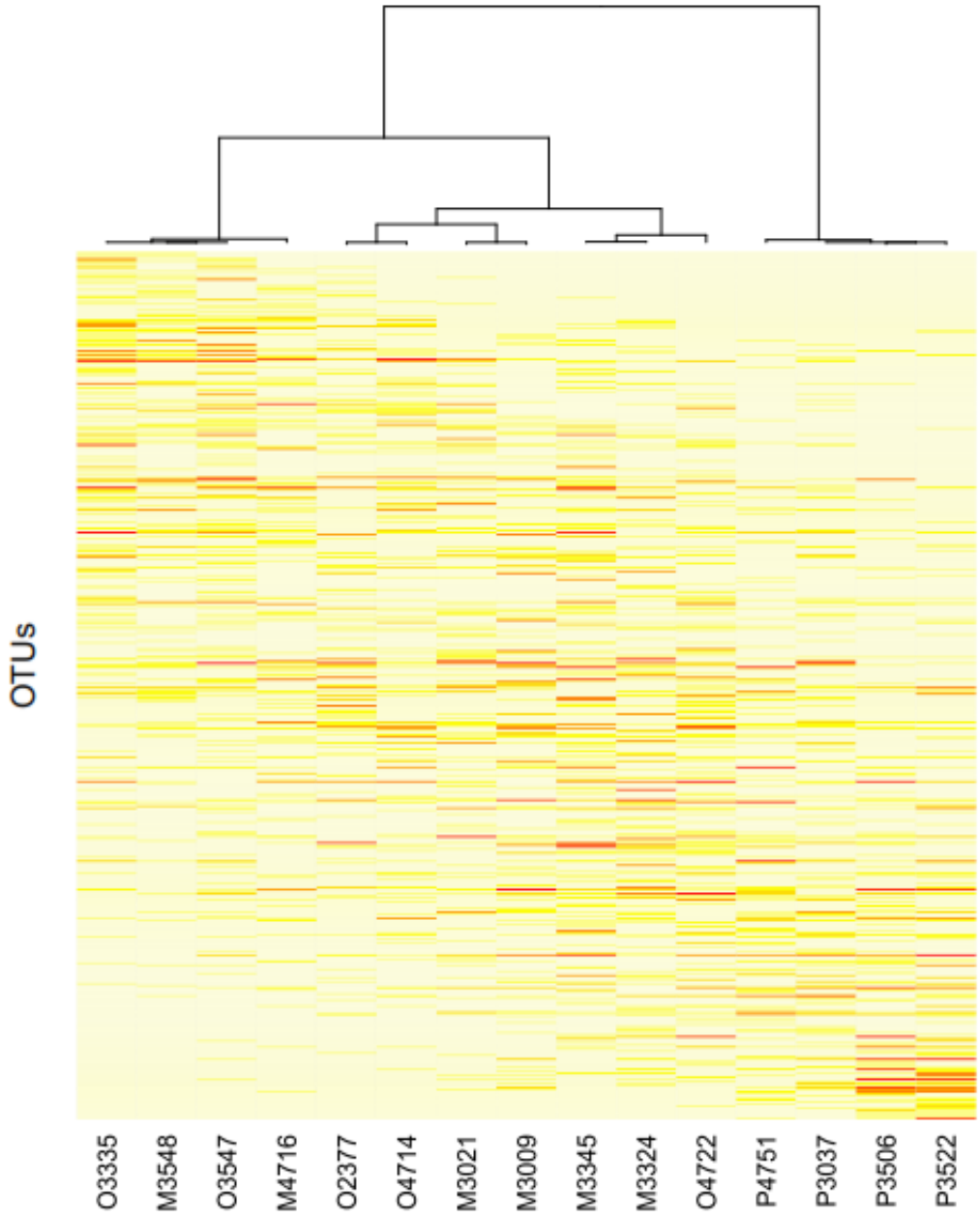

Figure 5.2: Heatmap showing the incidence of all OTUs at each site. Sites are arranged into clusters based on UPGMA clustering of the Jaccard distance matrix. Red represents high incidence, and the first letter of each site name indicates the crop type at that site (O for oak, P for pine, and M for mixed crop).

An *mvabund* analysis, which fits a GLM to every species to test its response to an environmental variable, identified 24 OTUs associated with Pine stands, 21 associated with Oak stands, and 18 associated with Mixed stands. These included members of a wide range of taxonomic groups, although the majority belonged to the Diptera (Table 5.4).

Table 5.4: Number of OTUs in each taxonomic group that are significantly associated with each forest crop type. Based on three separate multivariate LR tests in *mvabund* with binomial errors, pit.trap resampling and 999 bootstrap iterations. Each analysis tested one forest type against the other two (pooled). Numbers in parentheses in the 'Pine' column give the number of OTUs associated specifically with the two Pine monoculture compartments (P3522, P3506).

Taxonomic group	Oak	Pine (monoculture sites only)	Mixed
Arachnida	0	1 (2)	1
Coleoptera	0	4 (2)	1
Lepidoptera	0	1 (0)	0
Diptera	12	14 (7)	11
Hemiptera	2	3 (1)	2
Ephemeroptera	1	0(0)	0
Hymenoptera	0	0 (0)	1
Mecoptera	1	0(0)	0
Orthoptera	4	0 (0)	0
Unknown order	1	1 (0)	2

Variation across time

An unexpected result is the finely-grained temporal succession of arthropod communities along the second ordination axis, from Week 1 (8^{th}- 15^{th} August) to Weeks 7 and 8 (19^{th} September – 4^{th} October; linear regression, CA_axis_score_2 ~ week, $F_{1,118}$=346.5, R^2=0.74, p<0.001; NMDS axis score 2 ~ week, $F_{1,118}$=115.5, R^2=0.49, p<0.001), which suggests that metabarcode datasets can detect even subtle community shifts (Figure 5.3; Figure 5.4). Across the full sampling period, the change in arthropod community composition is considerable, such that the mean Jaccard dissimilarity between samples from Week 1 versus Week 7 (within site) is similar to that between samples from Oak versus Pine sites (within week) (week 1 vs. week 7: mean=0.87 ± 0.009 (s.e.); Oak vs. Pine: mean = 0.90 ± 0.003). This temporal turnover occurs at the same rate across the three forest types (Figure 5.3; PERMANOVA test, community ~ Type*Week, interaction effect: $F_{df=14}$=0.80, p=1.00).

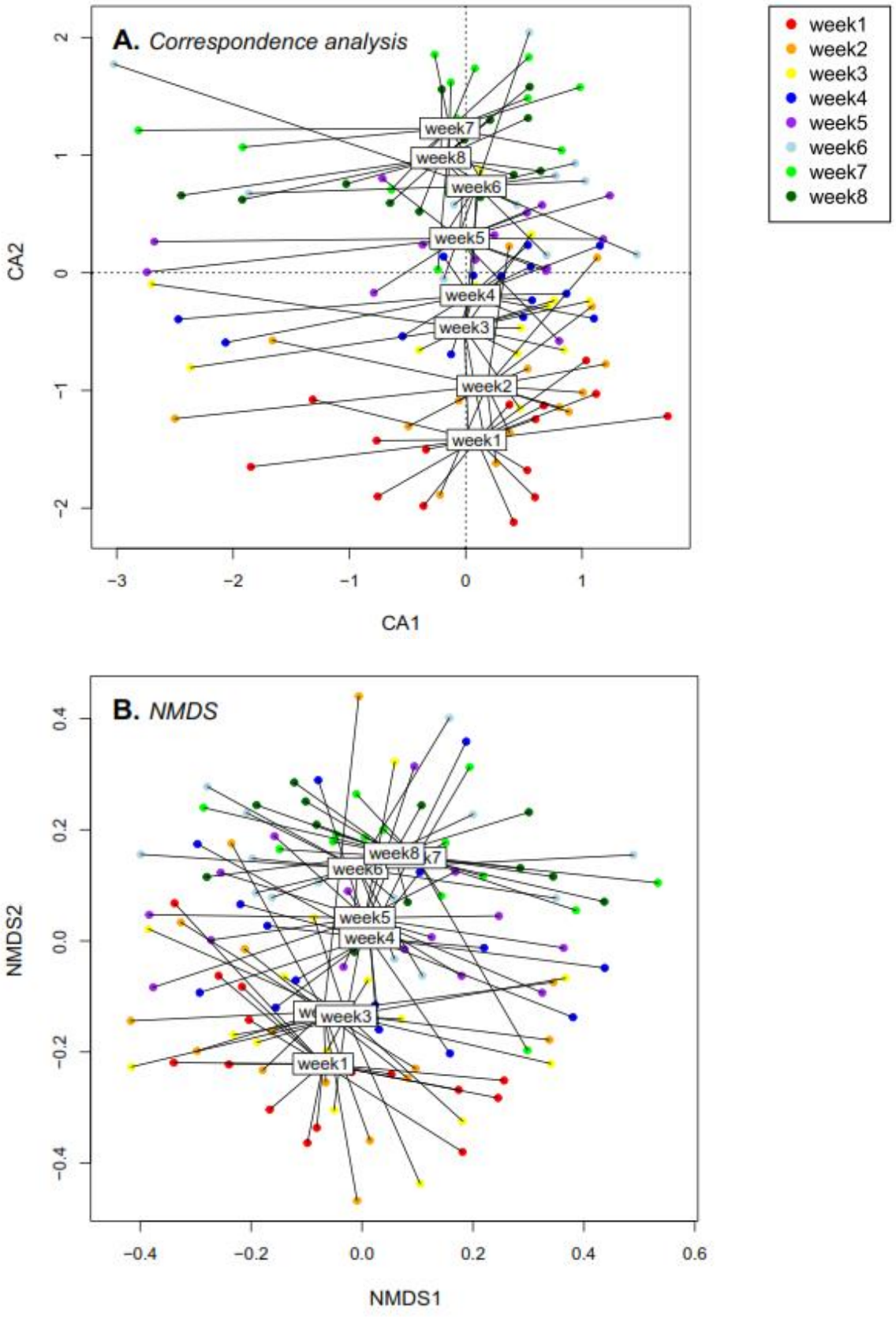

Figure 5.3: CA and NMDS ordinations showing all malaise trap samples, grouped and coloured according to sampling week.

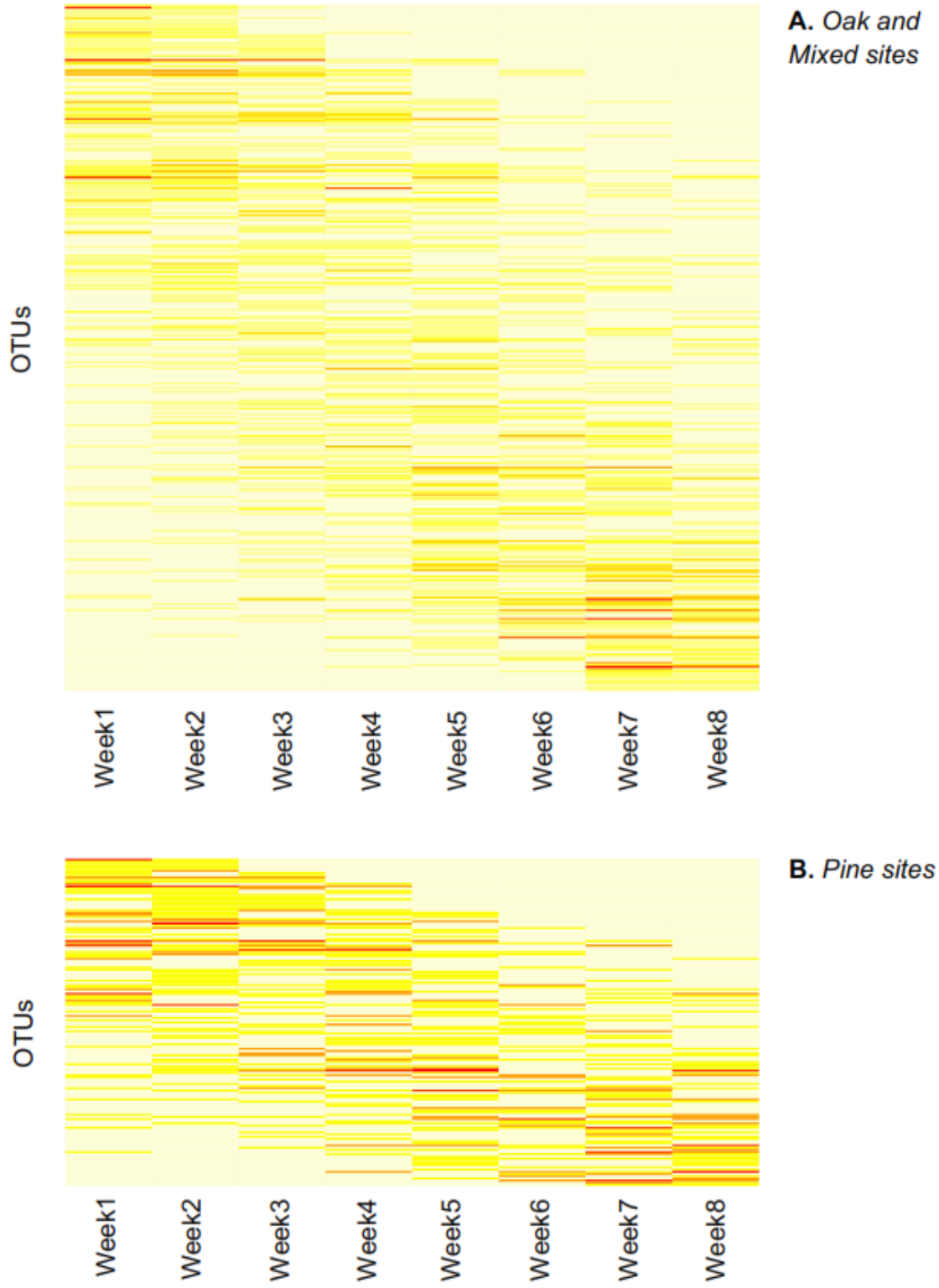

Figure 5.4: Heatmap showing the relative incidence of each OTU across the 15 sites for each sampling week for (A) Oak and Mixed sites, and (B) Pine sites. Red indicates high incidence

32 OTUs were identified by *mvabund* as being associated with the beginning of the trapping period in Oak and Mixed sites (pooled), and 23 associated with the end of the trapping period. In Pine sites, sixteen OTUs were associated with early weeks, and four with the late ones (Table 5.5).

Table 5.5: Number of OTUs belonging to each taxonomic group that show a significant temporal response (unadjusted *p*-value < 0.05) in Oak/Mixed sites and Pine sites. Based on Poisson multivariate likelihood-ratio tests conducted in *mvabund* with pit.trap resampling and 999 bootstrap iterations.

	Oak/Mixed		Pine	
	Early	*Late*	*Early*	*Late*
Arachnida	5	2	1	0
Coleoptera	1	0	1	0
Diptera	14	15	10	4
Hemiptera	3	2	1	0
Hymenoptera	1	1	1	0
Lepidoptera	3	1	1	0
Orthoptera	2	0	1	0
Psocoptera	1	0	0	0
Unknown order	2	2	0	0

Only five OTUs showed a significant temporal response in both Oak/Mixed and Pine. At the ordinal level, there was no clear taxonomic commonality in the OTUs associated with early or late sampling weeks; OTUs picked out by the *mvabund* analysis included members of Diptera, Hymenoptera, Hemiptera, Lepidoptera, Arachnida and Coleoptera (Table 5.5), indicating that a diverse range of arthropod taxa are involved in driving the observed pattern of temporal succession.

In summary, there is strong separation of communities both by plantation crop and by sampling week, with species from multiple taxonomic groups contributing to these effects.

In the following analyses, I use the pooled dataset for all beta-diversity analyses because existing methods of analysing the determinants of beta diversity are not able to correct for repeated measures. However, I do take advantage of repeated measures for analysis of alpha diversity.

5.4.3 Comparing metabarcode and standard datasets

Comparison of ordinations from the metabarcode (Malaise-trap) and standard (pitfall-trap) datasets found that they contained similar beta diversity information, with significant correlation between the Jaccard distance matrices and NMDS ordinations from the two datasets (Procrustes test R^2=0.85, p=0.001; Mantel test r=0.33, p=0.007). NMDS ordinations of both datasets separate sites by crop type, with the four Pine sites showing the greatest distinctiveness (Figure 5.5).

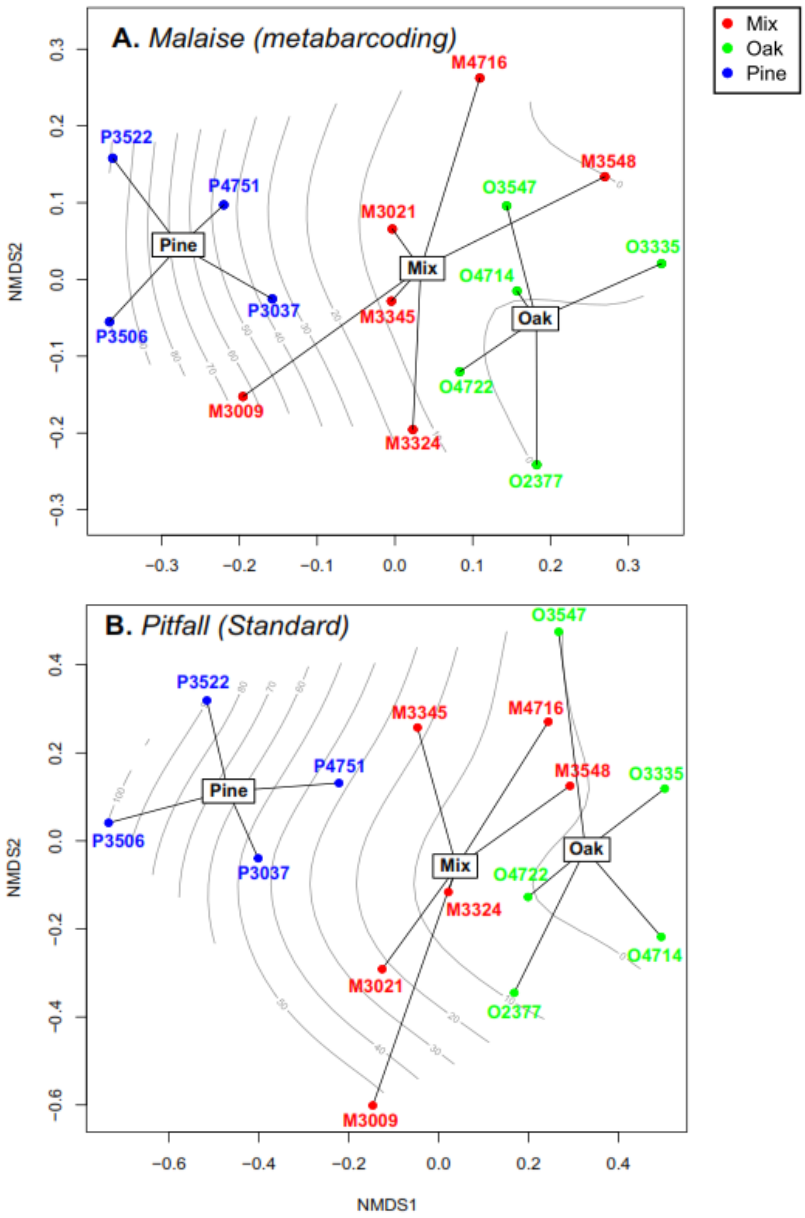

Figure 5.5: Quantitative Jaccard NMDS ordinations for (A) the Metabarcode dataset containing Malaise-trap-sampled arthropods, and (B) the Standard dataset containing pitfall-trap-sampled carabid beetles and spiders. Contours represent the continuous variable

Results of multivariate LR tests in *mvabund* support these observations: a significant overall effect of crop type (Oak/ Mix/ Pine) on community composition was found in both the metabarcode dataset (community ~ crop_type (Poisson errors), $LR_{df=2,12}$=15243, p=0.001) and the standard dataset (community ~ crop_type (negative binomial errors) $LR=338_{df=2,12}$, p=0.014). Pine sites differed from Mixed and Oak sites in both datasets, although this result became non-significant in the case of the Standard dataset when p-values were corrected for multiple tests. The species

composition of Oak sites differed marginally from that of Mixed sites in the Metabarcode dataset but not in the Standard dataset (Table 5.6).

Table 5.6: For both the Metabarcode (Poisson errors) and Standard (negative binomial errors) datasets, the results of multivariate likelihood ratio tests in *mvabund*, comparing each crop type with each other type. Results based on 999 bootstrap iterations with pit.trap resampling. Raw *p*-values are shown, but significance is assigned after correction for three tests using Benjamini and Hochberg's (1995) correction (*method="fdr"*).

Metabarcode	df	LR statistic	*p*-value
Pine vs. Oak	7	8906	**0.001****
Pine vs. Mixed	8	7976	**0.001****
Mixed vs. Oak	9	4189	**0.04***
Standard			
Pine vs. Oak	7	219.1	0.020
Pine vs. Mixed	8	176.9	0.049
Mixed vs. Oak	9	64.76	0.587

Similar to the fine-grained temporal resolution seen in Figure 5.3, community analysis of the metabarcode data also appears to detect fine-grained differences due to canopy-tree composition. Mixed sites with a high proportion of oak (M4716 and M3548; Table 5.2) have arthropod communities typical of Oak sites, while the only one that is dominated by Scots pine (M3009) has a community that more closely resembles those found in Pine sites. This effect can also be seen in the almost straight contour lines for the environmental variable %Pine (Panel A, Figure 5.5), which describes the broadleaf/conifer ratio of the sites. I examine the environmental determinants of arthropod community structure in more detail in section 5.4.6 below.

5.4.4 Does mixed-crop forest have biodiversity benefits?

Here, I consider whether a net positive effect on biodiversity would be achieved by replacing a mosaic of single-species plantations with continuous cover of a mixed-species crop.

Beta diversity

Ordinations (Figure 5.1, Figure 5.5) and Table 5.4 suggest that Mixed is the least distinct crop type in terms of arthropod species composition. In the ordinations, the Mixed sites cover less ordination space than does a combination of Oak and Pine sites, and they particularly fail to cover the space occupied by Pine sites. This tells us that greater regional diversity of arthropods can be achieved by planting a mosaic of pure-oak and pure-pine plantations than could be achieved by planting only mixed-crop plantations of the sort considered in this study, mainly because such a substitution would incur the loss of pine specialists.

Alpha diversity

Surprisingly, I did not find that the Mixed crop type had higher estimated total species richness of arthropods than did the Oak crop type, even though it seems intuitive that Mixed sites should be able to support greater diversity through catering for both oak and pine specialist species. Both Oak and Mixed forest had higher estimated richnesses than Pine forest (Table 5.7).

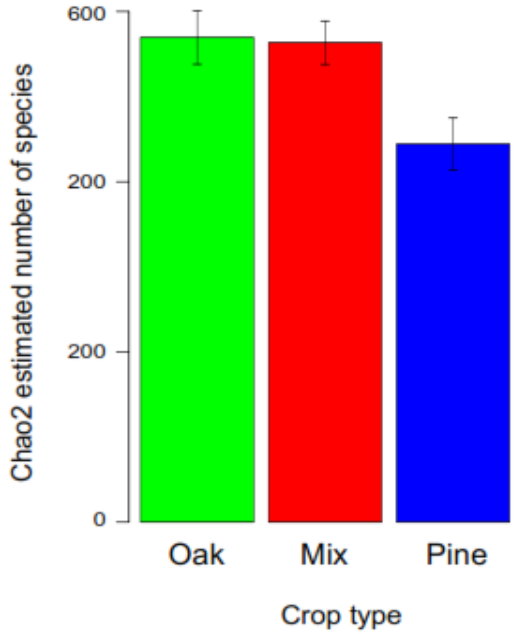

Figure 5.6: Bar chart showing the Chao2 estimated total number of arthropod species (± 1 s.e.) for each crop type.

Table 5.7: Results of pairwise comparisons of Chao2 estimated total species richness between crop types, using a manual Welch's *t*-test based on the Chao2 estimates and standard errors returned by function *specpool()* in *R* package *vegan*. *P*-values were derived from *t* –values and degrees of freedom using *R* function *pt()*, and corrected for 3 pairwise tests using *p.adjust(method="fdr")*.

	T	df	*P*
Mixed vs Oak	-0.15	79	0.559
Mixed vs Pine	2.98	79.9	**0.0019****
Oak vs Pine	2.85	85.6	**0.0027****

Overall, these data provide no evidence that mixed-crop sites are able to substitute fully for pure-pine and pure-oak sites in terms of either alpha or beta diversity of flying arthropods.

5.4.5 Are monocultures bad for biodiversity?

Beta diversity

Although nine of the fifteen study sites were characterised by a single-species forest crop, seven of these contained one or more additional (non-crop) tree species in the understorey or lower canopy layers. Non-crop tree species included beech (*Fagus sylvatica*), field maple (*Acer campestre*), ash (*Fraxinus excelsior*), hawthorn (*Crataegus monogyna*), holly (*Ilex aquifolium*), and silver birch (*Betula pendula*). Only two sites (P3522 and, probably, P3506) can be considered true monocultures, containing only Scots pine over-storey and no understorey trees (Table 5.9). These two Pine sites have previously been mentioned as having the most distinctive arthropod communities (Figure 5.1), featuring species that are not found in other sites (Figure 5.2; Table 5.4). The results of multivariate Poisson LR tests in *mvabund* support the observation that the Pine monoculture communities are distinct from those of other sites (Community ~ Monoculture status: $LR_{df=13}$=9098, p=0.001).

Order-specific NMDS ordinations show that the distinctiveness of the pine monoculture communities is not driven by a single group but can be observed in several insect orders, including Diptera, Hemiptera, Lepidoptera and Coleoptera (Figure 5.7). Furthermore, the twelve OTUs identified by the *mvabund* analysis as being specialists of Pine monocultures (Table 5.4, above) included members of the insect orders Diptera, Coleoptera and Hemiptera, as well as two species of spider.

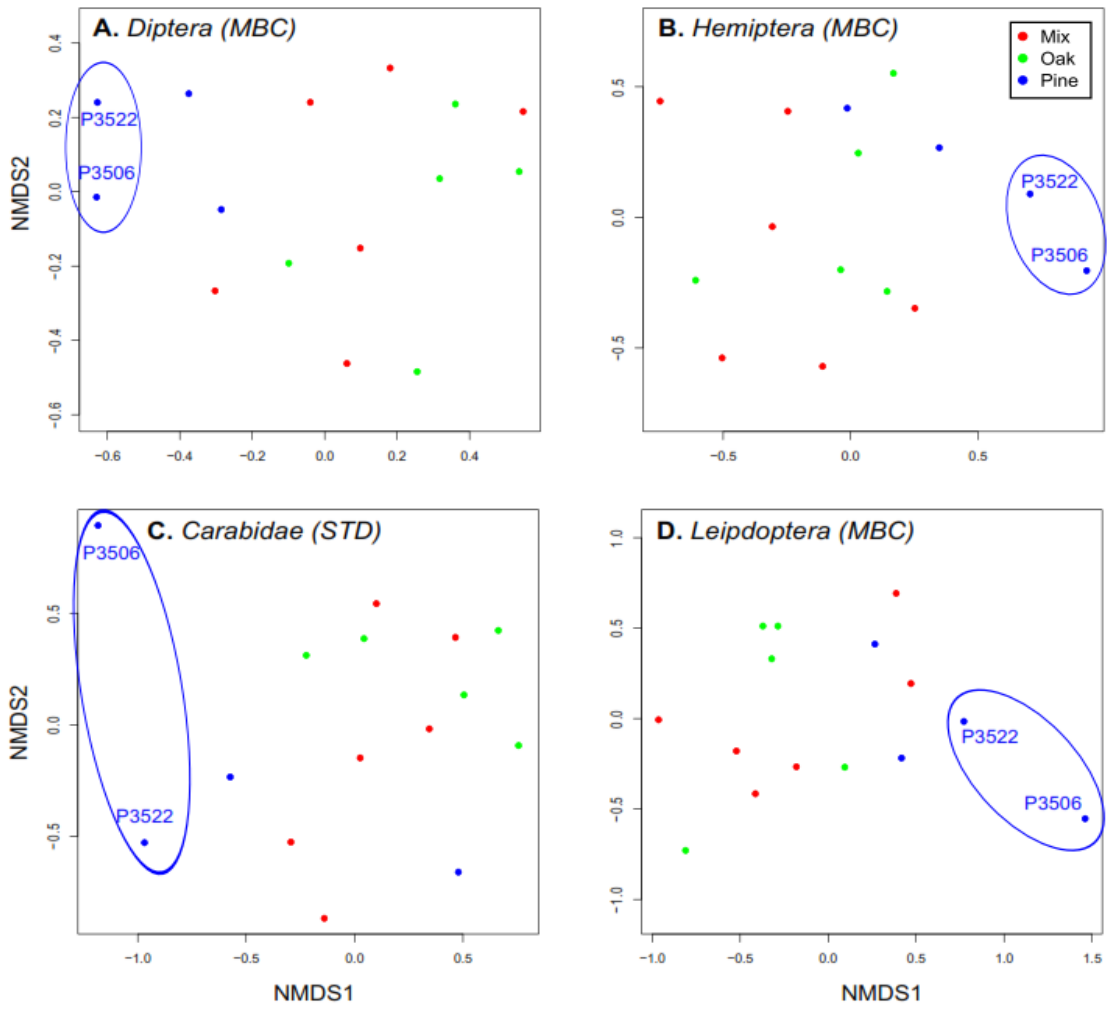

Figure 5.7: NMDS ordinations based on the pooled dataset for each of the four best represented insect groups, (A) Diptera, (B) Hemiptera, (C) Carabidae (Coleoptera), and (D) Lepidoptera. Carabidae community data come from the Standard dataset, while community data for the other three groups comes from the metabarcode dataset. Points are coloured by forest crop type, and the two pine monocultures are labelled and circled in blue.

Alpha diversity

Not only are the arthropod communities in pine monocultures distinctive, but they do not have significantly lower observed species richnesses than non-monoculture communities (Poisson GEE on 15 groups, richness ~ monoculture, $\chi^2_{df=1}$= 0.978, p=0.32), despite having the lowest plant species richness (*P. sylvestris* with a ground cover of bracken fern) and lacking structural complexity. Figure 5.8, which shows Chao2 estimated total species richness for each of the fifteen sites, also suggests that monoculture sites are not less species richness than non-monoculture sites. Interestingly, the sites that have the highest estimated total species richness are Oak sites rather than Mixed sites.

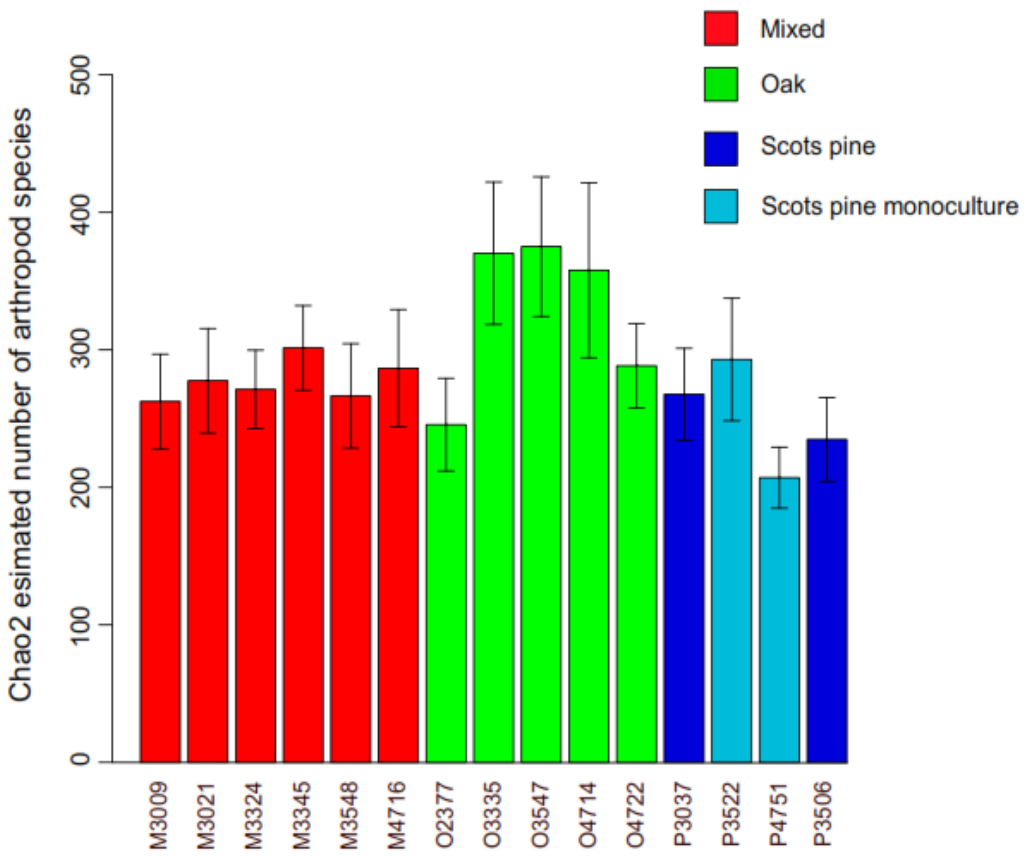

Figure 5.8: Barchart showing estimated total number of arthropod OTUs at each site. Calculated using the Chao2 estimator via *R* function *specpool()*, and the binary arthropod dataset. Error bars represent ±1 s.e.

Thus, there is no evidence that Pine monocultures are particularly bad for biodiversity; indeed, , they actually seem to make a positive contribution to regional biodiversity in this case, through supporting specialists.

5.4.6 How well do coarse indicators predict biodiversity?

Alpha diversity – all arthropods

Having a total of 16 candidate coarse indicator variables posed a model-selection problem. I took advantage of the fact that the indicators included several subsets of inter-correlated variables (Table 5.8), and chose one from each subset for initial inclusion in the full model, as explained in the Methods section. The eight variables chosen for inclusion in the initial model were Simpson_count, Crop_density, Stump_area, Deadwood_area, Stump_count, Deadwood_count, Non-crop_density, and %Pine (Table 5.3).

Table 5.8: Table showing adjusted R^2 (lower matrix) and un-adjusted *p*-values (upper matrix) for pairwise linear correlations of all coarse indicator variables. * indicates unadjusted $p \leq 0.002$, and header bar colours represent subsets of inter-correlated variables.**

	Stem_density	Tree_species	%Pine	Crop_density	Non-crop_density	Deadwood_area	Deadwood_count	Stump_area	Stump_count	DS_area	DS_count	Simpson_count	Simpson_area	SCI	ESCI_1	ESCI_2
Stem_density		0.1	0.44	0.08	***	0.95	0.69	0.9	0.01	0.91	0.01	0.37	0.2	0.17	0.19	***
Tree_species	0.2		0.46	0.57	0.06	0.55	0.38	0.81	0.52	0.92	0.24	***	***	***	***	***
%Pine	0	0.03		0.16	0.14	0.9	0.93	0.06	0.78	0.07	0.84	0.52	0.21	0.17	0.19	0.33
Crop_density	0.17	-0.1	0.09		0.62	0.46	0.95	0.98	0.39	0.85	0.39	0.89	0.57	0.88	0.85	0.1
Non-crop_density	**0.87**	0.2	0.1	-0.1		0.72	0.67	0.89	0.02	0.96	0.01	0.34	0.08	0.13	0.16	***
Deadwood_area	-0.1	-0.1	-0.1	0	-0.1		0.04	0.89	0.51	0.51	0.54	0.54	0.08	0.6	0.64	0.91
Deadwood_count	-0.1	0	-0.1	-0.1	-0.1	0.26		0.74	0.4	0.44	0.15	0.54	0.22	0.22	0.27	0.21
Stump_area	-0.1	-0.1	0.21	-0.1	-0.1	-0.1	-0.1		0.99	***	0.85	0.6	0.6	0.43	0.4	0.77
Stump_count	0.4	-0.1	-0.1	0	0.34	0	0	-0.1		0.87	***	0.83	0.74	0.8	0.75	0.27
DS_area	-0.1	-0.1	0.19	-0.1	-0.1	0	0	**0.94**	-0.1		0.74	0.51	0.36	0.51	0.48	0.79
DS_count	0.41	0.04	-0.1	0	0.4	0	0.1	-0.1	**0.58**	-0.1		0.52	0.28	0.61	0.69	0.05
Simpson_count	0	**0.65**	-0.1	-0.1	0	-0.1	-0.1	-0.1	-0.1	0	-0.1		***	***	***	0.02
Simpson_area	0.06	**0.55**	0.06	-0.1	0.17	0.17	0.05	-0.1	-0.1	0	0.02	**0.72**		0.01	0.01	0.04
SCI	0.08	**0.68**	0.08	-0.1	0.11	-0.1	0.05	0	-0.1	0	-0.1	**0.52**	0.4		***	***
ESCI_1	0.06	**0.67**	0.07	-0.1	0.09	-0.1	0.03	0	-0.1	0	-0.1	**0.52**	0.37	**1**		***
ESCI_2	**0.65**	**0.58**	0	0.14	**0.53**	-0.1	0.06	-0.1	0.02	-0.1	0.22	0.31	0.26	**0.58**	**0.55**	

Table 5.9: Values of the eight selected indicator variables at each site. For compartment P3506, * indicates that the value is based on the assumption that the site was a monoculture with similar crop properties to compartment P3522.

Site	%Pine	Crop density	Non-crop density	Deadwood count	Deadwood area (m^2)	Stump count	Stump area (m^2)	Simpson count
M3009	68	15	2	0	0	8	135.6	2.0
M3021	8	25	38	2	8.9	9	16.3	3.0
M3324	14	25	18	1	0.8	3	22.6	3.7
M3345	9	22	88	5	15.5	7	118.9	5.3
M3548	3	34	70	10	9.5	6	50.8	2.1
M4716	1	30	82	0	0	9	66.0	2.5
O2377	0	26	10	0	0	10	27.9	1.7
O3335	0	30	62	1	0.6	2	57.3	2.2
O3547	0	21	140	0	0	16	38.5	1.5
O4714	0	25	5	0	0	2	5.8	1.4
O4722	0	15	68	4	33.2	6	11.8	2.3
P3037	29	18	44	4	5.4	6	20.1	2.9
P3506	100*	48*	0*	NA	NA	NA	NA	1.0*
P3522	100	48	0	4	11.4	3	91.2	1.0
P4751	46	80	93	1	0.3	14	37.3	3.4

There was no effect of ESCI_2, the enhanced structural complexity indicator, on species richness in these plantation sites (GEE regression: Wald statistic=0.13, p=0.72).

In the stepwise GEE regression, all remaining terms were significant after the removal of variables Deadwood_area and Stump_area (Poisson GEE, n=14, richness ~ Simpson_count + Crop_density + Non-crop_density + %Pine + Deadwood_count + Stump_count; See Table 5.10 for gradients, Wald coefficients, and p-values).

Table 5.10: Table showing Wald statistics and *p*-values for each variable in the six-term arthropod GEE model.

Variable	Gradient	s.e.	Wald	*p*-value
Simpson_count	0.052	0.018	8.63	0.003
Crop_density	-0.006	0.001	27.47	<0.001
%Pine	0.003	0.0007	15.31	<0.001
Non-crop_density	0.003	0.0009	13.59	<0.001
Stump_count	-0.024	0.007	13.06	<0.001
Deadwood_count	-0.022	0.009	6.04	0.014

However, for variables Deadwood_count and Stump_count, the direction of the effect was negative (i.e. fewer species in sites with more deadwood and stumps), which makes little sense from a biological perspective. Combined with the fact that measurement of these particular variables was extremely imprecise, it seems likely that their effects are statistical artefacts. Removing Deadwood_count and Stump_count from the model caused the variables %Pine and Non-crop_density to become non-significant, revealing the instability of the six-term model given above, and leaving a minimal model with just two significant terms: a negative effect of Crop_density (Wald=3.96, p=0.046) and a positive effect of Simpson_count (Wald=9.68, p=0.0016; Figure 5.10).

Plotting the Pearson residuals for the minimal two-term GEE model showed them to be normally distributed, and reasonably free from heteroscedasticity (Figure 5.9). There was no correlation between the size of the residuals and predicted species richness (linear regression, Pearson residuals ~ predicted species richness, $F_{1,110}$=0.006, R^2=0.006, p=0.94).

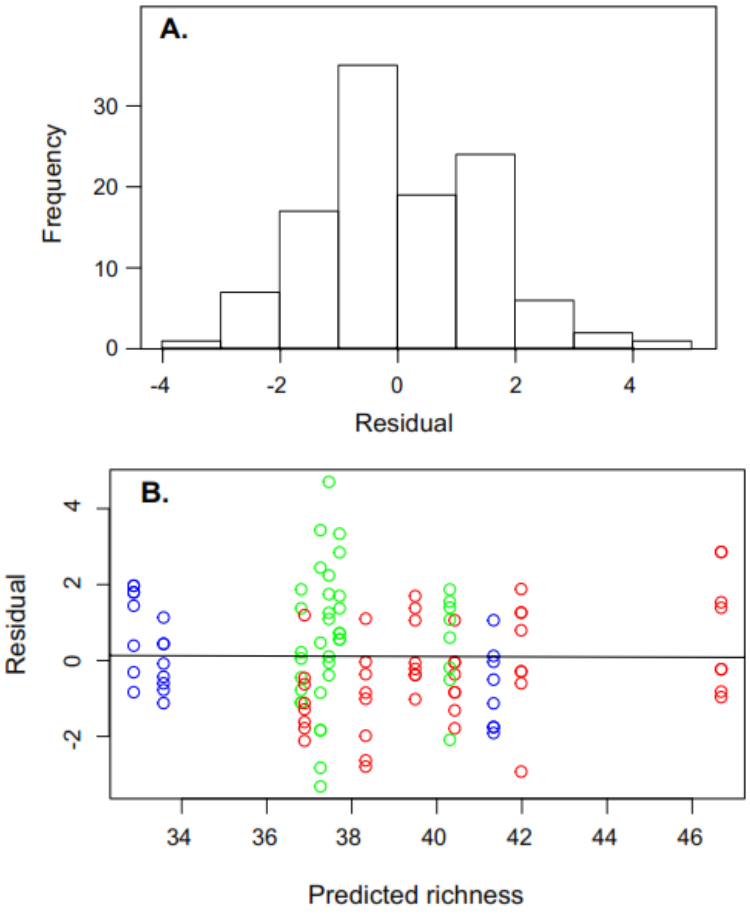

Figure 5.9: (A) Histogram of Pearson residuals for the minimal two-term GEE model for arthropod species richness (richness ~ Crop_density + Simpson_count). (B) Scatterplot showing the same residuals plotted against predicted arthropod species richness, with regression line shown.

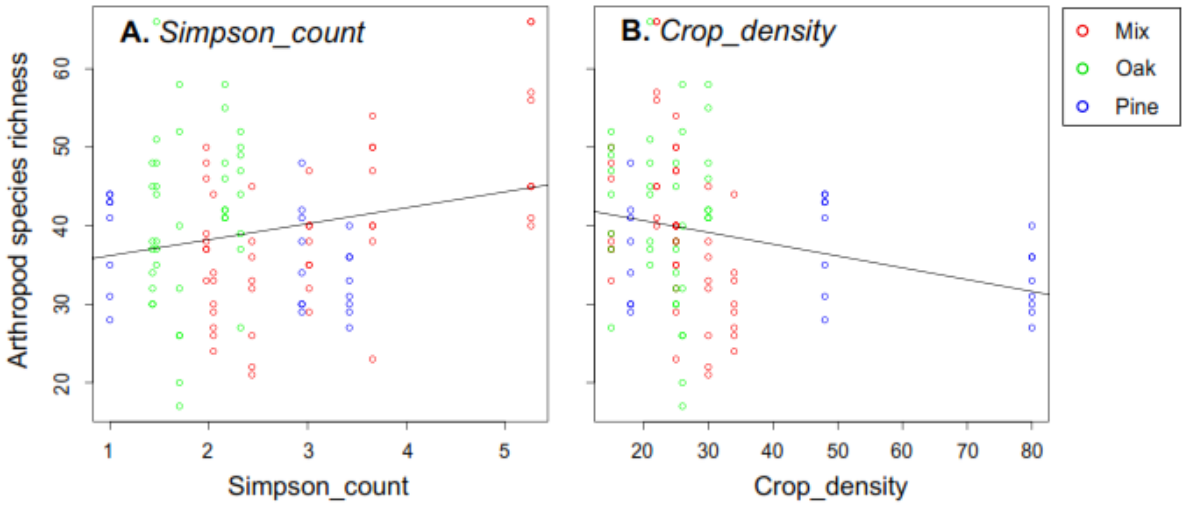

Figure 5.10: Scatterplots showing the relationship between number of arthropod species per Malaise-trap sample and each of the coarse indicator variables that features in the minimal GEE model for arthropods: (A) Simpson_count, and (B) Crop_density. Points are coloured by crop type.

The effect of Crop_density appears to be driven by a single site (P4751, far right hand side of Figure 5.10B), which has very high crop density and lower species richness of arthropods. Removing this one site caused the effect of Crop_density to become non-significant (Wald statistic = 0.67; *p*=0.67). Likewise, the positive effect of Simpson_count appears to be driven by site M3345 (far right hand side of Figure 5.10A), which has high tree species diversity and also high arthropod species richness. Once again, removing this site from the dataset resulted in the loss of significance for this variable (Wald=0.005, *p*=0.95).

In short, no structural indicator was found to have a robust relationship with arthropod species richness in the sites considered here.

Alpha diversity – order-level analyses

Considering Diptera alone, the minimal GEE model featured the same variables as did the model above for all arthropods: Simpson_count and Crop_density (Table 5.11, Figure 5.11), which is not surprising given that Diptera account for the majority of arthropod OTUs.

Table 5.11: Statistics and *p*-values for the two variables in the minimal GEE model for Diptera.

Variable	Gradient	s.e.	Wald	*p*-value
Simpson_count	0.079	0.020	15.2	<0.001
Crop_density	-0.005	0.001	21.9	<0.001

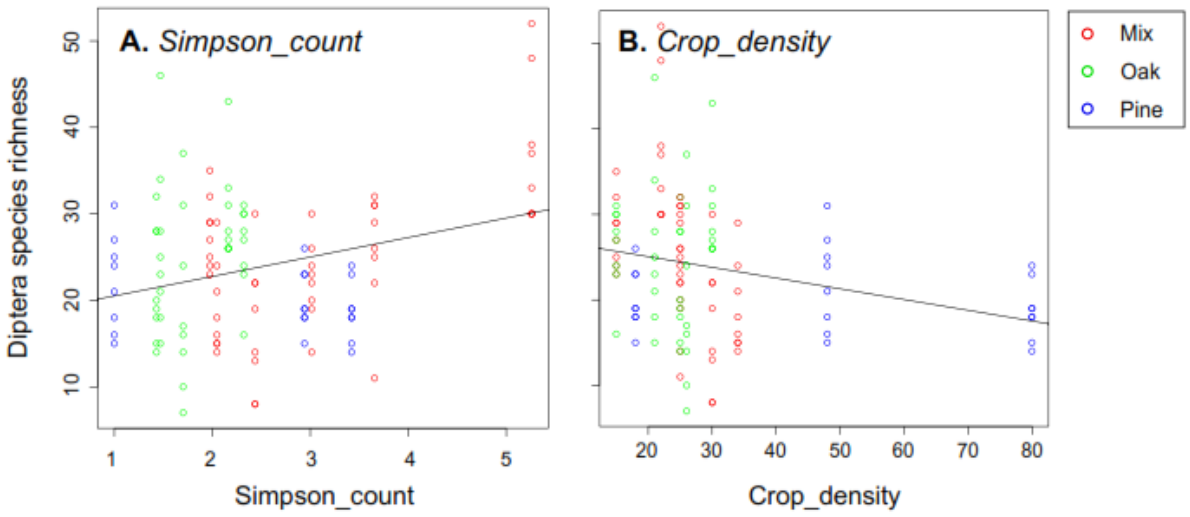

Figure 5.11: Scatterplots showing the relationship between the number of Diptera species per Malaise-trap sample and each of the coarse indicator variables that feature in the minimal GEE model for Diptera: (A) Simpson_count, and (B) Crop_density.

Once again, the observed effects of the Simpson_count and Crop_density variables were driven by sites M3345 and P4751 respectively, and exclusion of these sites resulted in loss of significance for both variables.

For all other orders, I used a Poisson GLM on the site-level species-richness data, since these groups were represented by few species in any one Malaise trap sample. For Coleoptera, the best-fit model given by both manual and automated step-wise regression included only the single term %Pine (Poisson regression: z_{13}=-2.05, p=0.041). Species richness declined in compartments with a higher frequency of pine trees (Figure 5.12; b=-0.005, AIC=76.13, $\chi^2_{1,12}$=4.54, p=0.03). The model is not ideal: variance in residuals increases with predicted species richness, since the %Pine variable cannot explain variation in richness among sites where pine is absent. However, it could not be improved upon using the variables that were measured in this study.

I found no variable, or combination of variables, that could explain variance in species richness for Lepidoptera, Hemiptera, Hymenoptera, or Arachnida.

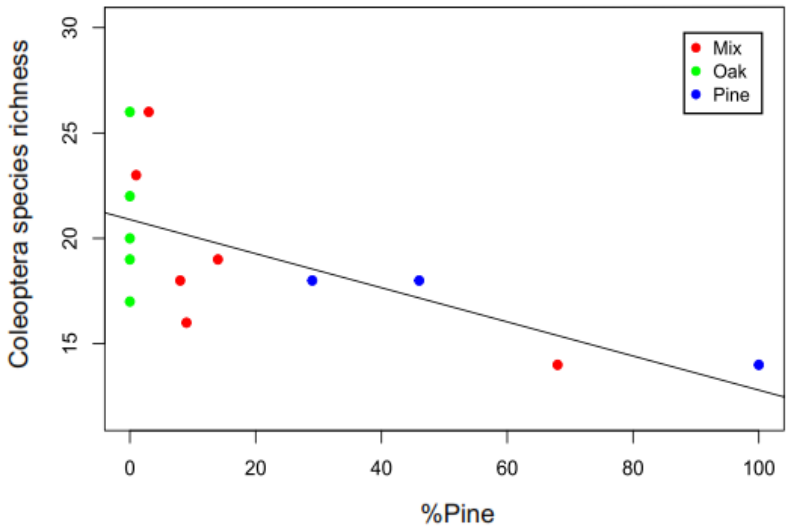

Figure 5.12: Scatterplot showing number of beetle species per site (pooled over the eight malaise trap samples) plotted against the coarse indicator variable %Pine.

These analyses suggest that it is difficult to find general predictors of species richness at the scale considered here.

Beta diversity

I used the pooled community dataset for exploring the effect of coarse indicators on arthropod community composition, testing the same variables that had been considered in the species richness model above (Table 5.9).

Of the eight variables tested, only %Pine was significant as a single term in an *mvabund* model, and this was also found to be significant in the PERMANOVA and CCA permutation tests (Table 5.12). Recall that crop types were separated along the primary axis in the pooled NMDS ordination (Panel A, Figure 5.5): Oak sites were at the opposite end of the axis to Pine sites, with Mixed sites positioned between them. The %Pine variable, which is defined as the percentage of all measurable stems that are Scots pine (contours in Figure 5.5, above) is a continuous measure of this difference between crop types. %Pine can also explain variation among individual Mixed and Pine sites, since it takes into account both planted (crop) and naturally regenerated (non-crop) trees. Clearly this variable cannot explain variation among Oak sites, since they contain no Scots pine. Nevertheless, %Pine correlates strongly with axis scores from NMDS axis 1 (linear regression, NMDS1 ~ %Pine, $F_{1,13}$=74.5, R^2=0.85, p<0.001), and I consider it the primary explanatory variable for beta diversity.

In terms of other variables, Non-crop_density (the density of naturally regenerated measurable stems) was found to be significant in the CCA test, and Crop_density in the PERMANOVA test (Table 5.12), but significance was detected for neither variable in the *mvabund* analysis, which is the most reliable method for significance testing. No method assigned significance to any other variable when considered as a single term.

Table 5.12: Results of three statistical tests for the ability of six coarse indicator variables to predict arthropod beta diversity: mvabund, using pit.trap resampling and 999 bootstrap permutations; PERMANOVA, using vegan package's adonis() function on a Jaccard distance matrix, with 999 permutations; ANOVA-type permutation test for significance of constraints in constrained Correspondence Analysis (CCA), with 999 permutations.

	Mvabund		***PERMANOVA***		***CCA***		
Variable	LR	*p*	*F*	*p*	*F*	*p*	**Df**
%Pine	841.1	**0.03**	1.96	**0.001**	1.93	**0.001**	1,13
Simpson_count	537.3	0.23	1.02	0.180	1.12	0.191	1,13
Crop_density	548.4	0.21	1.33	**0.013**	1.20	0.073	1,13
Non-crop_density	583.9	0.20	1.22	0.093	1.32	**0.021**	1,13
Deadwood_area	544.4	0.33	0.96	0.650	0.99	0.490	1,12
Stump_area	550.1	0.26	1.03	0.389	1.11	0.195	1,12
Deadwood_count	502.5	0.614	0.86	0.881	0.84	0.980	1,12
Stump_count	504.7	0.552	0.78	0.984	0.91	0.830	1,12

Multiple-term models. – Given the above results, I wanted to ensure that the %Pine variable was included in any multiple-term model for beta-diversity. Therefore, I used a forward selection approach in *mvabund*, adding each of the other variables as a second term to a model which had %Pine as its first term. The terms that were significant in these two-term models were Crop_density, Simpson_count, and Stump_area.

Species' responses to each of these variables were visualised via heatmaps, with sites arranged according to the variable of interest (Figures 5.13, 5.14, 5.15, and 5.16). For Stump_area, the heatmap showed that the effect was driven primarily by species that were absent when the total cross-sectional area of stumps was high (Figure 5.16). This makes little biological sense, and suggests, like in the alpha diversity analysis, that effects of the poorly measured stump and deadwood variables are artefactual, rather than real. Therefore, Stump_area was excluded from further analyses.

The other two variables (Crop_density and Simpson_count) were tested as terms in a three-term model with %Pine (Arthropod community ~ %Pine + Crop_density + Simpson_count). All terms in this model were highly significant (%Pine: LR=1230.5, p=0.001; Crop_density: LR=878.6, p=0.003;

Simpson_count: LR=911.2, *p*=0.001; overall result for three-term model: LR=2940, *p*=0.001), and a quantitative Jaccard distance matrix based on the three constituent indicators was significantly correlated with that based on the pooled community dataset of Malaise trap arthropods (Mantel test: *r* = 0.60, *p*=0.001), suggesting that the combination of these three variables can accurately predict patterns of compositional differentiation among sites.

LR coefficients and *p*-values were generated for the response of every OTU to each of the three significant variables (%Pine, Simpson_count, and Crop_density) to pick out the OTUs that were driving the effects. The variable to which the greatest number of species showed a significant response was %Pine (Table 5.13), which fits the fact that this was the only variable that all three types of test found to be a significant predictor of beta diversity. Furthermore, a greater number of OTUs were associated with high values of Simpson_count than with low ones, and vice versa for Crop_density. This is consistent with the results of the species richness analysis, which suggested that greater species richness *may* be associated with high Simpson_count and low Crop_density values (Table 5.10, Figure 5.10).

Table 5.13: Table showing the number of OTUs from each taxonomic group that responded significantly to each of the three variables affecting arthropod community composition, and the direction of the response. Based on Poisson likelihood-ratio tests in *mvabund* with pit.trap resampling and 999 bootstrap iterations.

	%Pine		**Crop density**		**Simpson count**	
	Positive	*Negative*	*Positive*	*Negative*	*Positive*	*Negative*
Arachnida	3	2	0	0	0	1
Coleoptera	1	1	1	0	0	0
Dermaptera	0	0	1	0	0	0
Diptera	14	18	6	13	15	6
Hemiptera	0	2	1	1	1	0
Hymenoptera	0	2	0	1	1	0
Lepidoptera	0	1	0	0	1	0
Odonata	0	0	0	0	1	0
Orthoptera	0	2	0	0	0	0
Unknown	0	0	0	0	0	1
Total	**18**	**28**	**9**	**15**	**19**	**8**

In summary, %Pine is the strongest predictor of Arthropod community composition, while there is some additional influence of Crop_density and Simpson_count, which is a measure of tree species diversity. Taken together, these three variables can predict patterns of arthropod community composition.

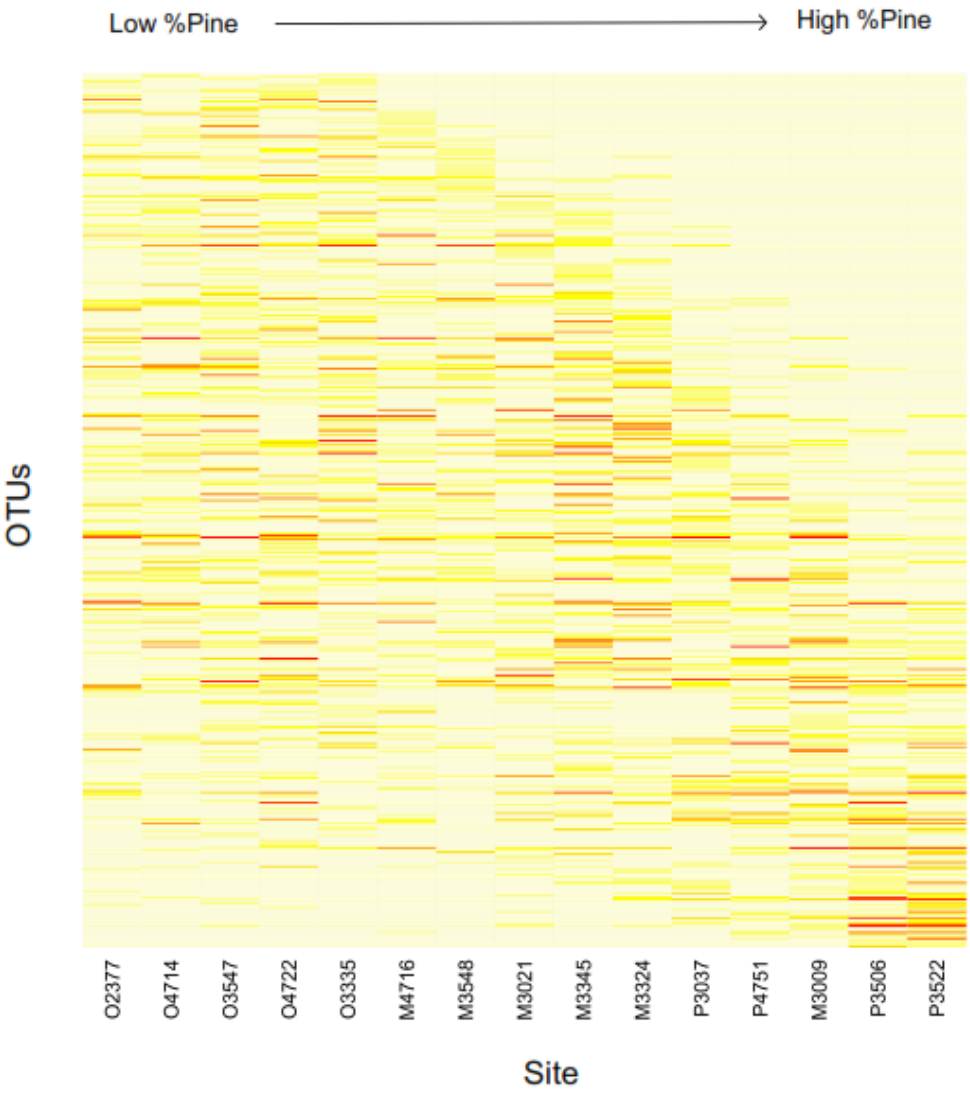

Figure 5.13: Heatmap of arthropod species, with sites sorted by the %Pine variable. Red shows high incidence.

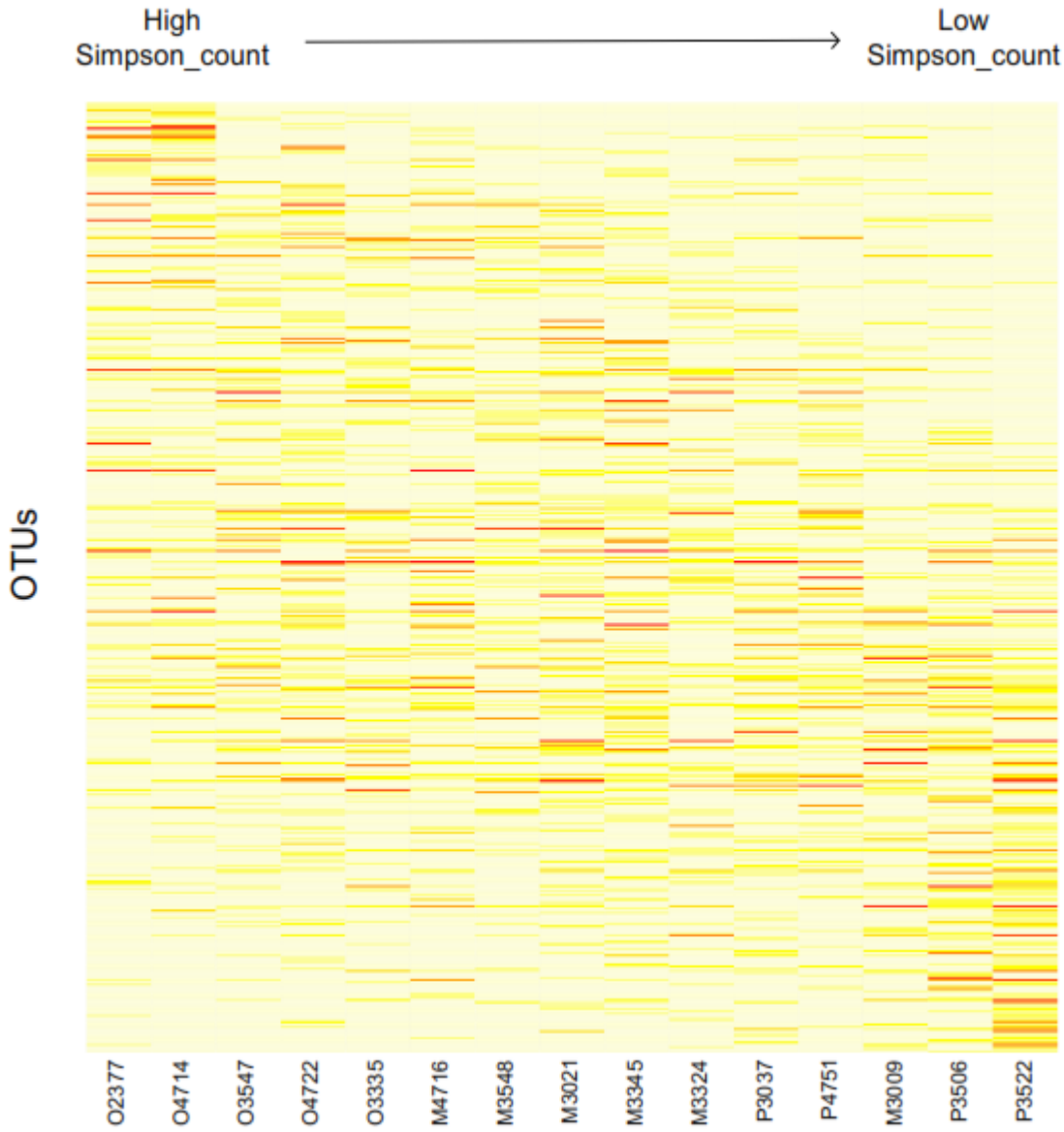

Figure 5.14: Heatmap of arthropod species, with sites sorted by the Simpson_count variable. Red shows high incidence.

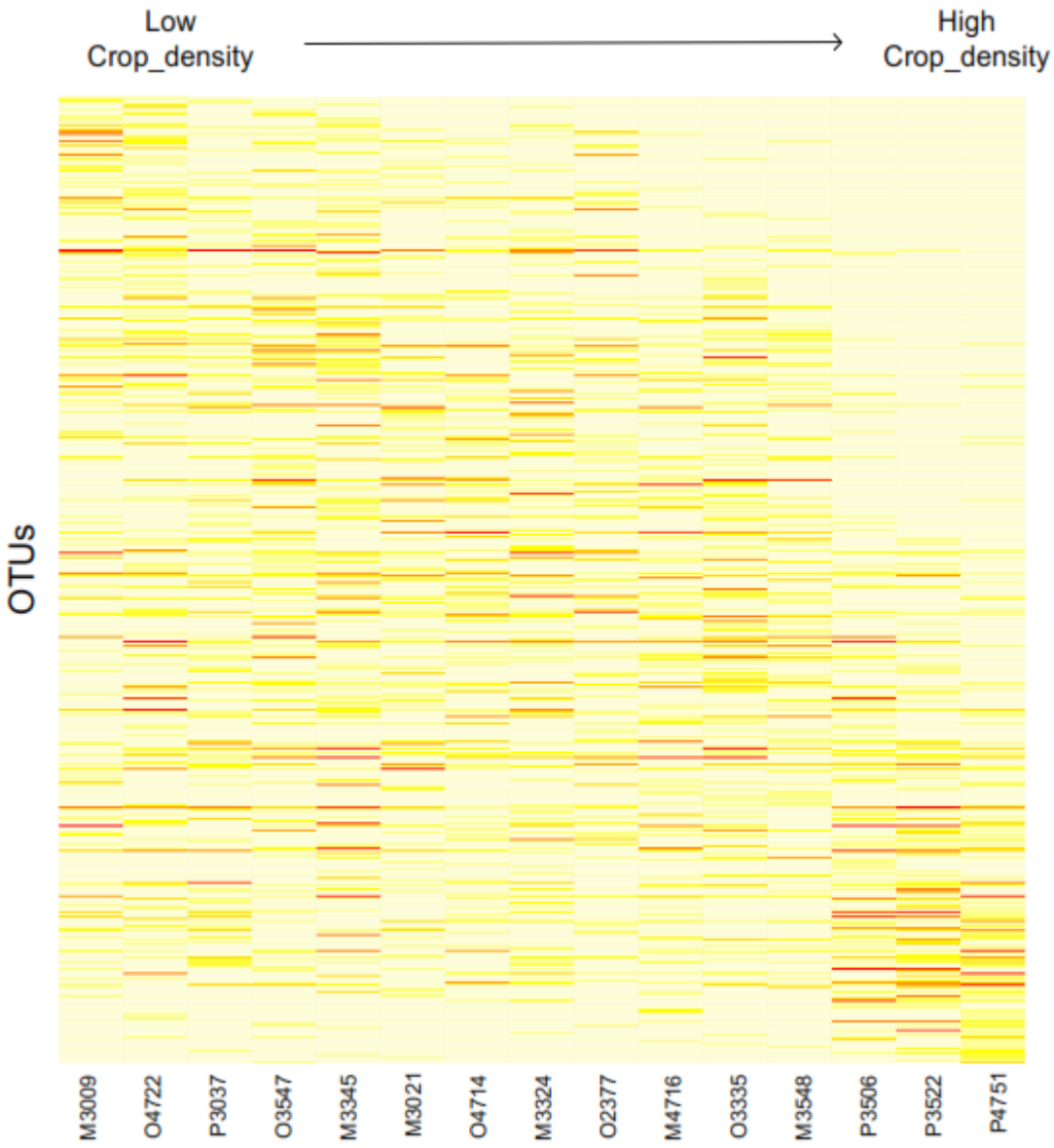

Figure 5.15: Heatmap of arthropod species, with sites sorted by the Crop_density variable. Red shows high incidence.

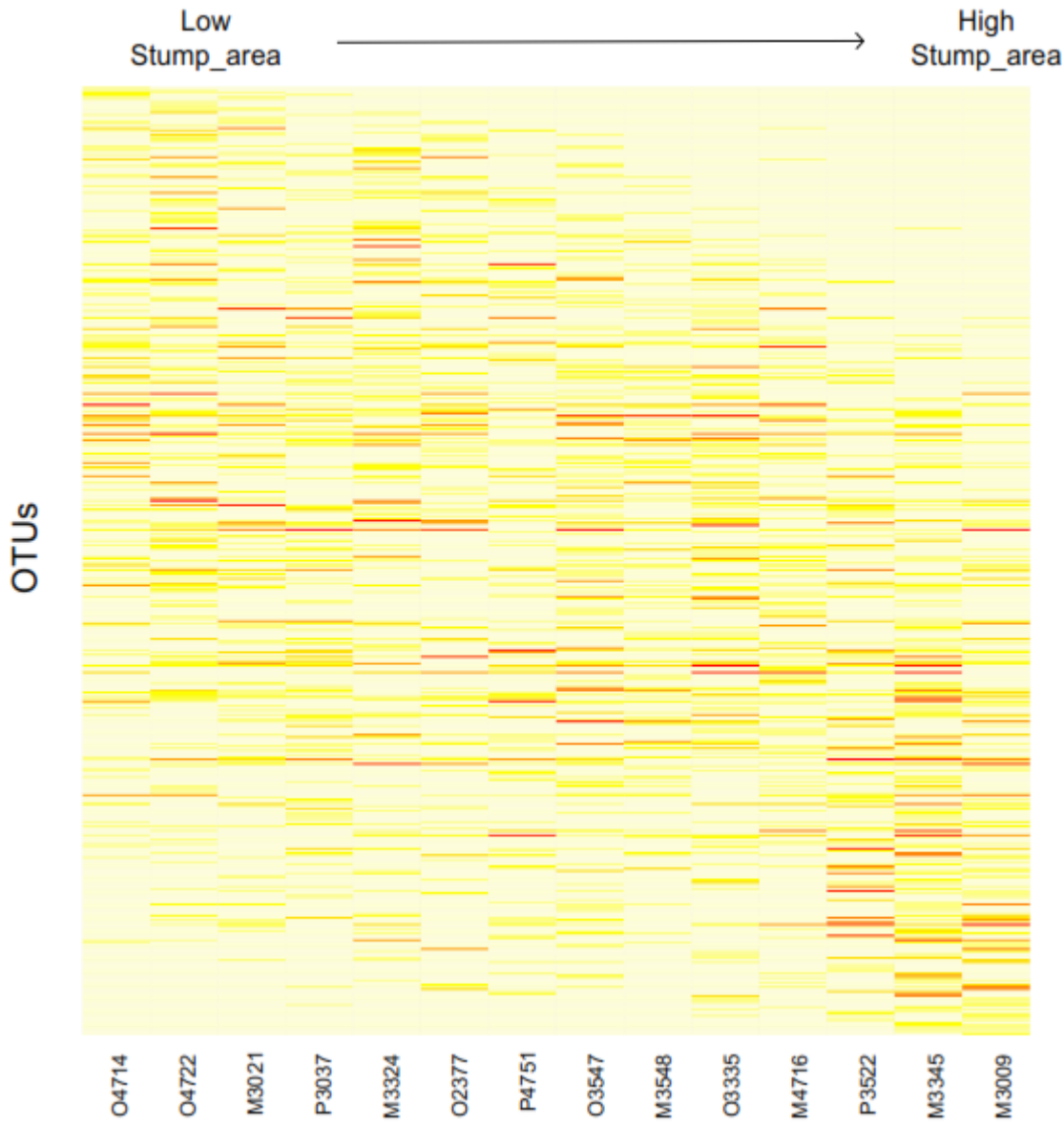

Figure 5.16: Heatmap of arthropod species, with sites sorted by the Stump_area variable. Red shows high incidence.

5.5 Discussion

5.5.1 Spatial and temporal variation in arthropod communities

Metabarcode data revealed fine-scale temporal and habitat variation between arthropod communities in a plantation forest ecosystem. The rapid turnover of species is detectable from one week to the next (Figure 5.3), and the differences in species composition between samples collected at the same location but seven weeks apart were similar to the differences between samples collected simultaneously in very different forest types (Oak versus Pine, Figure 5.1, Figure 5.3). Therefore, a key message is that biodiversity surveys across habitat types must collect in synchrony or at least block samples by week (i.e. collect a subset of all habitats in each week). Otherwise, surveys risk confounding temporal effects with environmental differences.

In terms of variation among sites, plantation crop type had a strong effect on community composition, with oak and Scots pine plantations supporting markedly different arthropod communities across all sampling dates, which is consistent with the findings of previous studies (e.g. Day *et al*., 1993). Here, inclusion of oak-pine mixtures revealed that compositional differences tracked with the ratio of broadleaf to conifer trees in a plantation stand (Figure 5.5). Factors associated with the different plantation types that are likely to directly influence species occurrence include association with a particular tree species; dependence on deciduous leaves or leaf-litter, which will occur in proportion with the number of broadleaf trees; and environmental factors associated with the different light regimes that occur in deciduous and coniferous plantations.

5.5.2 Validation with the Standard dataset

The high level of compositional correlation with the Standard dataset (Figure 5.5) helps to validate the Metabarcode data. Such a strong correlation was somewhat unexpected because Malaise trapping and pitfall trapping sample very different arthropod groups, with pitfall-sampled taxa more likely to be influenced by factors such as ground or field-layer vegetation and leaf litter. The fact that a pitfall trap dataset composed of ground beetles and spiders detected the same beta-diversity patterns as a Malaise-trap dataset dominated by Diptera provides some support for the use of indicator groups in assessing patterns of beta diversity; so too does the fact that several different insect groups independently showed the same two Scots pine stands as having the most distinct communities. However, these correlations between different groups may not hold at all spatial scales (Gaspar *et al*., 2010).

5.5.3 The effects of monocultures and mixed-species crops

I expected to find evidence that monocultures are biodiversity-poor, while mixed-species crops provide biodiversity benefits, but results did not entirely support these hypotheses. First, although Scots pine sites had the lowest species richness of the three crop categories considered in this study (Figure 5.6), the two true monocultures sites (P3522 and P3506) featured the most distinctive arthropod communities, including species that did not occur elsewhere (Figure 5.1, Table 5.4). Therefore, these stands, which seem lacking in biodiversity potential if judged on the basis of indicators such as tree species diversity and structural complexity, were actually found to make a unique contribution to regional arthropod biodiversity.

Second, I found no evidence that mixed-species stands support higher levels of biodiversity at a single point than do pure oak crops. Although this seems counterintuitive, a similar result was reported by Oxbrough *et al*. (2012), who also found no species richness benefit of a two-species crop compared with a single-species crop. Their explanation was that naturally regenerated understorey trees can make a greater contribution to structural complexity and tree diversity in the lower levels of the forest than can the addition of a second canopy (crop) species. Interestingly, Figure 5.14 shows that the sites with a pure oak crop have higher overall tree species diversity than do those with a mixed or pure Scots pine crop. This is likely explained by more light being able to reach the floor in broadleaf forest compared with in conifer forest, making it easier for understorey trees to establish, and it may be a contributing factor to the relatively high levels of arthropod diversity found in the pure oak sites.

Ordinations suggest that arthropod communities in sites with mixed-species crops are compositionally more similar to those in oak sites than to those in Scots pine sites (Figures 5.1, 5.5). However, this is likely to reflect the ratios of the different crop components in the limited number of stands that were sampled for this study. While four out of six mixed-crop sites were heavily dominated by oak (> 75% oak), and had arthropod communities that were more or less typical of pure oak stands, only one was dominated by Scots pine (Table 5.2), and this was compositionally similar to the pine sites (Figure 5.5). This suggests that, had the mixed-crop sites comprised a more even spread of oak/pine ratios, they would have featured more obviously intermediate arthropod communities, and would not have shown a greater overall resemblance to oak than to pine. In order to substitute for a matrix of single-species oak and Scots pine stands, substantial variation in the ratios of the different crop components would need to be included, or there is a risk that regional diversity will be reduced via the loss of specialists. In fact, the distinctiveness of the pine monocultures (Figure 5.1) suggests that mixed-species crops may never

be able to support the full range of species catered for in a matrix of single-species stands. However, considered from another perspective, results suggest that mixing a component of oak into what would otherwise be a (regional scale) conifer monoculture can indeed have a substantial effect in increasing arthropod diversity through the addition of broadleaf specialists.

5.5.5 Structural biodiversity indicators

Structural indicators were found to be more useful for predicting community composition (beta diversity) of Malaise-trap-sampled arthropods than for predicting species richness (alpha diversity). Species richness did not correlate with the enhanced structural complexity index (ESCI), despite a strong relationship having been reported in a subtropical forest in Yunnan, China (Beckschäfer *et al*., 2013). A likely explanation is that the range of complexity occurring in temperate plantation forests is much lower than is typical in tropical forest ecosystems; ESCI values for the plantation stands in Thetford Forest ranged from 6.8 to 49.2, while those for stands in Xishuangbanna (China) ranged from close to zero to over 1500. Although I found some suggestion that species richness may be affected positively by tree species diversity and negatively by the density of the plantation crop (Figure 5.10, Tables 5.10), more data are required from a greater range of sites if robust conclusions are to be drawn concerning the factors that determine species richness at the site level.

In contrast, species composition can be predicted fairly reliably from the broadleaf/conifer ratio, plantation crop density, and tree species diversity of a site. The fact that a greater number of species were associated with high tree species diversity and low plantation crop density (Table 5.13) supports the relationships suggested in the alpha diversity analysis. However, it is important to note that a number of species from a variety of taxonomic groups favoured the opposite conditions (i.e. low tree species diversity or high plantation crop density), which highlights the fact that individual arthropod species vary substantially in the ways in which they respond to the environment. This makes sense from an ecological perspective in terms of niche partitioning (Saetersdal and Gjerde, 2011), and has been illustrated previously in forest ecosystems (e.g. Davies and Margules, 1998). The importance of this point is that indicators seek to generalise, but it may often be impossible to do so in a meaningful way (Lindenmayer *et al*., 2008), particularly along weak environmental gradients (Saetersdal and Gjerde, 2011).

Because this study was focused on a small number of sites that comprised a limited range of forest types, it was not possible to consider the effects of variables such as stand age, location, or large-scale landscape patterns, all of which are likely to have had influenced the results. For instance, site P4751 was an influential site in the alpha diversity model because it drove the

negative effect of canopy density on species richness (Figure 5.10). This forest stand was planted more recently than others (1967 compared with 1930-1941; Table 5.2), and the difference in age might have affected both structural characteristics and the biological community of the site (Smith *et al*., 2008).

5.5.6 Conclusions

Implications for management of plantation forests

My results show that the responses of individual arthropod species to structural characteristics of plantation stands are highly variable, even within taxonomic groups. Thus, even if certain forest characteristics correlate to some extent with increased species richness, they are unlikely to represent the ideal conditions for all species, and forest types that have low species richness (e.g. even-aged conifer stands) are shown to be important for some species that may favour a less complex environment. Therefore, managers should be cautious in eliminating these types of forest from the landscape. Nevertheless, results support the importance of avoiding the dominance of conifer monocultures at large spatial scales, since these have relatively low alpha diversity and are unable to support broadleaf specialists.

Overall, these findings strongly support the assertion of Lindenmayer *et al*. (2006) that "*management for diversity calls for diversity of management"*. That is, maximum biodiversity at a landscape level is achieved not by standardising across all plantation stands to maximise the value of the structural characteristics associated with slightly higher alpha diversity, but by *maximising the variation* between stands in terms of structural characteristics and tree species composition (canopy and understory). Specifically, planting a variety of crop species at a variety of densities – leading in turn to variation in the density and species composition of the understory – should allow plantation forests to cater for the widest variety of arthropod species.

Where a plantation contains a range of commercial tree species, forest managers may prefer to plant them as a mixture, rather than as a matrix, for the sake of benefits unrelated to biodiversity, such as resistance to pests and disease, and higher social and recreational value (Felton *et al*.,2010; Taki *et al*., 2010). In this case, a full spectrum of ratios of the different crop species should be represented at the landscape level. This means including some stands that are highly dominated by each crop species, in addition to stands in which the species are more evenly represented.

Metabarcoding as a forest management tool

The fine-grained and interpretable variation between samples that was detected using the metabarcoding approach strongly suggests that metabarcoding could provide the kind of monitoring data that can (1) enable large-scale, controlled and replicated field experiments to be conducted, in order to test the effects of different management strategies, (2) detect and describe changes in communities that occur as a result of processes such as global warming and land-use change, and (3) detect the presence of economically damaging pest species while they are still at low population levels. This last potential application requires further advances in taxonomic assignment methods or the generation of high quality reference sequences, but it could provide the greatest economic justification for moving to a metabarcoding approach. Forest Europe *et al*. (2011) estimates that 13% of the forest area in Central Europe has been damaged by insects or disease, and the economic impact of this kind of damage can be considerable (Ayres and Lombardero, 2000).

Sampling invertebrates at a large scale should not be too onerous a task, since the majority of forests in the UK are regularly visited by managers, who can set traps for invertebrates and collect environmental samples. In general, the limiting factor in measuring arthropod biodiversity has always been the time and expertise required for the identification of specimens, and these recede as obstacles when the metabarcoding approach is employed. In short, by enabling evidence-based decision making and early detection of biological threats, metabarcoding represents a potentially powerful tool for improving our capacity to manage forests sustainably.

5.6 References

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Chapter 6: Using metabarcoding to compare UK farmland biodiversity under three models of agriculture

6.1 Summary

The use of the area of land covered by agri-environment schemes as a policy target and biodiversity indicator fails to recognise that the effectiveness of existing schemes in enhancing biodiversity is hardly well established. Testing the effectiveness of agri-environment schemes requires very large amounts of data at substantial spatial scales, and it has not been possible to generate such data except for a few groups (e.g. farmland birds). Metabarcoding now allows the rapid characterisation of invertebrate communities, which are vital for the functioning of agricultural ecosystems.

In this chapter, I show that metabarcoding can characterise arthropod communities with sufficient resolution to differentiate among three types of agri-environment scheme – Entry Level Stewardship, organic, and Conservation Grade. Malaise trap collections were made from twelve farms (four of each type), arranged geographically in triplets to control for large-scale spatial effects, and the samples were metabarcoded for COI. Arthropod communities were found to vary among farm types and geographic locations. This was also true for each of the four most prevalent insect orders considered individually, but patterns were not correlated among orders, suggesting differential responses to environmental variables. No difference was detected among farm types in terms of species richness, probably due to incomplete sampling.

In addition, soil samples were metabarcoded for 18S to investigate diversity patterns in soil invertebrates. Few OTUs were detected, which is likely a result of combining a DNA extraction method designed for extracellular DNA with the use of a relatively long amplicon. However soil arthropod communities also varied among farm types and among geographic locations, and there was significant correlation between the soil arthropod and Malaise trap arthropod datasets.

It is proposed that metabarcoding makes it possible for the first time to carry out large-scale monitoring of the effects of agri-environment schemes on arthropod diversity. However, improvements to current methods are required, particularly if reliable data are to be generated for the Hymenoptera.

6.2 Introduction

6.2.1 Biodiversity in agricultural ecosystems

There is overwhelming evidence that the intensification and homogenisation of agriculture during the second half of the 20th century has played a major role in driving the widespread decline in biodiversity in the developed world (Chamberlain *et al*., 2000; Donald *et al*., 2001; Robinson and Sutherland, 2002; Benton *et al*., 2003; Firbank *et al*., 2008; Tscharntke *et al*., 2012). In Europe, combatting biodiversity loss in agricultural ecosystems is a key aim of the Common Agricultural Policy (CAP), and member states are required to implement agri-environment schemes in order to achieve this (European Commission, 2010). These schemes are of particular importance for addressing the loss of native biodiversity in the UK, where agricultural ecosystems account for 70% of land cover (DEFRA *et al*., 2012).

The amount of land covered by agri-environment schemes is used as a key indicator of success in combating biodiversity loss at many political levels (Boccaccio *et al*., 2010; DEFRA, 2011; DEFRA *et al*., 2013). However, the use of this metric implicitly assumes that the schemes are effective, meaning that they have a positive effect on biodiversity in comparison with a 'business as usual' approach (Dudley *et al*., 2005). This is difficult to demonstrate for several reasons: agricultural landscapes are highly variable and comprise a multitude of managed and semi-natural habitats; farmers vary substantially in their attitudes to the environment, and so 'business as usual' is not easily characterised; and the biodiversity response to agri-environment measures is liable to vary among taxonomic and functional groups and to be influenced by confounding factors such as landscape and weather (Taylor and Morecroft, 2009). The sheer complexity of both the explanatory and response variables means that very large amounts of data are required across considerable temporal and spatial scales, if any conclusions are to be drawn about the impacts of sustainable management schemes on biodiversity. The danger of failing to provide this evidence is that ineffective schemes can act as a smokescreen, enabling political objectives and international biodiversity targets to be met on paper without actually having altered the rate of biodiversity loss. This means that there is little incentive to seek better management practices that might have a greater impact on biodiversity.

6.2.2 UK Entry Level Stewardship

In England, the most widely-implemented sustainable agriculture programme is the government Environmental Stewardship scheme, which is delivered by Natural England on behalf of DEFRA, in

response to the demands of the CAP. The most widespread strand of the scheme is the basic Entry Level Stewardship (ELS), which covered 61.4% of England's agricultural land as of May 2013 (Natural England, 2013a). Through this scheme, the government provides flat-rate payments of £30 ha^{-1} to farmers in compensation for loss of productivity caused by the implementation of measures that are putatively beneficial for the environment (Natural England, 2013b). Farmers are able to select from a wide range of options, which include maintenance of hedgerows, ditches, and other field boundaries, as well as the creation of uncropped field margins, areas of permanent grassland, or specific wildlife habitats such as nectar and pollen flower mixes, beetle banks, and skylark plots (Natural England, 2013b). A problem with this system is that the available options are equally weighted in terms of financial compensation but vary both in terms of how difficult they are to implement, and, probably, in terms of their benefits for nature. This means that the uptake of the different options is highly uneven, and skewed towards those that are least disruptive to 'business as usual', such as maintaining field boundaries (Natural England, 2009; Boccaccio *et al*., 2010). In general there is poor uptake of in-field management options and those that involve the creation of more complex wildlife habitats, and this is likely to limit biodiversity gains (Kleijn and Sutherland, 2003; Baker *et al*., 2012).

6.2.3 How effective is the ELS scheme?

Somewhat remarkably for a system that costs the public £400 million annually (Natural England, 2009), there is a conspicuous lack of standardised, large-scale biodiversity monitoring to assess the impacts of the measures that are implemented and to determine the extent to which aims of the CAP are achieved and value for money is obtained through the ELS scheme (Kleijn and Sutherland, 2003) (with the exception of indicator groups such as farmland birds, discussed below).

Far from being unique to the UK, this situation is reflected across Europe (Kleijn and Sutherland, 2003; Boccaccio *et al*., 2010), and the lack of data means that the overall impact of agri-environment schemes on biodiversity is extremely difficult to judge (Dudley *et al*., 2005; Kleijn *et al*., 2011) and continues to be a matter of debate amongst ecologists (Kleijn *et al*., 2001; Kleijn and Sutherland, 2003; Whittingham, 2007; 2011; Princé *et al*., 2012). Many studies have investigated the biodiversity effects of various scheme options, usually at the field level, using paired scheme and non-scheme fields (Kleijn *et al*, 2001; 2006; Kleijn and Sutherland, 2003; Feehan *et al*., 2005; Marshall *et al.,* 2006; Carvell *et al*., 2007; Pocock and Jennings, 2008; Merckx *et al*., 2009; Perkins *et al*., 2011; Roth *et al*., 2011), and results have been mixed. For instance, Kleijn *et al*. (2006) found that, across five European countries, many common species showed a

positive response to agri-environment schemes, but rare or endangered species generally did not benefit; Kleijn *et al*. (2001) found no positive effect of Dutch agri-environment schemes on plants or birds but modest increases in the abundance of bees and hoverflies; Feehan *et al*. (2005) found no evidence that an Irish agri-environment scheme benefitted surveyed taxa (field margin flora and ground beetles); Marshall *et al*. (2006) found a positive impact of wide field margins (a popular option under ELS) on flora, bees, and Orthoptera but no impact on birds, spiders or ground beetles; finally, Holland *et al*. (2012) found that grass field margins had a strongly positive effect on the control of field aphids by supporting increased populations of aerial natural enemies (predominantly Diptera), whereas Olson and Wäckers (2007) found that field margins acted as sinks, rather than sources, for natural enemies, and observed no effect on pest control. Many of the options available within the ELS framework are targeted specifically at increasing populations of farmland birds (Natural England, 2013b) but, with some local exceptions (e.g. Davey *et al*., 2010; Perkins *et al*., 2011), there has been little overall success in reversing declines (Whittingham, 2011; Baker *et al*., 2012).

Most of the studies mentioned above have focused on a narrow range of taxonomic groups, and all are limited in terms of the number and variety of farms considered. Furthermore, few experimental designs can account for the confounding effects of (1) the predisposition of an unknown proportion of participating farmers to manage farms in a nature-friendly way and (2) the tendency for scheme options to be implemented on fields which are naturally of lower value for production and higher value for biodiversity (Kleijn and Sutherland, 2003; Kleijn *et al*., 2004; 2006; 2009; Taylor and Morecroft, 2009). Therefore, the overall effectiveness of agri-environment schemes, including the UK ELS scheme, in achieving the CAP biodiversity aims remains largely unknown (Kleijn *et al*., 2001; Kleijn and Sutherland, 2003), and there is a need for comprehensive studies that cover larger spatial and temporal scales and consider a wider range of taxonomic groups, including rarely considered hyper-diverse groups such as the Diptera, parasitic Hymenoptera, and soil fauna.

6.2.4 The use of indicators

With a lack of comprehensive biodiversity data to guide the development of agri-environment policy, it is instead driven by the population trends of a narrow range of species that are considered to be indicators of ecosystem health (DEFRA, 2011). These tend to be species that are (1) conspicuous, (2) thought to be representative of agricultural ecosystems, and (3) known to have been declining as agricultural intensification has increased. The most influential such indicator is the farmland bird index, which tracks the populations of nineteen species of farmland

birds (Donald *et al*., 2001; Vickery *et al*., 2004; Birdlife International, 2006; Butler *et al*., 2007; Gregory *et al*., 2008; 2010; Boccaccio *et al*., 2010; Davey *et al*., 2010). As mentioned above, many options under the ELS scheme are specifically targeted at reversing the population declines of these birds (e.g. creation of Skylark plots, supplementary winter feeding, and planting wild bird seed mixes) (Natural England, 2013b).

Butterflies are also considered by Natural England to be good indicators of biodiversity, and the Farmland Butterfly Initiative, which was launched in May 2012 (Natural England, 2013a), will explore ways in which farmland can be managed to benefit seven endangered species of butterfly. Thus, these appear set to become key indicators alongside the nineteen species of farmland birds, and Natural England (2013a) claim that the results of the Farmland Butterfly Initiative will inform the design of any new agri-environment scheme. Populations of priority species are also widely monitored across the UK and are used to inform agricultural policy (DEFRA, 2011; Natural England, 2013a).

More complex cross-taxon indices have recently been developed by Butler *et al*. (2009; includes mammals, birds, plants, bees, and butterflies) and Overmars *et al*. (2012; includes vertebrates and plants), but these are not yet widely used.

Although birds, butterflies, and priority species have an important cultural role to play, they make up only a small proportion of the faunal diversity in agricultural landscapes. Balmford *et al*. (2005) presented a consultation with a hypothetical extra-terrestrial being, who observed that the taxa selected for monitoring should be representative of the total diversity. This seems obvious but is patently not the case. It is (non-butterfly) invertebrates that account for the vast majority of animal species, and which provide many of the ecosystem services that underpin the productivity of agricultural systems and, ultimately, food security, via nutrient cycling, pollination, and pest control (Thrupp, 2000; Lavelle *et al*., 2006; Tscharntke *et al*., 2012; Baker *et al*., 2012). Therefore, any effort to judge the effectiveness of agri-environment schemes should make extensive reference to invertebrates.

The assumption that patterns can be extrapolated from birds to invertebrate taxa is unsound, since many studies have demonstrated that birds and arthropods show contrasting responses to environmental variables in agricultural ecosystems (e.g. Marshall *et al.,* 2006; Kleijn *et al*., 2006; Pocock and Jennings, 2008; Roth *et al*., 2011), with invertebrates generally being more sensitive than vertebrates are to environmental change (Pocock and Jennings, 2008). Moreover, the many conservation actions targeted at farmland birds serve to decouple them from the rest of farmland

biodiversity, lessening their reliance on lower trophic levels and compromising what usefulness they may have had as indicators. This is a phenomenon known as Goodhart's Law: "...*once an indicator or other surrogate measure is made into a policy target, then it will lose the information content that would qualify it to play its role as an indicator*." (Newton, 2011, pg 265).

Nor can patterns necessarily be extrapolated from butterflies to other invertebrate taxa (despite the claims of some authors – e.g. Merckx *et al*., 2009). The most detailed agricultural biodiversity studies have found that there is variation among arthropod groups in the way in which they respond to variables associated with agricultural management (Kleijn *et al*., 2006; Marshall *et al*., 2006; Pocock and Jennings, 2008; Billeter *et al*., 2008; Gabriel *et al*., 2010; Roth *et al*., 2011; McMahon *et al*., 2012). Therefore, if indicators are to be used to gain a broad understanding of the response of wildlife to agri-environment measures, they should ideally include a wide range of arthropod groups, and the limitations of each group as an indicator of others should be clearly understood (Dudley *et al*., 2005; Pocock and Jennings, 2008; Billeter *et al*., 2008). However, any taxonomically comprehensive sample would seem to require unfeasibly large amounts of time, resources, and taxonomic expertise, especially given that a comprehensive monitoring scheme would need extensive spatial and temporal replication if it is to determine the overall biodiversity impacts of agri-environment measures. In short, the taxonomic impediment has been an important, perhaps the *most* important, constraint on designing and managing effective agri-environment schemes.

The aim of this chapter is not to assess the biodiversity benefits of any particular agri-environment scheme, but rather to demonstrate a method for rapidly generating large amounts of data on invertebrate communities in agricultural ecosystems, thereby overcoming the taxonomic impediment.

6.2.5 Other types of agri-environment scheme

I consider three agri-environment schemes that operate in the UK: ELS (described above), Conservation Grade, and organic farming.

Conservation Grade

The Conservation Grade (CG) protocol is implemented by a private company, Conservation Grade Producers Ltd (St Neots, UK) and links farmers who conform to a strict sustainability protocol with food brands that will pay – and pass on to their customers – a premium price for the crop. This is a market-driven scheme, relying on the customer recognising and valuing the importance of responsible environmental stewardship. The CG trademark serves as a clearly recognisable

certification stamp, conveying information to the customer about the environmental ethics that have governed production of the item.

The primary concern of the CG scheme is the support of wildlife on farms. The protocol is far more prescriptive than that of the ELS scheme, with farmers required to allocate 10% of their farm area to the creation of specific wildlife habitats. This includes 4% dedicated to the plantation of pollen and nectar mixes, 2% to wild bird food crops, and 2% to tussock and fine grass mixtures, with the remaining 2% dedicated to other habitats specific to each individual farm (CG, 2011). The protocol also promotes the maintenance of border habitats, including hedgerows and watercourses, and the creation of skylark plots. The use of certain pesticides, including organophosphates and synthetic pyrethroids, is prohibited on CG farms. These chemicals are approved for use in the UK, but are believed to be harmful to wildlife (CG, 2011). The use of other chemicals, including the controversial neonicotinoids (Goulson, 2013), is allowed.

There has been much debate in recent years about the relative benefits of land-sharing versus land-sparing approaches for reconciling biodiversity and food production (Green *et al*., 2005; Fischer *et al*., 2008; Hodgson *et al*., 2010; Phalan *et al*., 2011). Although, at a landscape scale, all agri-environment schemes represent a land-sharing approach to biodiversity conservation, at the farm scale, the CG protocol (and also ELS) can be considered an example of a land-*sparing* approach: intensive farming is encouraged in production areas, while areas that have the least value for production are specifically targeted for the establishment of high quality wildlife habitats.

Although there are research projects now underway, aimed at determining the biodiversity effects of the CG protocol, they focus on a narrow set of taxonomic groups, primarily bees and birds. Bees are a natural focus in light of their importance as pollinators and the widespread population declines that they appear to be suffering (Potts *et al*., 2009; 2010; Breeze *et al*., 2010; Lebuhn *et al*., 2013), but it would be of interest to ascertain the extent of the wider biodiversity effects of the CG approach in comparison with other agri-environment schemes.

Organic farming

The organic model of agriculture promotes ecosystem sustainability by making use of natural processes (Jonason *et al*., 2011). Organic farming differs from the other two models in that it focuses on making the whole farmed area more hospitable to wildlife, rather than on creating discrete patches of high quality habitat (Hole *et al*., 2005). In other words, at the farm scale, organic farming is an example of a land *sharing* approach to reconciling biodiversity with food

production (Hodgson *et al*., 2010). It is also the model under which the use of chemicals is most strictly regulated, with all synthetic fertilisers and pesticides avoided (Winqvist *et al*., 2011; Geiger *et al*., 2010). In general, production costs are higher than in conventional farming (Odefey *et al*., 2011), yields are often lower (Mäder *et al*., 2002; Seufert *et al*., 2012; Gabriel *et al*., 2013), and there is a greater species richness of non-crop plants (Gabriel *et al*., 2006). Like CG, this is a market-driven approach in which the cost of implementing the protocol is passed on to customers who are prepared to pay a premium price for ethical environmental management. The organic farming system is the best studied of the three systems considered in this study, and there is strong support for the claim that it has positive biodiversity effects across a wide range of taxonomic groups (Bengtsson *et al*., 2005; Gabriel *et al*., 2006; 2010; Hodgson *et al*., 2010; Smith *et al*., 2011; Winqvist *et al*., 2011; Jonason *et al*., 2011). In a recent meta-analysis, Rahmann (2011) found that 83% of 396 relevant studies reported that organic farming had a positive effect on biodiversity in comparison with conventional farming. Several long-term studies of organic farming have also been carried out in Europe and North America (e.g. Mäder *et al*., 2002; Felefyn-Szewczyk, 2008; Hepperly *et al*., 2006; Rahmann *et al*., 2006; Mazzoncini *et al*., 2010), and all have reported positive biodiversity effects (Rahmann, 2011).

6.2.6 Study sites and research questions

In this study, I make use of a network of twelve farms located in the UK Midlands. These are currently being used by researchers at the University of Reading (Prof. Simon Potts, Ms Chloe Hardman) to study the effectiveness of the CG biodiversity protocol compared with the ELS and organic farming models. In order to control for large-scale spatial effects, which are known to have a strong influence on biodiversity (Gabriel *et al*., 2006), farms are grouped into four geographical triplets, each including one of each farm type (CG, ELS, Organic). Within a triplet, all farms occur within the same National Character Area (NCA), which means that they share common features of landscape and geodiversity (Natural England, 2013c).

An unavoidable complication of studying agri-environment schemes is that farms can participate in more than one scheme, and enter them at different times. This is partly driven by the fact that farms meeting the requirements for the more demanding schemes, such as the CG and organic protocols, generally also meet the requirements for ELS with little or no extra effort and thereby qualify to receive ELS payments (Conservation Grade, 2011). Thus, all twelve farms in this study participate in the ELS scheme (Table 6.1), which means that the ELS-only farms represent the lowest level of environmental management and can be considered the baseline farming method, while CG and organic farms can be thought of as 'ELS+' farms. In addition, two of the CG farms

(Crux Easton and Codicote Bottom), and all four organic farms, also participate in the Higher Level Stewardship (HLS) scheme (Table 6.1), which is the upper tier of the government Environmental Stewardship programme. HLS prescribes more targeted conservation measures coupled with a greater degree of monitoring and a more detailed assessment of the biodiversity opportunities of the particular farm (Natural England, 2010). It has much in common with the CG scheme, and so the two CG farms that are non-participants in HLS can be expected not to differ in any fundamental way from those that are HLS participants.

Table 6.1: Details of the twelve farms, including triplet name, National Character Area (NCA), location, the primary type of agri-environment scheme in which the farm participates, and the date at which each farm entered each of the ELS, HLS and CG schemes. Dates at which farms became organic were not available.

Farm	Triplet	NCA	Latitude	Longitude	Type	ELS	HLS	CG
Copt Hall	Chilterns North	Chilterns	51.86642	-0.36818	ELS	2007	-	-
Shiplake	Chilterns South	Chilterns	51.49811	-0.90292	ELS	2010	-	-
West Woodhay	Hampshire	Hampshire Downs	51.36719	-1.44818	ELS	2002	-	-
Tismans	Low Weald	Low Weald	51.0848	-0.47926	ELS	2003	-	-
Codicote Bottom	Chilterns North	Chilterns	51.84562	-0.24938	CG	2006	2012	2006
Vines	Chilterns South	Chilterns	51.51396	-1.03072	CG	2011	-	2004
Crux Easton	Hampshire	Hampshire Downs	51.30444	-1.38995	CG	2009	2009	2006
Malham	Low Weald	Low Weald	51.04828	-0.48869	CG	2010	-	2006
Hammond's End	Chilterns North	Chilterns	51.79691	-0.37144	Organic	2007	2013	-
Collings Hangar	Chilterns South	Chilterns	51.69283	-0.74016	Organic	2010	2012	-
Box Farm	Hampshire	Hampshire Downs	51.31707	-1.52368	Organic	2011	2011	-
Barlavington	Low Weald	Low Weald	50.93597	-0.61864	Organic	2007	2007	-

Because time and resources allowed the collection of only one sample per farm, I focus on beta diversity rather than alpha diversity. I ask two questions. First, I ask whether arthropod assemblages differ between (1) the three farming models and (2) the different geographical areas as delineated by Natural England's NCAs. Second, I ask whether the observed patterns of differentiation are consistent across different taxonomic groups.

The main focus of this study is flying arthropod diversity, which was sampled using Malaise traps. However, soil fauna provide important ecosystem services in agricultural environments (Altierei, 1999), and several studies have reported that soil invertebrate communities can differ among farm management systems (e.g. Mazzoncini *et al*., 2010; Cluzeau *et al*., 2012). Therefore, I also

consider whether soil invertebrate communities vary according to agri-environment scheme or geography, using an approach outlined by Taberlet *et al*. (2012) to extract extracellular metazoan DNA from soil.

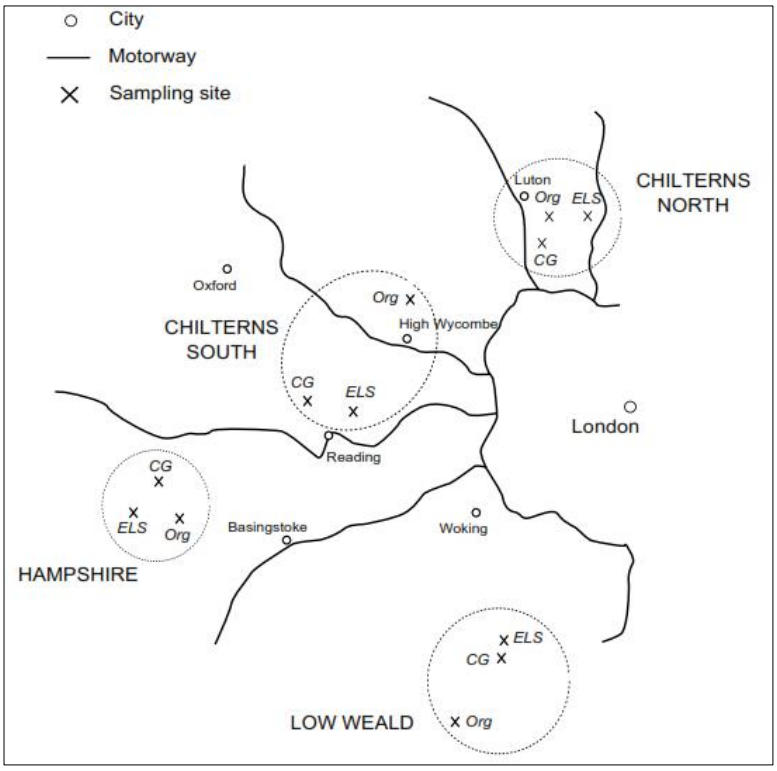

Figure 6.1: Map showing the locations of the twelve sampling sites. Circles with dotted lines delineate the four triplets, each of which lies in a different NCA, except Chilterns North and Chilterns South which both lie in the Chilterns NCA. The farm management type (CG/ELS/Org) is indicated beside each sampling point.

I hypothesise that the greatest difference in flying arthropod community composition will occur between organic and non-organic farms for the reason that sampling was conducted in each farm on the margin of a cereal crop, rather than in the specially created habitats found in ELS and CG farms, and so these management schemes may have little effect on biodiversity in the sampling location. In contrast, the organic protocol, being applied across the farm, should more directly affect the sampling sites. Organic farms are also likely to have the most distinct soil fauna because this farming model actively promotes soil biodiversity by banning chemical inputs (Hole *et al*., 2005; Thiele-Bruhn *et al*., 2012).

6.3 Methods

6.3.1 Sampling methodology

Farms in the Chilterns North and Chilterns South triplets were visited on 21st-22nd August 2012 to to erect Malaise traps and to collect soil samples. Farms in the Hampshire and Low Weald triplets were visited one week later.

Malaise trap sampling

Due to limited time and resources, it was only possible to sample with one Malaise trap at each farm. This raised a site selection problem, and it was decided to standardise as far as possible across all farms. Therefore, at each farm, the Malaise trap was erected in a grassy field margin between a hedgerow and a cereal crop (usually wheat), and was oriented such that it lay perpendicular to the hedgerow. Specially created wildlife habitats and areas of woodland were avoided, as were public footpaths and other areas where the trap was likely to be disturbed by passers-by or agricultural activities. In all cases but one (Box Farm; Organic – Hampshire), harvesting of the crop had occurred prior to erection of the trap. Collection bottles were filled 2/3 full of 100% ethanol, and traps were left to gather samples for seven fine weather days (less than three hours of rainfall during daylight hours) before being dismantled.

Soil sampling

Soil samples were collected at the same time as the Malaise traps were erected. Previous research has shown that a high degree of heterogeneity exists between the biological communities from individual soil cores, but combining multiple cores from a 10 x 10 m area gives a sample that is representative of the area (Taberlet *et al*., 2012). Therefore, a soil auger was used to take ten small cores from the area immediately surrounding the Malaise trap. The ten cores combined to give approximately 1 kg of soil per farm, and this was collected into a heavy duty rubble bag, which was sealed securely with strong adhesive tape to prevent leakage and avoid cross-contamination. The soil auger was also cleaned with bleach between farms to avoid cross-contamination. Soil samples were stored at -20 ^{o}C until DNA extraction was carried out.

6.3.2 Laboratory steps and bioinformatic processing

Malaise trap samples

Bulk DNA extraction and high-throughput sequencing of the Malaise trap samples was carried out by collaborators at the Kunming Institute of Zoology (KIZ), following the protocols described in

Chapter 4. The 12 samples were sequenced on two 1/16 regions of a Roche GS FLX sequencing plate, with each sample processed as a separate MID. Subsequently, I used the bioinformatic pipeline described in Chapter 4 to perform quality control, OTU picking, and taxonomic assignment. The resulting OTU table was filtered to exclude single-read and non-arthropod OTUs, forming the 'Malaise Arthropod' dataset, which was the basis for the majority of analyses. Taxonomic subsets of this table were created for the most prevalent insect orders: Diptera, Hemiptera, Hymenoptera, and Lepidoptera. As a check, the original OTU table was also filtered for vertebrate taxa to make sure that the species recorded were native to the UK.

Soil samples

Laboratory steps. – Soil DNA extractions were performed following the method outlined in Taberlet *et al*. (2012). Extracellular DNA, which was the target of the soil analysis, tends to form bonds with negatively charged soil particles and organic matter (England *et al*., 2004; Pietramellara *et al*., 2009). Therefore, the first step in DNA extraction was to break down these bonds and release the DNA into solution using a phosphate buffer (NA_2HPO_4). For each sample, the soil was weighed and mixed with an equal weight of phosphate buffer in a 3.6 L plastic barrel with a screw-top lid (CJK Packaging Ltd, Chinley, Derbyshire). The phosphate buffer was characterised by a pH of 8, which was achieved by dissolving 1.97 g of NaH_2PO_4 and 14.7 g of Na_2HPO_4 into each litre of distilled water. The soil and buffer were mixed vigorously for 20 minutes in order to fully homogenise the sample. Two DNA extraction replicates were obtained for each soil sample. For each replicate, a 2 ml aliquot of the homogenised sample was centrifuged for 10 minutes at 10,000 g, and 400 µl of the resulting supernatant was retained for DNA extraction.

The following steps were carried out using the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany), largely following manufacturer's instructions but skipping the initial DNA lysis steps. First, 200 µl NucleoSpin SB buffer was mixed with the 400 µl of sample supernatent, and then 550 µl sample + buffer was loaded onto a NucleoSpin Soil column and centrifuged for 1 minute at 11,000 g. Flow through was discarded, and the silica membrane was washed, first with 500 µl SB buffer, next with 550 µl SW1 buffer and, finally, twice with 700 µl SW2 buffer. At each washing step, the appropriate buffer was added to the column and centrifuged at 11,000 g for 30 seconds so that it passed through the membrane. Flow-through was discarded after each step. After washing, the silica membrane was dried by centrifuging at 11,000 g for 2 minutes, and 100 µl elution buffer was then loaded into the column and incubated at room temperature for one

minute before centrifuging for 30 seconds at 11,000 g. The eluted DNA was stored at -20 ^{o}C and later transported to Kunming, China in a vacuum flask packed with frozen peas.

PCR and sequencing was carried out at the KIZ, as described in Chapter 4, with each of the 24 DNA extractions processed as a separate MID. Subsequently, I carried out bioinformatics processing following the steps outlined in Chapter 4 for 18S data. Following assignment of taxonomy, an OTU table was created, which showed the number of sequence reads assigned to each OTU in each of the 24 DNA extractions (two extraction replicates per soil sample). The OTU table was filtered to remove single-read and non-metazoan OTUs, and the remaining OTUs formed the main 'Soil Metazoa' dataset.

Data analysis for the COI Malaise trap datasets

6.3.3 Ordination – Malaise trap data (COI)

For analysis of beta diversity, OTU read numbers were converted to binary presence-absence data in order to negate the effect of taxon-specific bias that can arise during the amplification and sequencing steps. This was achieved using function *decostand()* in *vegan* v. 2.0-7 (Oksanen *et al*., 2013). To check the robustness of the ordination results, both nonmetric multidimensional scaling (NMDS; function *metaMDS()* in *vegan*) and correspondence analysis (CA; function *cca()* in *vegan*) were used to ordinate the Malaise Arthropod dataset, and the results were compared statistically using a Procrustes test with 999 permutations. The NMDS ordination was based on the binary Jaccard dissimilarity index, which counts only presence and absence and gives no weight to shared absences. In the ordination plots, points were coloured and grouped by farm management type (CG/ELS/Organic), and also grouped by triplet (Chilterns N, Chilterns S, Hampshire, Low Weald), to aid visualisation of beta diversity patterns.

6.3.4 Significance testing – Malaise trap data (COI)

Statistical tests were performed to examine the effects of Triplet and Type on arthropod community composition. I was mostly interested in the effect of Type, since this was the relevant variable for answering research questions about the effect of different farm management approaches. Statistical tests had limited power because each Type/Triplet contained low replication (4 samples per Type, 3 per Triplet). Therefore, I used four methods to test the statistical significance of the Type and Triplet variables in explaining community-composition variation. The methods were (1) multivariate binomial likelihood ratio (LR) tests in the *R* package *mvabund* (Wang *et al*., 2012a), (2) *envfit*, which is the *vegan* package's permutation test for

community data, based on the Jaccard distance matrix; (3) PERMANOVA permutation tests (function *adonis()* in *vegan*), also based on the Jaccard distance matrix, and (4) canonical correspondence analysis (CCA; function *anova.cca()* in *vegan*), which is also a permutation testing method but is based on eigenvalues instead of a distances. The permutation testing methods (2-4) were each performed using 2000 permutations, with the effect of the Triplet variable taken into account when testing for an effect of Type (strata = Triplet), and vice versa. All *mvabund* analyses were performed with 999 bootstrap iterations and pit.trap resampling (an unpublished resampling method designed by the authors of *mvabund*). Here, the effect of Triplet was taken into account when testing for the significance of Type by specifying the formula as "community ~ Triplet + Type", and vice versa when testing for an effect of Triplet (community ~ Type + Triplet). Highest confidence was attributed to the *mvabund* analysis because it takes into account the mean-variance relationship of a given dataset, and is therefore less likely than other methods to confuse location effects with dispersion effects (Wang *et al*., 2012a; Warton *et al*., 2012).

An additional advantage of the *mvabund* analysis is that it fits an independent GLM to each OTU to test its individual response to the predictor variable(s), returning OTU-specific *p*-values and coefficients in addition to the overall test result. This allows the user to identify the OTUs that show the strongest responses to the predictor variable, and to identify the direction of the response. However, when multiple explanatory variables are included in the model, interpretation of the LR coefficients becomes difficult. Therefore, although Triplet was included as a factor in the overall test for the effect of the Type variable, I performed a second test for Type alone ("community ~ Type" instead of "community ~ Triplet + Type"), and the un-adjusted OTU-specific *p*-values and LR coefficients from this test were used to pick out the OTUs that responded most strongly to farm type (unadjusted $p < 0.05$). Of these OTUs, those that had a high/positive coefficient for one type and a low/negative coefficient for the other two types were taken to be positively associated with the first type, meaning that they were more likely to occur in this farm type than in the other two farm types. Conversely, OTUs that had a low/negative coefficient for one type and high/positive coefficients for the other two types were taken to have be negatively associated with the first farm type, meaning that they are rarely found in that farm type compared with the other two types.

6.3.5 Comparing among taxonomic subsets – Malaise trap data (COI)

I next asked whether different taxonomic groups within the Arthropoda varied in terms of beta diversity patterns. Jaccard NMDS ordinations were produced for each of the four largest taxonomic subsets of the Malaise Arthropod dataset (Diptera, Hemiptera, Hymenoptera, and

Lepidoptera). Ordinations were plotted with points coloured by Type, and grouped first by Triplet and then by Type. To test whether beta diversity patterns varied among taxonomic groups, pairwise comparisons of Jaccard dissimilarity matrices were carried out between taxonomic subsets using Mantel tests with Spearman's rank correlation and 999 permutations.

For each taxonomic subset, *mvabund* analyses were used to test for significant effects of Type and Triplet on beta diversity, controlling for Triplet when testing for Type, and vice versa, as described above. To investigate effect sizes, the mean Jaccard dissimilarity between each pair of farm types (within Triplet) was calculated for each taxonomic group by averaging the relevant dissimilarity values picked out from the dissimilarity matrix of that group. A one-way ANOVA test was performed for each group to test whether or not all pairs of types were equally dissimilar.

6.3.6 Alpha diversity – Malaise trap data (COI)

The Chao2 incidence-coverage method (Chao, 1987; Gotelli and Colwell 2011; function *specpool()* in *R* package *vegan*) was used to estimate the total species richness of each farm type across the four farms belonging to that Type. The resulting estimates and standard errors were then used to perform a Welch's *t*-test for pair-wise differences between farm types

Data analysis for the 18S Soil datasets

6.3.7 Ordination – soil data (18S)

To check the performance of the soil DNA extraction method, an NMDS ordination was produced in which the two DNA extraction replicates from each soil sample were treated as separate samples. This was based on the Soil Metazoa dataset, with sequence read numbers transformed to presence-absence. Points were coloured by soil sample, and *vegan*'s *ordispider()* function was used to connect points that represented pairs of extraction replicates from the same soil sample.

For downstream analyses of the Soil Metazoa and Soil Arthropoda datasets, the number of sequence reads per OTU was summed over the two DNA extraction replicates for each soil sample. A subset of this dataset was also created, including only the OTUs that were assigned to Arthropoda. This is referred to as the 'Soil Arthropoda' dataset. Both datasets were transformed to presence-absence, and CA and Jaccard NMDS ordinations were created for each. Three sets of comparisons were carried out: (1) CA and NMDS ordinations were compared within each dataset, using Procrustes tests with 999 permutations, to check that they revealed similar patterns in the

data; (2) Jaccard dissimilarity matrices were compared between the two soil datasets, using Mantel tests with 999 permutations and Spearman's rank correlation, to investigate whether the inclusion of non-arthropod metazoan OTUs altered the overall beta diversity patterns; and (3) the Jaccard dissimilarity matrix for the Soil Arthropoda was compared with that of the Malaise Arthropoda dataset, using Mantel tests.

6.3.8 Significance testing – soil data (18S)

The four methods of significance testing described above (*envfit*, PERMANOVA, CCA, and *mvabund*) were applied to the Soil Metazoa and Soil Arthropoda community datasets to test for the effect of the Type and Triplet variables

6.4 Results

6.4.1 Detection of OTUs and assignment of taxonomy

Pyrosequencing returned 102,439 raw COI sequence reads across the 12 Malaise trap samples. Quality-control and initial clustering at 99% similarity reduced this number to 10,065, and Bayesian clustering in CROP at 98% similarity gave a total of 2027 molecular OTUs. Of these, 822 were assigned to Arthropoda and contained > 1 sequence read. These were retained for downstream analysis, forming the Malaise Arthropod dataset. The majority of the Malaise Arthropod OTUs were assigned to Diptera (N=511), with lower prevalence of other groups, including Hemiptera (70), Hymenoptera (65), Lepidoptera (45), Coleoptera (34), and Arachnida (21). Using SAP, taxonomic identification to ordinal level was achieved for 94% of arthropod OTUs, and 20% were identified to species. As a check, OTUs assigned to Chordata were inspected, and four species were detected. These were field vole (*Microtus agrestis*), common pheasant (*Phasianus colchicus*), yellow-necked mouse (*Apodemus flavicollis*), and a rat (*Rattus* sp.), all of which are commonly encountered in agricultural ecosystems in the UK.

6.4.2 Ordination – Malaise trap data (COI)

Correspondence analysis (CA) of the Malaise Arthropod dataset extracted 24% of the total inertia in the first two axes, and stress levels in the two-dimensional Jaccard NMDS analysis were acceptable at 0.14, with a convergent solution achieved after 24 iterations. The two methods produced very similar ordinations, which were highly correlated (Procrustes test with 999 permutations: R^2=0.89, p=0.001).

Both ordinations show that communities cluster by triplet (panels 1A and 2A in Figure 6.2) , which indicates a geographical effect on community composition. Note that the two triplets that are separated by the greatest distance in the ordinations (Low Weald and Chilterns North) are also separated by the greatest geographical distance (Figure 6.1, above). Communities also cluster by farm type, with centroid positions suggesting that ELS communities are intermediate to those of CG and Organic farms (panels 1B and 2B in Figure 6.2). The CA ordination (panel 1A) shows more overlap between types than does the NMDS ordination (panel 2B) does. Contrary to my expectation, the patterns in these ordinations do not suggest that that organic farms are the most distinct Type.

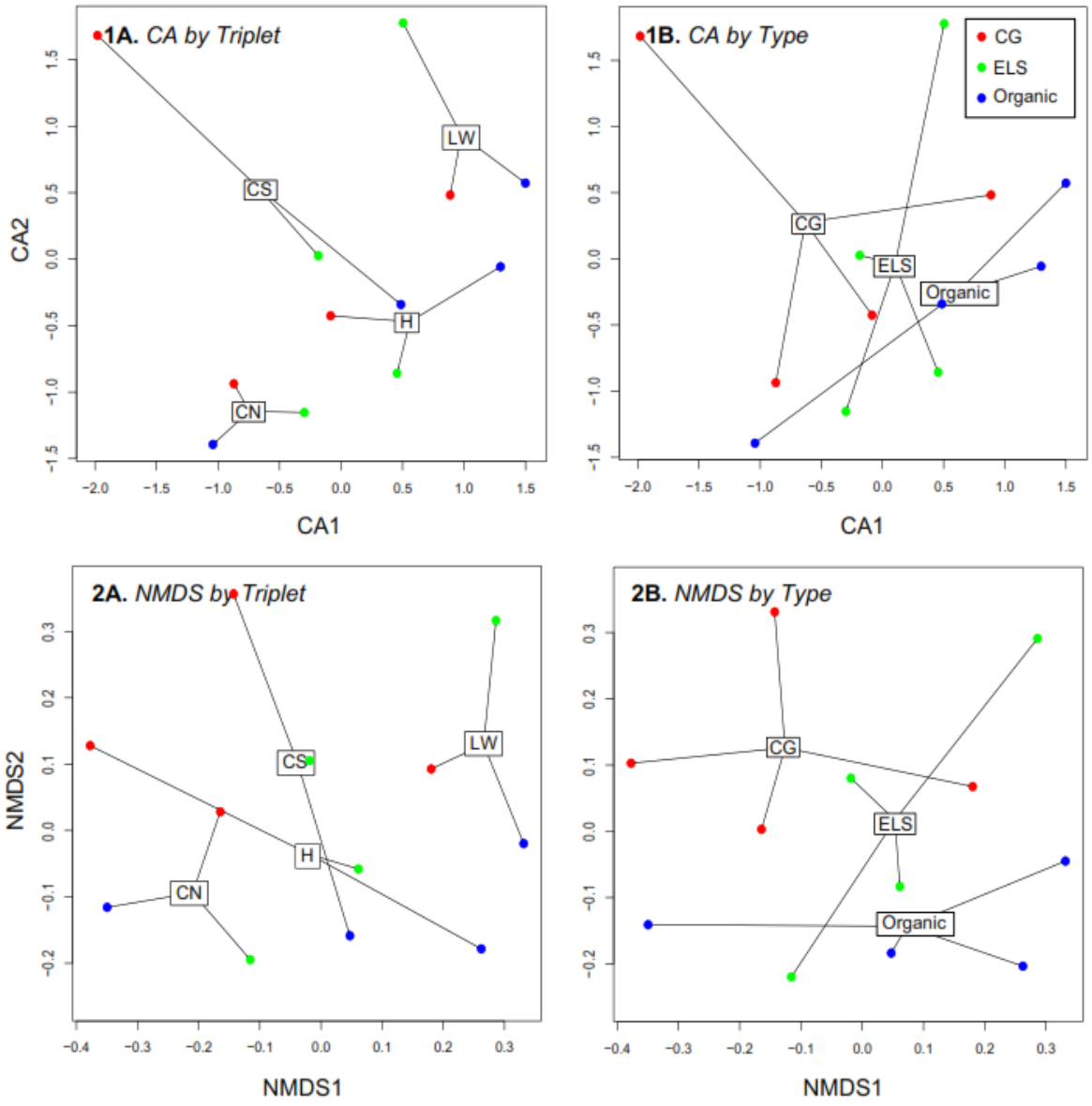

Figure 6.2: (1) Correspondence analysis and (2) NMDS ordinations of the twelve farms based on presence-absence data for Malaise trap arthropods. Points are coloured by farm type and grouped by (A) Triplet – Chilterns North (CN), Chilterns South (CS), Hampshire (H), Low Weald (LW); and (B) Type.

6.4.3 Significance testing – Malaise trap data (COI)

mvabund, which is the method to which the greatest amount of confidence is attributed (see methods section), found significant compositional differences among both farm *triplets* and farm *types* (Table 6.2). Post-hoc pairwise comparisons also found that each farm type is significantly different from each of the other two types. No other method found significant differences among both types and triplets, nor between all pairs of farm types (Table 6.2). However, each test that

was significant in the *mvabund* analysis was also significant in at least one other test, with the exception of the post-hoc comparison between ELS and Organic farms. Therefore, only the *mvabund* method will be used for beta-diversity analyses henceforth.

Table 6.2: Results of four statistical tests for the effects of farm Type and Triplet on community composition of Malaise trap arthropods. Original p-values are reported here, but significance is determined after correction for three tests, using p.adjust(method="fdr") * $p < 0.05$, ** $p < 0.01$.

		Mvabund		*envfit*		PERMANOVA		CCA	
	Df	*LR*	*P*	R^2	*p*	*F*	*p*	χ^2	*p*
Type (Overall)	2,9	3330	**0.002****	0.20	**0.029***	0.97	0.191	1.06	0.17
Type: CG vs ELS	1,6	1833	**0.002****	0.15	**0.001****	0.96	0.373	1.04	0.15
Type: CG vs Organic	1,6	1827	**0.001****	0.16	**0.001****	0.96	0.244	1.11	0.27
Type: ELS vs Organic	1,6	1780	**0.001****	0.14	0.242	1.00	0.265	1.11	0.24
Triplet	3,9	4513	**0.003****	0.28	0.157	1.23	**0.001****	0.73	**0.005***

The inclusion of two predictor variables (Triplet + Type) in the *mvabund* model made it difficult to pick out OTUs that were associated with the different farm types. Therefore, I ran a model with Type only. Although the main effect of Type was non-significant when Triplet was not included ("community ~ Type", $LR_{df=2,9}$=2087, p=0.202), the OTU-specific p-values from that analysis identified a small number of species that showed a significant association with specific types. I used the LR coefficients for these species to infer the direction of the individual responses. A handful of species, spread over multiple arthropod orders, were identified as showing a significant response to each farm type (Table 6.3), although recall that these identifications are based on unadjusted p-values.

Table 6.3: Table showing the number of OTUs belonging to each taxonomic group that showed a significant (un-adjusted *p*-value < 0.05) positive or negative association with each farm type. A positive association means that the OTU is found primarily in that farm type; a negative association means that the OTU is rarely found in that type compared with the other two types.

	CG		**ELS**		**Organic**	
	+ve	*-ve*	*+ve*	*-ve*	*+ve*	*-ve*
Diptera	5	1	3	0	2	0
Lepidoptera	0	0	1	0	1	2
Coleoptera	1	0	0	0	0	0
Hemiptera	1	0	0	0	0	0
Arachnida	0	0	0	0	1	0
Unknown	0	0	1	0	0	0

In summary, arthropod community composition differs significantly over local geography (triplet) and management types, driven by species from a variety of taxonomic groups. However, there is no evidence to support the hypothesis that organic farms have the most distinct arthropod communities.

6.4.4 Comparing among taxonomic subsets – Malaise trap data (COI)

Analysis in *mvabund* found that community composition varied both by triplet and by type for each of the four most prevalent insect orders (Diptera, Lepidoptera, Hemiptera, and Hymenoptera; Table 6.4). Furthermore, post-hoc pairwise comparisons of farm types found that, across all orders, each type differed significantly from each other type.

Table 6.4: Results of *mvabund* analyses for each of the four most OTU-rich insect orders, including tests for the overall effect of Triplet and Type as well as pairwise comparisons between each pair of farm types. Original *p*-values are shown but significance of pairwise comparisons is assigned after correction for three tests using p.adjust(method="fdr"). Analyses are performed with 999 bootstrap iterations and using pit.trap resampling. Df=2,9 for tests of Type, df=3,8 for test of Triplet. * $p < 0.05$, ** $p < 0.01$.

	Triplet		Type		CG vs Org		CG vs ELS		Org vs ELS	
	LR	*p*	*LR*	*p*	*LR*	*p*	*LR*	*p*	*LR*	*p*
Diptera	2852	**0.001****	2093	**0.001****	1186	**0.002****	1171	**0.001****	1095	**0.001****
Hemiptera	388	**0.007****	284	**0.003****	151.1	**0.001****	157.3	**0.001****	153.8	**0.001****
Hymenoptera	336	**0.008****	257	**0.007****	119.9	**0.005****	152.5	**0.002****	135.1	**0.001****
Lepidoptera	264	**0.003****	203	**0.001****	94.26	**0.001****	102.6	**0.002****	122.7	**0.001****

When Jaccard distance matrices were compared between orders, no order was was found to be significantly correlated with any other (Table 6.5), suggesting that each responds differently to geographic and farm management variables.The contrasting patterns can be observed in the order-specific NMDS ordinations in Figure 6.3.

Only the distance matrix for Diptera was significantly correlated with that of the Arthropoda (Table 6.5), which indicates that the pattern observed in the main Malaise Arthropod dataset (Figure 6.2, Table 6.2) was driven only by the Diptera.

Table 6.5: Mantel *r* statistics based on Spearman's rank correlation (lower matrix) and *p*-values (upper matrix) from pairwise comparisons of Jaccard dissimilarity matrices between taxonomic subsets of the Malaise Arthropoda dataset. Original *p*-values are shown, but significance is determined after correction for ten tests, using p.adjust(method="fdr") * $p < 0.05$.

	Arthropoda	**Diptera**	**Hemiptera**	**Hymenoptera**	**Lepidoptera**
Arthropoda		**0.001***	0.202	0.146	0.024
Diptera	**0.89**		0.694	0.222	0.258
Hemiptera	0.12	-0.09		0.636	0.273
Hymenoptera	0.16	0.10	-0.05		0.123
Lepidoptera	0.28	0.10	0.08	0.15	

The Lepidoptera ordination in Figure 6.3 appears to separate out the organic farms, as originally hypothesised. However, there is no statistical support for this observation, with post-hoc one-way ANOVA tests finding no difference between the pairwise Jaccard distances among farm types (Table 6.6).

Table 6.6: Mean and s.e. pairwise Jaccard distances between farm types (within triplet) for each of the four most OTU-rich insect orders, and the results of a one-way ANOVA to test whether the distances vary between pairs. *P*-values are unadjusted.

	CG vs Org		CG vs ELS		Org vs ELS		ANOVA		
	Mean	**s.e.**	Mean	**s.e.**	Mean	**s.e.**	df	F	*p*
Diptera	0.73	0.032	0.73	0.019	0.71	0.017	2,9	0.3	0.75
Hemiptera	0.85	0.041	0.83	0.033	0.84	0.063	2,9	0.05	0.95
Hymenoptera	0.92	0.03	0.91	0.026	0.92	0.032	2,9	0.05	0.95
Lepidoptera	0.83	0.067	0.83	0.03	0.89	0.052	2,9	0.43	0.66

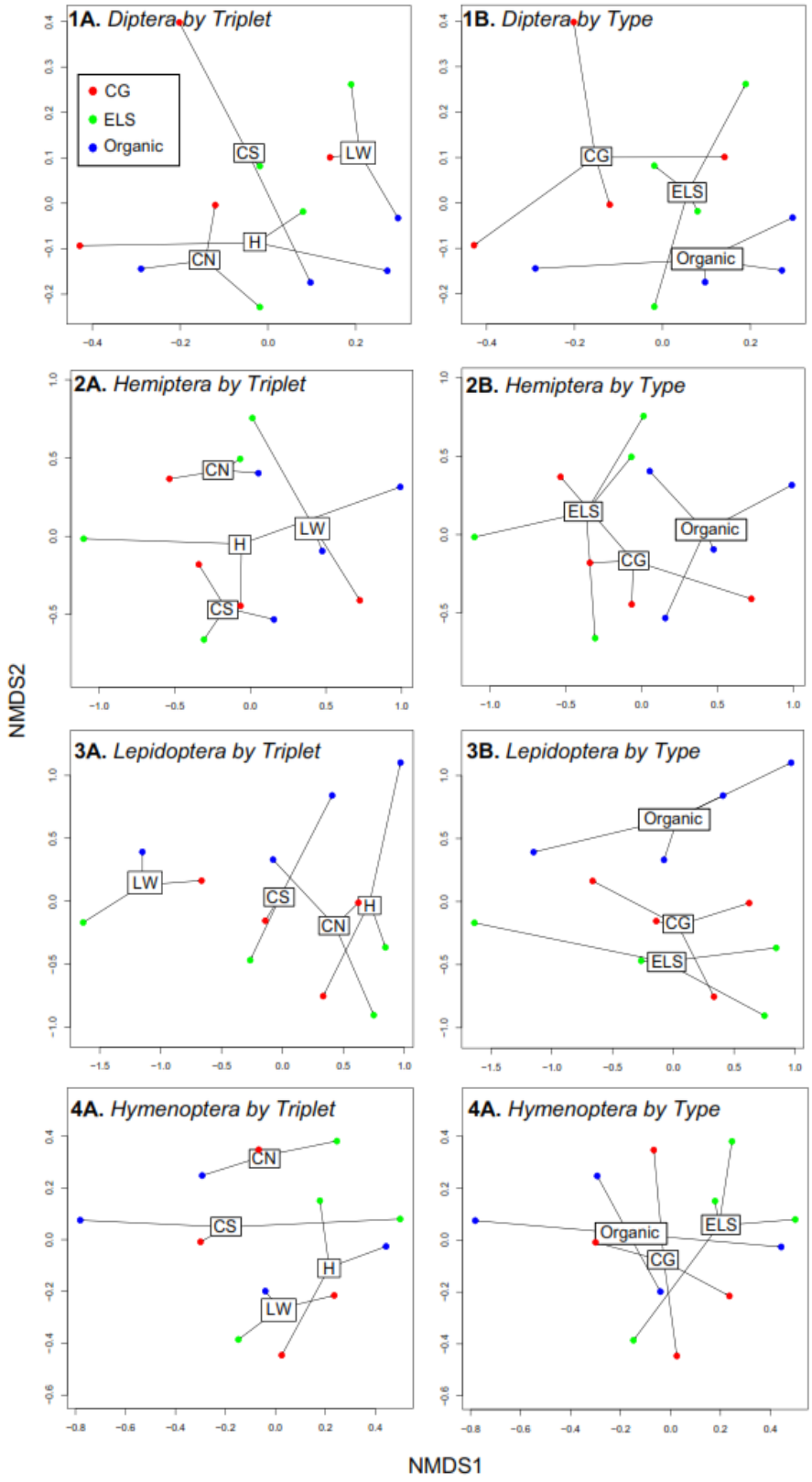

Figure 6.3: NMDS ordinations of the twelve farms based on presence-absence data for (1) Diptera, (2) Hemiptera, (3) Lepidoptera, and (4) Hymenoptera. Points are coloured by farm type and grouped by (A) Triplet – Chilterns North (CN), Chilterns South (CS), Hampshire (H), Low Weald (LW); and (B) Type.

In summary, there is evidence that the community composition of four arthropod orders differs significantly over geography and farm type. That is, for all orders considered here, both the CG and organic agri-environment schemes have an additional effect on community composition compared with the ELS scheme but the effect differs between the two schemes. Importantly, results also suggest that the four orders differ in how they respond to agricultural management.

6.4.5 Alpha diversity – Malaise trap data (COI)

The number of arthropod OTUs per malaise trap sample ranged from 158 at Collings Hangar Farm (Organic; Chilterns South) to 253 at Shiplake Farm (ELS; Chilterns South), with Diptera accounting for the majority of species at all farms (Figure 6.4).

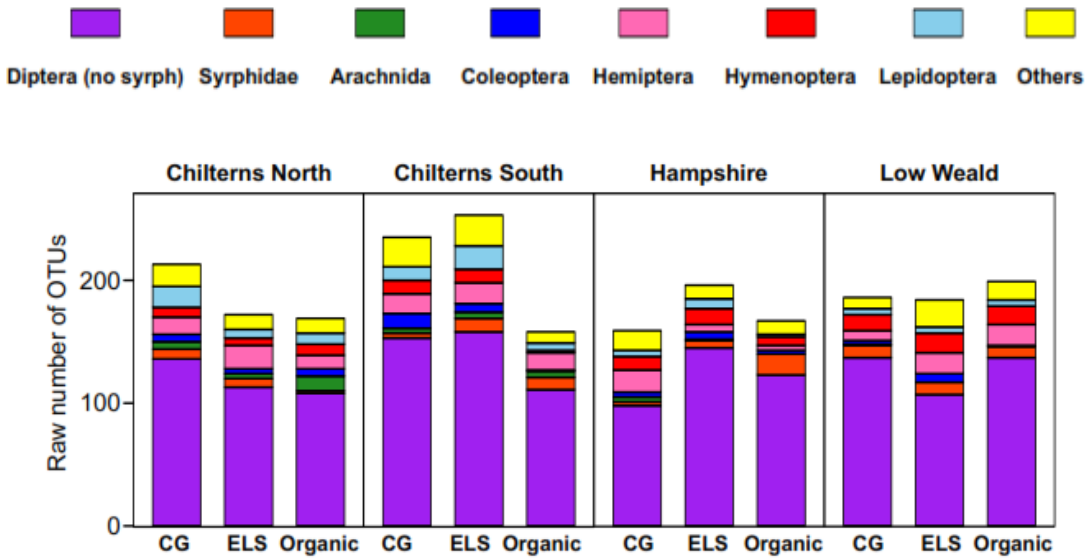

Figure 6.4: Species richness divided into taxonomic groups for each farm Type within each Triplet. Groups are orders except in the case of Syrphidae, which is a family within Diptera, and Arachnida, which is a class including spiders, mites and harvestmen. 'Others' includes low-prevalence orders and Arthropoda taxa for which identification to order level was not achieved.

Chao2 estimated total species richness was 945.9 ± 67.1 for ELS farms, 958.7 ± 69.9 for CG farms, and 863.1 ± 68.5 for organic farms. Post-hoc pairwise comparisons between types found no differences in estimated total species richness (Table 6.7).

Table 6.7: Results of post-hoc Welch's *t*-tests for a difference in Chao2 estimated total OTU-richness between each pair of farm types. Unadjusted *p*-values are shown.

	df	*t*	*p*
CG vs Organic	6.0	0.98	0.879
ELS vs Organic	6.0	0.86	0.818
CG vs ELS	6.0	1.30	0.789

6.4.6 Detection of OTUs – Soil data (18S)

Pyrosequencing of the soil DNA samples returned a total of 19,007 raw 18S sequence reads, which were reduced to 2,429 following quality control (removal of sequences < 100 bp in length, with > 2 mismatches in the primer sequence, or with a homopolymer run > 6 (4605 sequences removed), removal of chimeras (142 sequences removed), and preliminary clustering at 99% similarity). Bayesian clustering in CROP at 96% similarity gave 367 molecular OTUs, of which 71 of were assigned to Metazoa and contained more than one read. These 71 OTUs were retained to form the 'Soil Metazoa' dataset.

Arthropoda was the best represented phylum, with 42 OTUs. Annelida and Nematoda were less species rich, but OTUs in these two taxa were characterised by high numbers of sequence reads and were represented in all samples. Other groups included molluscs, tardigrades and platyhelminths (Figure 6.5), all of which are typical soil fauna. Within the Arthropoda, Insecta had the highest richness (36% of OTUs), followed by the Arachnida (24%) (Figure 6.5). Within the Insecta, seven OTUs were identified as Coleoptera, including two carabid and three staphylinid species, while five were Hemiptera, two were Diptera, and one was assigned to the Hymenoptera.

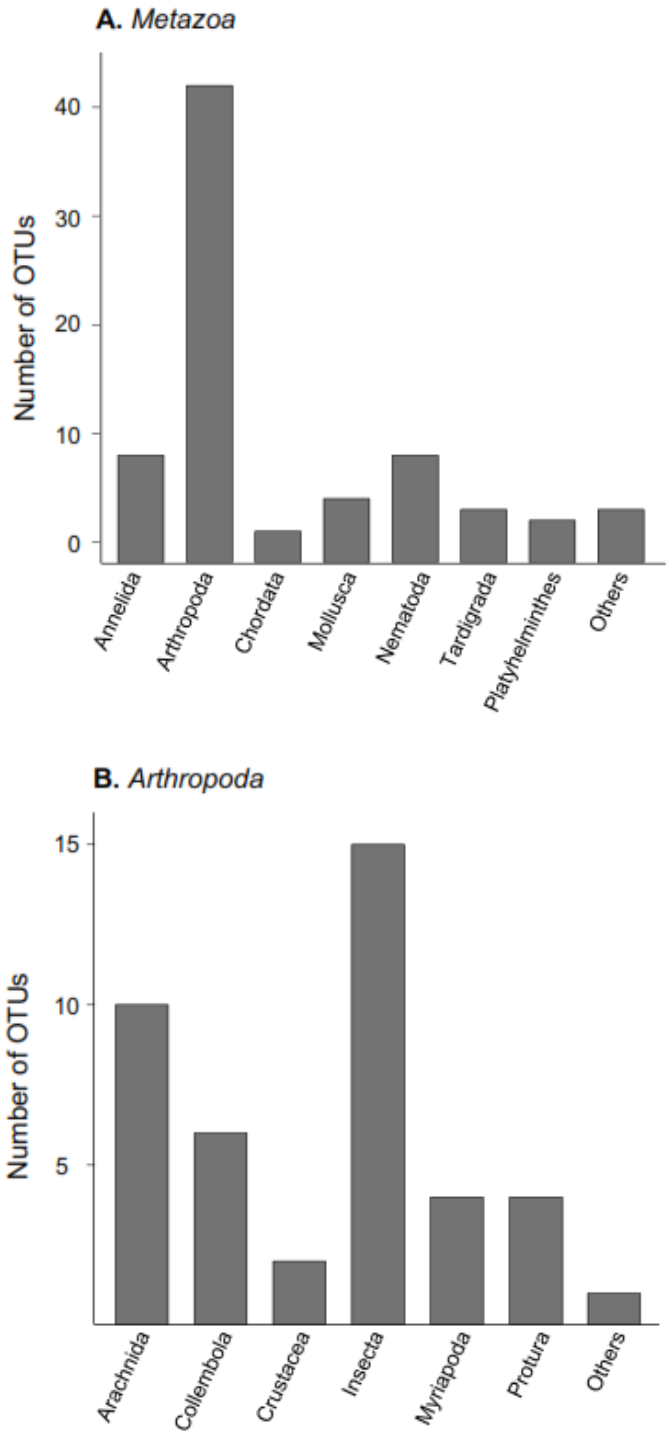

Figure 6.5: Bar chart showing the number of OTUs assigned to each taxonomic group within (A) the Soil Metazoa and (B) the Soil Arthropoda.

6.4.7 Ordination – soil data (18S)

The two-dimensional NMDS ordination of the 24 samples (2 replicates x 12 soil samples) had high stress values (0.26). Therefore, the ordination in Figure 6.6 shows the first two dimensions of a

three-dimensional ordination, which had improved stress (0.17). The two DNA extraction replicates from each soil sample were significantly correlated (Mantel test on Jaccard distance matrices with 999 permutations and Spearman's rank correlation (extraction A vs. extraction B), r=0.44, p=0.004) and clustered together in the ordination, although not tightly, which indicates some compositional variation between replicates. (Figure 6.6).

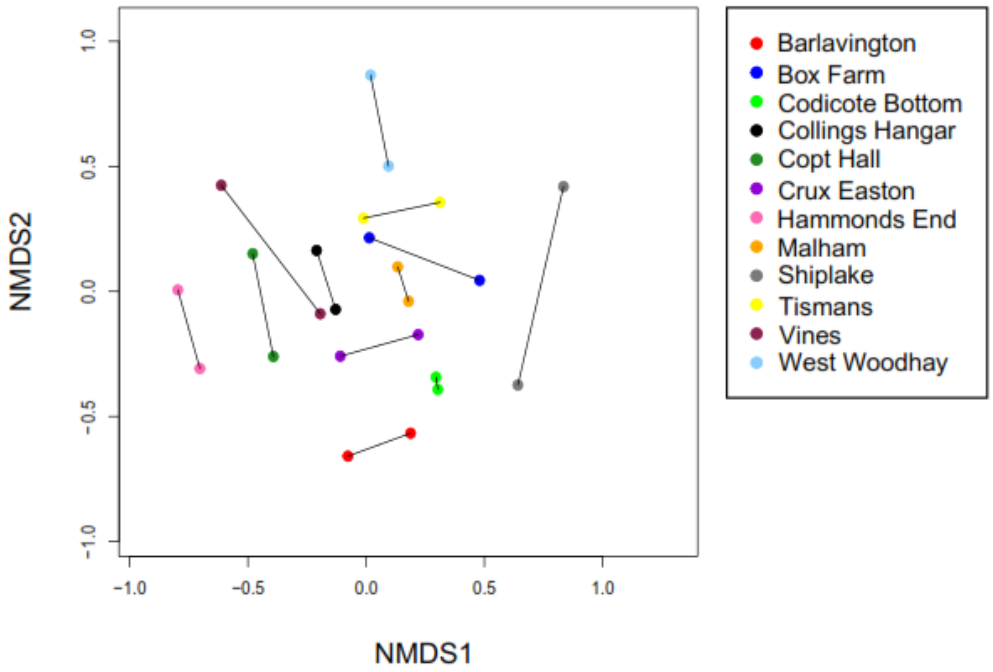

Figure 6.6: First two axes of a three-dimensional NMDS ordination based on a quantitative Jaccard distance matrix of the Soil Metazoa dataset, showing all 24 soil DNA extractions (2 replicates per soil sample). Points are coloured and grouped by farm.

Multivariate binomial LR tests in *mvabund* provided support for the clustering of extraction replicates (community ~ replicate, $LR_{df=23}$=716 , p=0.01). Therefore, replicates from the same soil sample were pooled for downstream analysis of the Soil Metazoa. Parallel analyses were performed on the Soil Arthropoda dataset, which comprised the arthropod-OTU-only subset of the Soil Metazoa dataset.

For the Soil Metazoa dataset, stress levels were again quite high (0.21) in a two-dimensional NMDS ordination. Therefore, as above, ordination plots for this dataset (Figure 6.7) show the first two dimensions of a three-dimensional ordination, which had improved stress levels (0.15). Stress levels were acceptable (0.14) for the Soil Arthropoda dataset using two dimensions. Within each

dataset, the first two dimensions of the NMDS ordination (Figure 6.7) were compared with a CA ordination (not shown), and significant agreement was found between the two methods in both cases (CA versus NMDS, Procrustes test with 999 permutations; Soil Metazoa: R^2=0.58, p=0.017; Soil Arthropoda: R^2=0.64, p=0.003). This suggests that the ordinations shown in Figure 6.7 are robust. Like for the Malaise Arthropoda dataset above (Figure 6.2), ordinations provide no evidence to support my hypothesis that organic farms would have the most distinct communities of soil fauna.

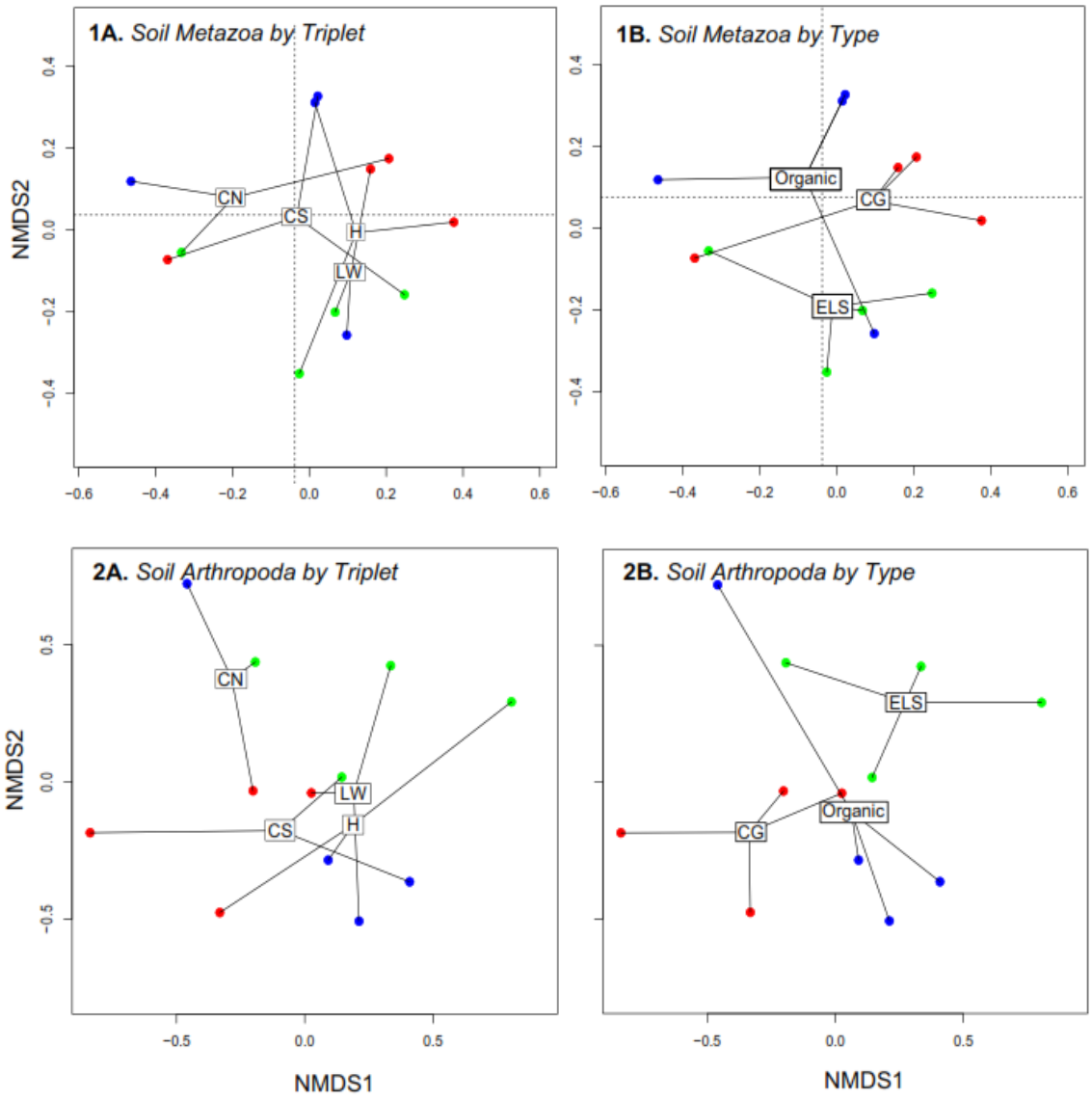

Figure 6.7: Binomial Jaccard NMDS ordinations of (1) Soil Metazoa and (2) Soil Arthropoda communities from the twelve farms. Points are coloured by farm type and grouped by (A) triplet – Chilterns North (CN), Chilterns South (CS), Hampshire (H), Low Weald (LW); and (B) farm type.

The Soil Metazoa and Soil Arthropoda datasets reveal broadly similar beta diversity patterns, despite the Soil Metazoa dataset containing a broader range of taxa (Mantel test: r=0.69, p=0.001) (Figure 6.7), and the Soil Arthropod dataset was also found to correlate significantly with the Malaise Arthropoda dataset (Mantel test, Soil Arthropoda vs. Malaise Arthropoda: r=0.36, p=0.014). This is surprising since there is probably little to no taxonomic overlap between the two datasets, and we have already seen that community composition patterns vary significantly even between taxonomic orders within the Malaise Arthropod dataset. There was no correlation between the dissimilarity matrices for Soil Metazoa and Malaise Arthropoda (r=0.14, p=0.132).

6.4.8 Significance testing – soil data (18S)

Analysis in *mvabund* detected significant compositional differences among farm types and triplets for both datasets, while the other three statistical methods only detected significance for the effect of Type on the Soil Arthropoda dataset (Table 6.8). This fits with the Type groupings for Arthropoda appearing to be the most clearly separated of the groups in the NMDS ordinations (Panel 2B, Figure 6.7).

Table 6.8: Statistics and *p*-values for beta diversity significance testing on the Soil Metazoa and Soil Arthropoda community datasets. Significance of Type and Triplet variables tested using four methods: (1) multivariate LR tests in *mvabund*, based on 999 bootstrap iterations with pit.trap resampling; (2) permutation tests in *envfit*; (3) PERMANOVA tests; and (4) CCA tests. Methods 2-4 are based on 2000 permutations.

			mvabund		*envfit*		PERMANOVA		CCA	
Farm	Dataset	*Df*	*LR*	*p*	R^2	*p*	*F*	*p*	*F*	*p*
Type	Metazoa	2,9	293	**0.007**	0.24	0.232	1.14	0.255	1.27	**0.019**
	Arthropoda		204	**0.003**	0.31	**0.038**	1.44	**0.005**	1.42	**0.005**
Triplet	Metazoa	3,8	350	**0.025**	0.18	0.912	0.99	0.384	1.03	0.62
	Arthropoda		219	**0.032**	0.21	0.678	1.00	0.211	0.98	0.70

6.5 Discussion

6.5.1 Malaise trap arthropods

This study demonstrates that a single week of sampling effort across 12 farms, in just one arable field margin per farm (and away from the specially created habitats that define the ELS and CG protocols), produces enough informative data to be able to detect compositional differences amongst landscapes (triplets) and agri-environment schemes (Figure 6.2, Table 6.2). Moreover, the metabarcode datasets are sufficiently comprehensive that it is possible to detect differences among taxa (here, insect orders) in how they respond to farm management (Figure 6.3, Table 6.4).

Overall, for the Malaise trap samples, the prediction that the organic farms would have the most distinctive arthropod communities was not upheld. This hypothesis was based on the fact that sampling was conducted in areas that were not directly affected by the agri-environment scheme in CG and ELS protocols (with the exception of field margins and hedgerows, which were present at all twelve sampling sites). In contrast, the more holistic nature of the organic protocol meant that all areas were likely to be influenced to some extent by the management scheme. The fact that this hypothesis was not fulfilled, but that the CG and Organic communities were distinct both from ELS communities and from one another (Figure 6.2. Table 6.2), suggests that (1) some of the effects of the targeted measures from the CG scheme do filter through from specially created habitats to influence invertebrate assemblages in the wider farmland area, including production areas, and (2) although the CG (land-sparing) and Organic (land-sharing) schemes each has an effect beyond that of the baseline ELS scheme, the effects are different in nature (i.e. they favour different species).

Breaking down the dataset by taxonomic group yielded a more complex picture, with patterns of differentiation varying among groups. Patterns observed in the overall arthropod dataset were found to be driven almost entirely by the Diptera, which accounted for the majority of arthropod OTUs in the Malaise trap dataset, while other groups showed contrasting patterns. This is consistent with other studies in revealing variation among arthropod taxa in their responses to environmental variables (e.g. Kleijn *et al*., 2006; Marshall *et al*., 2006; Pocock and Jennings, 2008; Billeter *et al*., 2008; Gabriel *et al*., 2010; Roth *et al*., 2011; McMahon *et al*., 2012).

Although compositional differences among farm types were detected, this study did not find that the CG and Organic farms had higher species richness of Malaise-trap arthropods than did the

baseline ELS farms (Figure 6.4). Therefore, there is no evidence here that either scheme results in 'improved' biodiversity compared with the ELS scheme, unless the species that are associated with CG/organic farms (Table 6.3) are particularly valued, either for their rarity or for their provision of ecosystem services.

In addition to the effect of farm management scheme, there was also a landscape effect on community composition, which was captured by the triplet variable. This does not seem to be entirely attributable to regional geology, since the Chilterns North and South triplets, which are located within the same National Character Area, are not always more similar to one another than to the Hampshire triplet, which is located nearby but in a different NCA. The landscape (triplet) effect is likely combining many factors that operate at regional scales, such as weather, geology, history, and distance per se, and other authors have reported similar regional biodiversity effects (e.g. Gabriel *et al*., 2006; 2010 Baselga *et al*., 2013).

6.5.2 Soil fauna using 18S amplicons

The taxa detected in the soil samples were reassuringly typical of ground and soil fauna, including arthropod groups such as Collembola, Myriapoda, and the coleopteran families Carabidae and Staphylinidae, which generally do not feature in Malaise trap samples.

Soil fauna are recognised as providing important ecosystem services and are affected by anthropogenic land uses (Mazzoncini *et al*., 2010; Cruzeau *et al*., 2012; Thiele-Bruhn *et al*., 2012). They are also taxonomically challenging since body size is often small, and it is common for there to be little morphological variation within groups to enable species identification (Stork and Eggleton, 1992). As a result, most studies have focused on the abundance or biomass of invertebrate groups at higher taxonomic levels (Stork and Eggleton, 1992; Cluzeau *et al*., 2012). A DNA-based approach has the advantage of being able to separate clusters of genetically similar organisms into molecular OTUs, which are broadly equivalent to species, even in the absence of morphological differences or species names. This allows community composition to be compared between samples at a far higher resolution than has previously been possible.

This study has shown that, in addition to species richness and abundance (Cluzeau *et al*., 2012), community composition at the species level is also affected by farm management factors. This is unlikely to be wholly attributable to differences in agricultural intensity, since CG and ELS farms both promote intensive farming in production areas, which is where the sampling was conducted. An alternative hypothesis is that the observed patterns are a result of differences between farm types in the use of agrochemicals.

Certain chemicals, including the controversial neonicotinoid pesticides, are known to accumulate in the soil during prolonged periods of use (Goulson *et al*., 2013). The impact of neonicotinoids on bees (Hymenoptera: Apidae) has recently been a subject of much discussion in light of their alarming population declines (e.g. Cresswell *et al*., 2012; Whitehorn *et al*., 2012; Laurino *et al*., 2013; Matsomoto, 2013; Thompson *et al*., 2013), but the environmental accumulation of the chemicals is such that invertebrate communities in soil and surface water habitats are likely to be even more strongly affected. Indeed, there is substantial evidence that many aquatic and soil invertebrates, including important taxa such as earthworms, are adversely affected by the presence of these chemicals, even at fairly low concentrations (Cluzeau *et al*., 2012; Roessink *et al*., 2013; Van Dijk *et al*., 2013, Wang *et al*., 2012b). The use of neonicotinoids is allowed under the ELS and CG protocols, but not on organic farms, which means that this factor cannot (on its own) explain why CG farms are distinct from ELS farms in the Soil Metazoa dataset (Figure 6.7). However, the use of organophosphate pesticides is prohibited in CG and Organic farms but unrestricted on ELS farms, and these are also known to affect soil invertebrates (Frampton and van den Brink, 2007; CG, 2011).

*S*oil fauna appeared to show a weaker response to geographic location than did the flying arthropods, with the Chilterns South, Hampshire, and Low Weald triplets all clustering together, especially for the Soil Arthropod dataset (Figure 6.7). Soil communities are perhaps less likely to be affected in the short term by factors such as the weather, which might have driven the strong triplet effects in the Malaise trap samples.

The difficulty of studying the effects of pesticides on soil invertebrate communities in the field has already led to the adoption of DNA-based approaches via the field of ecotoxicogenomics (Snape *et al*., 2004; Spurgeon *et al*., 2008). However, the requirement for custom cDNA microarrays for each species (Spurgeon *et al*., 2008) has limited the number of taxa that can be considered using this approach. Metabarcoding has the potential to be an important tool for expanding our understanding of the ecological effects of factors such as agricultural intensity and agrochemical usage, since it allows the rapid and highly resolved characterisation of soil invertebrate communities.

The soil metabarcoding method used here could be much improved upon. In particular, the 830 bp 18S amplicon used in this study is appropriate for live-sampled soil invertebrates (as in Yang *et al*., 2013), but it is too long for extracellular DNA, which occurs only in short fragments. Indeed, Taberlet *et al*. (2012) used a barcode region that was less than 100 bp in length for metabarcoding extracellular DNA in soil samples, and it is almost certain that this would have detected a greater

number of metazoan OTUs (but at lower taxonomic resolution), since the DNA extraction protocol used here included a phosphate buffer step to detach extracellular DNA from organic material and make it available for sequencing. Sequencing the long barcode region was an error, and means that it is likely that only those organisms that were live in the soil at the time of sample collection were detected. A second factor that may have limited the detection of OTUs is the transportation of samples from the UK to China in between the DNA extraction and PCR amplification steps. Although efforts were made to keep the DNA frozen during transportation, the fact that the two extraction replicates were grouped together in the ordination less tightly than perhaps expected suggests that some degradation probably occurred. Furthermore, the total number of metazoan OTUs is low compared with Yang *et al*. (2013)'s comparable study of soil samples from a subtropical forest (71 OTUs from 12 samples compared with 222 OTUs from2 samples). Although this is partly attributable to the lower diversity of temperate ecosystems compared with subtropical ones, it may also owe something to degradation during transportation. Again, sequencing a shorter amplicon would have allowed information to be gained even from heavily degraded DNA.

59% of metazoan OTUs were assigned to the Arthropoda, which is consistent with the study by Yang *et al*. (2013), in which arthropods accounted for 60-70% of metazoan OTUs in soil samples from Yunnan province, China. Insecta, Arachnida, and Collembola represent the three most prevalent groups in both studies, although the Yang *et al*. (2013) found that the Arachnida was more species rich than the Insecta, in contrast to the results presented here, where insects are the dominant group. The proportion of eukaryote OTUs assigned to the Metazoa seems very low in comparison with the Yang et al. (2013) study, with metazoan OTUs accounting for only 30% of non-single-read eukaryotes, compared with almost 100% in the Chinese study. This is principally due to the fact that Yang et al. (2013) filtered invertebrates from the soil, removing most plant, fungi and other non-metazoan material before sequencing intracellular DNA from specimens that were sampled live. In contrast, the method employed here targeted extracellular DNA bound to organic material in addition to the intracellular DNA from live-sampled organisms, and so did not include a sample filtering step, resulting in the detection of a much greater range of organisms.

6.5.3 Sampling limitations

Sampling effort was minimal due to time and resource limitations, and this limits the conclusions that can be drawn from the study. In particular, it is almost impossible to form conclusions about alpha diversity in the absence of repeated measures, and for this reason I have made little reference to species richness in this chapter. As stated in the introduction, this study did not aim

to assess the biodiversity benefits of any particular agri-environment scheme, which would require a far more comprehensive sampling design. The aim was rather to demonstrate the potential of this approach for future use in such studies by showing that it allows the detection of differences among arthropod assemblages corresponding with differences in management strategy.

Because of the low number of Malaise traps available for this study, it was necessary to standardise the sampling location across all farms, meaning that it was not possible to compare the biodiversity found in different microhabitats at the within-farm scale. Habitat heterogeneity is likely to influence the amount of biodiversity at the farm or landscape level (Benton *et al*., 2003; Concepción *et al*., 2008), and so future studies should aim to sample repeatedly across the range of habitats present on each farm. This is currently being done in the same farm system by Chloe Hardman, a doctoral student at the University of Reading. However, that study has been limited by the ability to process and identify specimens, and so has focused solely on bees, butterflies, and birds. The metabarcoding approach would allow a much wider range of taxa to contribute to such studies in the future, particularly with the incorporation of additional sampling methods, such as pitfall trapping and light trapping, to ensure thorough sampling of all groups.

Another result of having a limited number of Malaise traps was that sampling had to be conducted in two rounds, with the Hampshire and Low Weald triplets sampled a week after the two triplets in the Chilterns. This was not ideal, particularly in light of the strong temporal effect on arthropod assemblages that was detected over an eight week period in Chapter 5. Since the difference between successive weeks in that study was generally small, it is unlikely that this had a strong effect on the results presented here, but it is something that should be considered and controlled for if farmland arthropods are to be surveyed on a larger scale in the future.

6.5.4 Conclusions and further work

Despite over a decade of intensive research (Kleijn *et al*., 2001; 2004; 2006; Kleijn and Sutherland, 2003; Marshall *et al*., 2006; Pocock and Jennings, 2008; Taylor and Morecroft, 2009; Gabriel *et al*., 2010; 2013), we still struggle to determine which agri-environment measures result in genuine and cost-effective biodiversity gains in agricultural ecosystems (Whittingham, 2007; 2011; Matzdorf and Lorenz, 2010; Kleijn *et al*., 2011; Princé *et al*., 2012). There are many open questions, the answers to which require overcoming the taxonomic impediment. One of the most important questions is how local conservation gains scale up to national or continental biodiversity trends. There is currently a disconnect between policy objectives and evaluation of

results because policy objectives are usually set at the national level or above, but the implementation and evaluation of conservation measures tends to occur at the scale of individual fields (Kleijn *et al*., 2011). At this scale there is the additional problem that effects are liable to be complicated by factors such as landscape complexity and metapopulation dynamics (Gabriel *et al*., 2010; Kleijn *et al*., 2011).

Metabarcoding provides a way to overcome the taxonomic impediment and to rapidly gather large amounts of invertebrate community data, This offers an opportunity to consider the biodiversity effects of different agri-environment measures at spatial and temporal scales that have previously been impractical, and to start to make the link between local actions and regional or national trends.

It also allows a wider range of taxa to be considered – including those, like the Diptera, that are so diverse and so time-consuming to sort and identify that they are ignored in most biodiversity surveys (with the occasional exception of the hoverflies) (Kleijn and Sutherland, 2003; Kleijn *et al*., 2004; Gabriel *et al*., 2010). The non-hoverfly Diptera warrant a far higher level of attention than they typically receive in the evaluation of agri-environment measures. This is because (1) they are important from a biodiversity conservation perspective since they account for so many species, and (2) they provide important ecosystem services, principally in their role as natural enemies of common agricultural pests such as aphids (Holland *et al*., 2012).

Easier access to representative biodiversity data could also enable different models of environmental stewardship to be implemented. The vast majority of current agri-environment schemes are action-oriented – that is, farmers receive financial compensation for implementation of a scheme, rather than for a successful increase in local biodiversity – but the idea of results-oriented schemes is gaining traction in Europe (ENRD and EC, 2010; Burton and Schwarz, 2013), and has in some cases received support from farmers (de Sainte Marie, 2013). This approach is regarded as likely to be more cost-effective and to yield better results, at least in some types of landscape (Matzdorf and Lorenz, 2010; Gibbons *et al*., 2011), but it brings the issue of monitoring to the fore. The approach demonstrated here has the potential to facilitate the kind of large scale monitoring that would be required to make payments conditional on biodiversity outcomes.

Current limitations

A continuing limitation of metabarcoding is the ability to assign taxonomies at the species level. In this study, for example, only 20% of Malaise trap OTUs were identified to species, despite the fact that the British fauna is relatively well known. Reference databases will improve over time as

more species are added, and much ecological information can be retrieved even without putting names to OTUs. However, given the importance of invertebrate species in agri-ecosystems for the pollination, nutrient cycling, and pest control services that they provide, it is often desirable to achieve a higher level of taxonomic resolution. Furthermore, we generally want to be able to detect the presence of rare or endangered species where they occur. The method of taxonomic assignment used here (SAP) is known to be very conservative (Zhang *et al*., 2012), and can certainly be much improved upon. Efforts are on-going to adapt a method based on fuzzy set theory (Zhang *et al*., 2012) for use with metabarcode datasets such as this one. This method has been shown to perform much better than SAP (Zhang *et al*., 2012), and will enable better assignment of taxonomy at the species level.

There is currently a high level of interest in monitoring bee populations because of their important role as pollinators and the global population declines that have been documented (Potts *et al*., 2009; 2010; Breeze *et al*., 2010; Lebuhn *et al*., 2013). Metabarcoding is a tool that could reduce the time involved in processing samples collected as part of a large-scale bee monitoring programme such as the one proposed by Lebuhn *et al*. (2013). However, an on-going problem with the metabarcoding method used in this study is that the Hymenoptera do not amplify well with the degenerate PCR primers that are currently used (Yu *et al*., 2012). The lack of success with Hymenoptera must be addressed if this method is to be a realistic option for large-scale agricultural biodiversity studies. A potential solution is to incorporate the PCR-free, ultra-deep sequencing method described by Zhou *et al.* (2013). Although this method has limitations in its applicability to very large arthropod collections (see Chapter 8), it would nevertheless be possible to sequence the mitochondrial genomes of a reference collection of important pests, pollinators, and natural enemies, and then employ a PCR-free shotgun *re*-sequencing approach to make high confidence species identifications. The PCR-free approach would also allow inferences to be made about the abundance or biomass of key species, the scope for which is limited under the current approach. The amplicon-based metabarcode approach demonstrated here could be used alongside to provide data on wider biodiversity trends.

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Chapter 7: Metabarcoding as a tool for informing ecological restoration projects

7.1 Summary

Habitat restoration is often undertaken but rarely evaluated for success in meeting ecological goals, which limits the potential for improving outcomes by learning from past successes and failures. Here, I build on an existing 'standard' dataset from a heathland restoration study to show that COI metabarcoding can be used to inform decision-making and evaluate success in restoration projects.

Pedley *et al*. (2013a) applied six candidate disturbance treatments to grassy plots in Thetford Forest, UK, and analysed the responses of three arthropod indicator groups (carabid beetles, spiders, and ants, identified morphologically) over a two-year period, with control and reference sites also sampled for comparison. For this study, collections from year 2 of the project were metabarcoded for both COI and 18S. Taking advantage of the high-quality reference dataset containing species abundance data, I explore the effect of using read-count data in community analysis and test the relationship between read count and abundance in a common ant species. Results suggest that read count data do contain some useful information for the purposes of community analysis, and so counts were not converted to presence-absence for downstream analysis, as they were in previous chapters.

COI data yielded alpha and beta diversity patterns that closely matched those of the standard dataset. This included identifying the most aggressive treatments as those that were most successful in generating heath-like arthropod communities. Reference-based OTU-picking was performed in addition to the *de-novo* approach, allowing patterns of occurrence of particular species to be compared across datasets. For COI, occurrence patterns generally matched those in the standard dataset, although a high level of drop-out was detected, which is likely the result of long-term storage in suboptimal conditions. 18S data also recovered key beta diversity patterns, but it performed less well than COI in almost every respect.

COI metabarcoding is concluded to be a useful tool for tracking the success of restoration projects and facilitating adaptive management. This is particularly important in the context of biodiversity offsetting, which seems set to become incorporated into government policy in the UK.

7.2 Introduction

7.2.1 Ecological restoration of habitats

As undisturbed natural habitats have become increasingly scarce over recent decades, there has been a corresponding increase in efforts to restore ecological function and valued biodiversity to areas of already-degraded habitat (Suding, 2011). The widespread acceptance of restoration as a tool for biodiversity conservation is emphasised by the fact that among the Aichi Biodiversity Targets agreed at the 2010 meeting of the Convention of Biological Diversity (CBD) in Nagoya, Japan was a commitment to restore 15% of degraded ecosystems by 2020 (Secr. Conv. Biol. Divers., 2010). This kind of international mandate, together with the increasing uptake of biodiversity offsetting as a conservation tool at national and regional scales (Maron *et al*., 2012; DEFRA, 2013), makes it likely that restoration projects will continue to proliferate.

Restoration is undertaken in a wide variety of circumstances with a corresponding range of practitioners, stakeholders, and goals (Clewell and Aronson, 2006). These can be summarised as follows:

1. Private companies may undertake restoration in order to mitigate their environmental impact where temporary activity (e.g. resource extraction) has caused ecological damage (Cooke and Johnson, 2002). This is usually motivated by a need to comply with the terms under which permission was granted to carry out the activity.
2. Where permanent habitat loss is unavoidable due to development, companies may take responsibility for the creation of an equivalent habitat elsewhere, under the conceptual framework of biodiversity offsetting, which aims to achieve 'no net loss' (NNL) or 'net gain' of biodiversity (Bekessey *et al*., 2010; Maron *et al*., 2012; IFC, 2012; Bull *et al*., 2013). Although some companies engage in voluntary offsetting in order to demonstrate environmental credentials and derive an associated reputational benefit (ten Kate and Inbar, 2008; Maron *et al*., 2012), offsetting is increasingly being incorporated into government policy. A Green Paper is currently in the consultation phase in England (DEFRA, 2013), which would make offsetting compulsory under many circumstances where other forms of mitigation are not possible.
3. Restoration may feature in government programmes designed to deliver national or international policy targets, such as those set by the Convention on Biological Diversity (Clewell and Aronson's (2006) "technocratic rationale").

4. Habitat or species restoration projects may be established and overseen by conservation NGOs.
5. Ecological restoration may be undertaken privately for commercial purposes, in order to enhance the profitability of natural resources (e.g. in aquaculture, to improve fish production) (Saenger and Siddiqi, 1993; Smokorowski *et al*., 1998; Secor *et al*., 2008; Ovando *et al*., 2013).
6. Land managers may seek to restore natural landscapes for cultural and recreational purposes (e.g. in National Parks and other protected areas).

Hereafter, I focus on restoration projects that have primarily ecological goals; those with purely commercial or cultural goals are not considered further.

7.2.2 Measuring success in habitat restoration

Despite thousands of restoration projects having been conducted to date, many authors have noted that there remains a conspicuous lack of consensus on how the success of such projects should be measured (Chapman and Underwood, 2000; Palmer *et al*., 2005; Jähnig *et al*., 2011; Suding, 2011), and the question of how to measure biodiversity losses and gains is recognised as one of the principal challenges facing the effective use of biodiversity offsetting (BBOP, 2009; Bull *et al*., 2013; Maron *et al*., 2013). In an ideal scenario, success would be evaluated by comparing the total species composition of the restored habitat with that of nearby control and reference (target) habitats (Lake, 2001), but this has never been a realistic option because carrying out detailed biodiversity assessment is prohibitively demanding of time and expertise (Lytzau Forup *et al*.,2008; Gollan *et al*., 2010), especially when repeated measures are required across time and space, as is the case in a well-designed restoration study (Lake, 2001; Baasch *et al*., 2010).

The 'field of dreams' hypothesis

Instead, all too often, restoration success is simply not evaluated post-implementation (Bernhardt *et al*., 2005; Ruiz-Jaen and Aide, 2005; Palmer *et al*., 2007; Tischew *et al*., 2010; Suding, 2011). This represents either a lack of interest in the success of the restoration project on the part of stakeholders (for instance, if restoration is regarded as a tick-box exercise to gain approval for ecologically damaging activities) (Tischew *et al*., 2010), or an implicit or explicit reliance on the 'field of dreams' hypothesis (Palmer *et* al., 1997) that 'if you build it, they will come'. The problem here lies with the fact that there are many examples of restoration projects where the field of dreams hypothesis has failed to be upheld (Bond and Lake, 2003; Hilderbrand *et al.*, 2005; Ahlering and Faaborg, 2006; Choi *et al*., 2008; Palmer *et al*., 2010; Brudvig, 2011; Sudduth *et al*., 2011); that is, a structural resemblance to the target habitat has not resulted in the expected

biotic changes. As an example, Smokorowski *et al*. (1998) found that, although 98% of aquatic habitat restoration projects considered in their meta-analysis succeeded in meeting structural habitat targets, the biotic goal (increase in fish production) was achieved in only 5% of projects. This highlights the importance of direct post-intervention biodiversity monitoring and adaptive management if the ecological goals of restoration projects are to be met.

Unfortunately, metrics for measuring biodiversity losses and gains in the context of government offsetting schemes also rely implicitly on the 'field of dreams' hypothesis. Although they attempt to evaluate success, they do so by measuring broad surrogates of biodiversity, which are largely structural in nature, with no direct consideration of any aspect of species diversity on a site-by-site basis beyond the broad classification of vegetation (DEFRA, 2012). The danger here is that natural habitats are substituted for recreated ones that appear superficially to be in good condition, while in fact lacking key elements of their natural biological community (see Munro *et al.*, 2007). This would allow for high background rates of biodiversity loss despite the appearance of having achieved NNL.

The use of indicator taxa

Another common way of evaluating success in restoring biological communities is the explicit use of indicator taxa. This approach is problematic because the selection of meaningful indicators is extremely complex and yet constrained by the need to be easily measurable (Lake, 2001; Lindenmayer *et al*., 2002; Doren *et al*., 2009; Brudvig, 2011). Lake (2001) states that the desirable properties of indicators include "*their being relatively easy and inexpensive to measure…; they must have no taxonomic difficulties or measuring uncertainties; they need to be sensitive to the restoration measures; they need to respond at different rates over different time spans; and preferably they need to be linked with each other in their ecological functioning*". In reality, with some notable exceptions (e.g. Rosenthal, 2003; Doren *et al*., 2009), indicator properties tend not to be considered beyond ease of measurement. The majority of indicator-based evaluations measure just one group of organisms, most commonly vascular plants (Ruiz-Jaen and Aide, 2005; Brudvig, 2011), with the implicit – and usually invalid (Lindenmayer *et al*., 2002; Munro *et al*., 2007) – assumption that other groups will follow the same trends as the indicator group. Thus, the responses of many elements of biodiversity to habitat restoration efforts usually remain unknown (Brudvig, 2011). This includes groups such as terrestrial invertebrates and soil biota, which are likely to be of functional importance in the ecosystem, and, therefore, to affect its ability to persist in the long-term (Middleton and Bever, 2010; Ohsowski *et al*., 2012).

The consequences of insufficient monitoring

The general lack of monitoring and reporting of the results of habitat restoration projects is damaging at two levels: at the project level, it reduces the potential for adaptive management, which is likely to be required for a truly successful outcome, given the complexity of the natural ecosystems that projects seek to replicate (Cooke and Johnson, 2002; Hilderbrand *et al*., 2005; Martin *et al*., 2005; Palmer *et al*., 2007); at a higher level, it hampers the development of a conceptual framework for restoration ecology by limiting the ability of the scientific community to learn from the successes and failures of past projects (Chapman and Underwood, 2000; Lake, 2001, Cooke and Johnson, 2002; Bond and Lake, 2003; Palmer *et al*., 2007; Suding, 2011). For habitat restoration to provide the biodiversity benefits that it promises, there is a need to be able to generate direct biodiversity data across a wide range of taxonomic groups and at large spatial and temporal scales, so as to improve our understanding of the factors that influence restoration success (Majer, 2009).

7.2.3 Heath restoration in Thetford Forest

Pedley *et al*. (2013a) conducted a well-designed heath restoration experiment in Thetford forest, located on the Norfolk/Suffolk border in the UK, where isolated fragments of a rare lowland heath habitat are distributed among plantation forest stands. It has been proposed that the modification of an existing network of grass-covered trackways within the forest might enhance connectivity between heath fragments by allowing the percolation of dispersal-limited taxa such as ground arthropods, many of which are highly stenotopic (i.e. specialist in their habitat requirements) and do not occur on the trackways in their unmodified form (Pedley *et al*., 2013b).

The project aimed to determine the most effective way of modifying the trackways to allow them to support arthropod heath specialists and, ultimately, to act as corridors between heath fragments. It considered the response of three groups of ground arthropods (spiders, carabid beetles, and ants) to a gradient of six increasingly severe disturbance treatments over a period of two years. Control (no intervention) and reference (natural heath) sites were also sampled for comparison (see Methods section for full details of experimental design). A total of 17,498 individual specimens belonging to focal taxa were collected in the second year alone, requiring 496 person-hours of sorting and identification effort (Ji *et al*., 2013).

A key finding of the Pedley *et al*. (2013a) study was that different taxonomic groups of arthropods showed markedly different responses to disturbance treatments, with plants showing different patterns again. This supports the concerns of Lindenmayer *et al*. (2002) that different indicator

groups can give conflicting information about the success of habitat restoration efforts, and reaffirms the danger of judging outcomes on the basis of the response of a single group.

7.2.4 Aims of this study

In this chapter, I use arthropod collections from the second year of the Pedley *et al*. (2013a) study to demonstrate the usefulness of metabarcoding for tracking the success of habitat restoration projects. I show that a metabarcode approach can replicate results obtained via traditional morphological identification of specimens, while being far less demanding of time and taxonomic expertise.

I also take advantage of the high-quality standard (STD) dataset to explore the performance of some aspects of the metabarcode data.

The use of sequence count data

First, I consider the usefulness of sequence count data as a proxy for species abundance information. In previous chapters, I have followed the conservative approach of Yu *et al*. (2012) and Ji *et al*. (2013) in converting sequence read counts per Operational Taxonomic Unit (OTU) to simple presence-absence. This is because it is unknown to what extent factors such as PCR and sequencing biases might have affected the quality of quantitative data. Although some correlation between sequence read number and abundance of individuals has been occasionally been reported (Amend *et al*., 2010; Porazinska *et al*., 2010; Hajibabaei *et al*., 2011; Deagle *et al*., 2010; 2013), the relationship is usually noisy, and the use of wide confidence intervals is generally advised when making direct inferences about relative abundance or biomass from sequence count data (Amend *et al*., 2010; Deagle *et al*., 2013). A factor that makes my data particularly susceptible to interspecific amplification bias, both here and in previous chapters, is the inclusion of a large number of species across a wide range of taxonomic groups. This necessitates the use of highly degenerate primers, which inevitably amplify some taxa more readily than others (Hajibabaei *et al*., 2011; Yu *et al*., 2012).

Nevertheless, it remains possible that biases occurring during metabarcoding do not render sequence count information entirely useless for community analysis. This is because taxon-specific biases are often conserved within species, such that variation *across samples* is meaningful (Amend *et al*., 2010; Hajibabaei *et al*., 2011; Deagle *et al*., 2013). Therefore, using the true abundance data in the STD dataset as a reference, I ask whether retaining sequence count information improves or impedes the ability of metabarcode data to recover beta-diversity patterns.

Reference-based OTU-picking

Second, I explore the use of reference-based OTU-picking for elucidating patterns of occurrence for particular species – in this case the spider, carabid and ant species that make up the STD dataset. Reference-based OTU-picking is preferable to a *de novo* approach where there is interest in a particular set of species for which reference sequences exist. This is because (1) requiring a query sequence to match a reference sequence within a specified similarity threshold acts as a strict quality filter; (2) rare species (for which there are reference sequences) can be distinguished from sequencing errors and retained, when they might have been discarded from *de-novo* datasets; (3) greater certainty can be attributed to taxonomic assignments than is the case for *de-novo* OTU-picking with taxonomic assignment based on global reference datasets; and (4) OTU identifiers are stable because they are linked to named reference sequences, which means that OTUs can be matched across different studies (Bik *et al*., 2012a). In this study, a library of reference sequences is generated for the species that make up the STD dataset. The distribution of individual species is compared between the reference-based metabarcode dataset and the STD dataset in order to examine how well patterns are recovered by metabarcoding.

Comparing the performance of COI and 18S

Finally, I compare the ability of the COI and 18S barcode regions to characterise biodiversity patterns, using the STD dataset as reference. COI and 18S are two of the most commonly-used amplicons for metabarcoding studies of metazoan communities. COI is the standard barcode for animals (Hebert *et al*., 2003), and, as such, it is linked with the most extensive reference libraries of any barcode region, which is a strong argument in favour of its use (Hajibabaei *et al*., 2011; Yu *et al*., 2012; Leray *et al*., 2013). COI also tends to have a faster substitution rate than nuclear rRNA genes (e.g. 18S; Brown *et al*., 1979), which allows a higher level of taxonomic resolution to be achieved in most groups (Emerson *et al*., 2011; Dettai *et al*., 2012). Although this is generally a desirable property, it carries an inherent risk of overestimating species diversity by subdividing species (Machida and Tsuda, 2010).

Conversely, use of the more conserved 18S gene carries the risk of underestimating diversity due to the inability to differentiate between closely-related species (Porazinska *et al*., 2009), and many authors have argued that 18S is unsuitable for the study of species-level diversity (Creer *et al*., 2010; Derycke *et al*., 2010; Tang *et al*., 2012; Leray *et al*., 2013). Nevertheless, 18S has become the barcode of choice for studies of meiofaunal diversity (e.g. Chariton *et al*., 2010; Creer *et al*., 2010; Fonseca *et al*., 2010; Bik *et al*., 2012b; Bradford *et al*., 2013). One reason is that amplification of COI in nematodes, a key meiofaunal group, is inconsistent using the usual

barcoding primers (Folmer *et al*., 1994; Creer *et al*., 2010); another is that 18S occurs in tandemly repeated, multiple copies, which facilitates amplification from organisms of very small body size (Creer *et al*., 2010). While reference libraries for 18S are more limited than for COI, they are more comprehensive than those of other candidate rRNA genes, such as 28S, due to having a greater history of use in molecular studies of nematodes (Porazinska *et al*., 2009).

In this chapter, I test the hypothesis that COI overestimates diversity, while 18S underestimates it. I also ask which barcode performs best in terms of replicating the results of the STD dataset.

7.3 Methods

7.3.1 Field methods

All field work was carried out by Dr Scott Pedley as part of his Doctoral thesis at the University of East Anglia, and is described in Pedley *et al*. (2013a). Here, I summarise the elements of the field protocol that are of relevance to this study.

Experimental design

In February 2009, forest-trackway plots in Thetford Forest were subjected to one of six physical disturbance treatments, covering a range of severity from simple mowing (Swipe) to complete removal of vegetation and top-soil (Turf Strip) (Table 7.1; Figure 7.1).

Table 7.1: Details of the disturbance treatments applied to forest trackway plots, and the number of samples from each type of plot that contributed to the datasets in this chapter. Treatments are listed in order of severity from the mildest (Swipe) to the most disruptive (Turf Strip) and descriptions are taken from Pedley *et al*. (2013a).

Treatment	Disturbance Type	Number	Description
Control	None	8	No disturbance
Swipe	Vegetation disturbance	8	Sward cut with tractor-mounted blades, clippings left in-situ
Harvest	Vegetation disturbance	9	Sward cut and removed with silage harvester
Disc Plough	Soil disturbance	9	Tractor-pulled disc plough. Disrupts but does not destroy vegetation, with shallow soil disturbance, 10-20 cm deep
Forest Plough	Soil disturbance	9	Soil and litter inverted in plough lines producing bare mineral substrate in furrows 30-40 cm wide and 40-50 cm deep, alternating with strips of intact vegetation 40-50 cm wide
Agricultural Plough	Soil disturbance	9	Turf and top-soil inverted producing bare substrate across the plot, with biomass retained and buried to 20-30 cm depth
Turf Strip	Turf removal	9	Removal of vegetation, root mat, litter and organic soil exposing mineral subsoil at a depth of 15-30 cm
Heath	None (Target)	7	Natural lowland heath

Experimental plots were located on trackways that were at least 9 m wide and located within forest plantations aged 10-25 years. The plantations themselves comprised closed-canopy stands that lacked open-habitat carabid, spider, or plant species. In order to ensure that samples were

not capturing open-habitat species from adjacent areas, each plot was located at least 100 m away from other treatment plots, open areas, or felled plantation stands.

To minimise shading effects, plots were established at the widest point of North-South oriented trackways, or at the northern verge of trackways that had an East-West orientation. Treatments were randomly allocated to plots, stratifying between calcareous and acidic soils. There was also stratification between acidic soils that (1) lacked, or (2) were dominated by bracken (*Pteridium aquilinum*).

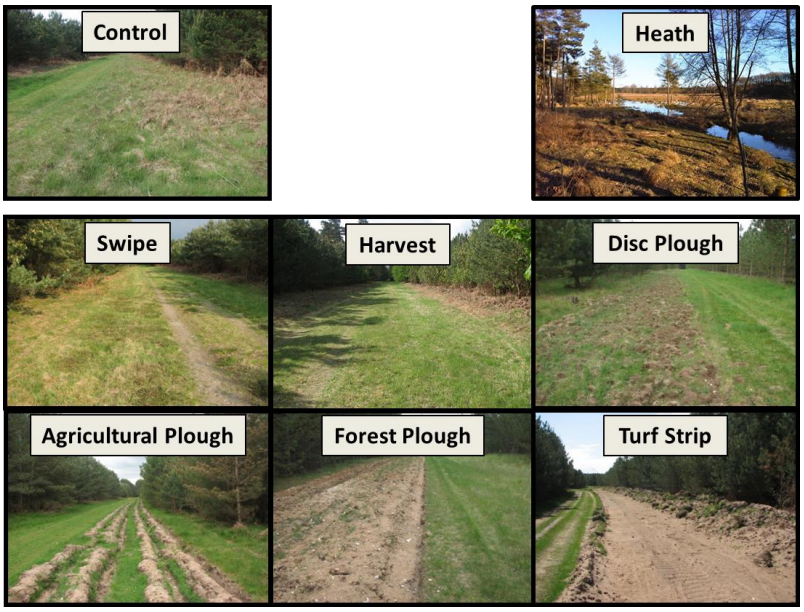

Figure 7.1: Photographs showing control and target (Heath) habitats and experimental plots with each of the six disturbance treatments applied.

Each treatment was initially applied to nine trackways, with plots measuring 150 m in length and 4-5 m in width. An additional nine plots acted as controls, receiving no disturbance treatment. Transects were blocked in sets of seven, so that all treatments plus the control were applied to a transect in each block. Blocks were arranged across the Thetford Forest landscape.

Invertebrate sampling

Ground-active invertebrates were sampled using pitfall trapping. Six pitfall traps were arranged in a single transect along the centre of each plot (beginning 37.5 m from each end, with intervals of 15 m between traps). Each trap was 7.5 cm deep and 6.5 cm in diameter and was filled with 50 ml of 70% ethylene glycol. Traps were opened for seven consecutive days on three separate occasions during spring/summer in each of the two years following the application of treatments ((1) May, (2) June, and (3) late July/ early August in 2009 and 2010). For each year, trap samples

were pooled within plots across the three sampling periods, resulting in one composite sample per plot-year.

In 2009, pitfall trapping was also conducted at seven heath reference sites, all of which were located within 8 km of the treatment plots. Three transects of six traps were set at each heath site, with transects separated from one another by a distance of at least 50 m. Like the traps in the treatment plots, those in the heath sites were also open for seven consecutive days on three occasions (May, June, and August). Trap samples were subsequently pooled within sites. Sampling was not repeated at heath sites in 2010 because these sites were expected to be ecologically stable. In contrast, the sites where disturbance treatments had been applied were expected to show ecological changes over time as the new habitats developed.

Morphological identification of specimens

Undergraduate students were employed to sort the indicator taxa to carabids, spiders, and ants. Other taxa – and immature specimens of reference taxa – were not considered further due to the difficulty of making morphological identifications. Subsequent to initial sorting, adult spiders were identified to species by S. Pedley following Roberts (1987; 1996), carabids by the Norfolk beetle recorder and an amateur coleopterist following Luff (2007), and ants by the Norfolk county ant recorder following Bolton and Collingwood (1975), Skinner and Allen (1996), and Blacker and Collingwood (2002).

The STD dataset used for comparison with the metabarcode datasets in this study includes abundance data for these three indicator groups.

Samples for metabarcoding

Samples from 69 of the 70 sites from the 2010 trackway and 2009 heath collections were compiled for metabarcoding, which was carried out in 2012. Note that samples had not been stored with genetic analysis in mind but had been kept densely-packed in dilute (70%) ethanol and handled without consideration of cross-contamination issues. Each sample contained all the indicator taxa (spiders, carabids, and ants) plus the unidentified bycatch, which included insect orders such as Orthoptera, Diptera, and non-carabid Coleoptera, plus other arthropod groups including Myriapoda, Isopoda and Collembola. Some of the rare indicator species had been pinned for reference collections, and two legs from each of these specimens were added to the sample for metabarcoding. Additionally, to save on DNA extraction costs, the bodies of large individual specimens were removed from the bulk samples with just a leg retained for DNA

extraction. One control site was subsequently omitted from analysis because the plot was discovered to have been mowed before sampling. This left 68 sites for analysis (Table 7.1).

7.3.2 Laboratory steps and bioinformatic processing

Bulk DNA extraction, PCR amplification of the COI and 18S barcode regions, and high-throughput sequencing of the pitfall trap samples was carried out by collaborators at the Kunming Institute of Zoology (KIZ) following the steps described in Chapter 4. Each of the 68 samples was processed as a separate MID.

COI and 18S de novo metabarcode datasets

Following sequencing, the COI and 18S *de novo* datasets were produced following the bioinformatics steps described in Chapter 4. Briefly, this includes elimination of low quality sequences and detection and removal of chimeras, followed by *de novo* OTU-picking in CROP, which was performed with a 97% similarity threshold for both COI and 18S. Taxonomy was assigned using SAP (Munch *et al*., 2008) for COI and by BLASTing against the Silva 108 reference dataset for 18S. For each dataset, the raw OTU table was filtered to exclude non-arthropod and single-read OTUs, with the remaining OTUs retained for downstream analysis and forming the 'COI *de novo*' and '18S *de novo*' datasets (Table 7.2).

Generating a local reference library for indicator groups

COI and 18S reference sequences were produced via Sanger sequencing for each spider, carabid, and ant indicator species that featured in the STD dataset. DNA was extracted from whole ant specimens and from a leg of each spider and carabid specimen using the Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany), following manufacturer's instructions. PCR amplification was performed in 30 µl reaction volumes using the same COI and 18S primer pairs that were used for the metabarcode datasets (detailed in Chapter 4). For COI, each reaction contained 3 µl of 10x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, 1 µl Taq DNA polymerase (TaKaRa Biosystems, Ohtsu, Shiga, Japan), and approximately 100 ng genomic DNA. For 18S, 0.6 µl Taq polymerase and 30 ng DNA were used, while other reagent volumes were the same as for COI. The thermocycling profile for COI consisted of an initial denaturation phase of 2 minutes at 95 ^{o}C, followed by 35 cycles of 15 seconds at 95 ^{o}C, 30 seconds at 49 ^{o}C (annealing), and 1 minute at 72 ^{o}C, with a final extension phase of 7 minutes at 72 ^{o}C. For 18S, cycling conditions consisted of 15 seconds at 95^{o}C, 45 seconds at 52 ^{o}C (annealing), and 1 minute at 72 ^{o}C, with a final extension phase of 10 minutes at 72 ^{o}C. PCR products were visualised on 2% agarose gels before being

bidirectionally sequenced using BigDye version 3.1 on an ABI 3730xl DNA Analyser (Applied Biosystems, Carlsbad, California, USA) at the KIZ.

COI-ref and 18S-ref metabarcode datasets

For each of the two amplicons (COI and 18S), a second metabarcode dataset was produced based on the library of reference sequences. Following initial quality control (removal of sequences < 100 bp, with > 2 primer mismatches or a homopolymer run > 6 for 18S, or > 9 for COI; removal of sequences that fail to align with the reference dataset; and correction of frameshift mutations detected using MACSE (Ranwez *et al*., 2011) for COI; see Chapter 4 for full details), the reference sequences were used as seeds for reference-based OTU picking in UCLUST (Edgar, 2010) via the *pick_otus.py* command in QIIME (Caporaso *et al*., 2011), with a similarity threshold of 97% for both COI and 18S. Sequences that did not cluster with a seed sequence were eliminated. For the species that were detected, an OTU table was created giving the number of sequence reads for each species in each sample. The resulting datasets are referred to henceforth as the 'COI-ref' and '18S-ref' datasets (Table 7.2).

Table 7.2: Details of the five main datasets considered in this study, including one based on standard morphological identification of specimens (STD) and four based on metabarcoding.

Dataset	Data type	Groups included	Taxonomic assignment	OTU picking	Similarity threshold
STD	abundance	Spiders, carabids, ants	Morphology	NA	NA
COI *de novo*	COI sequence reads	All arthropods	SAP	de-novo	97%
18S *de novo*	18S sequence reads	All arthropods	BLAST	de-novo	97%
COI-ref	COI sequence reads	Spiders, carabids, ants	Ref. sequences	reference-based	97%
18S-ref	18S sequence reads	Spiders, carabids, ants	Ref. sequences	reference-based	97%

Taxonomic subsets

From each of the five OTU tables (Table 7.2), taxonomic subsets were extracted for each of the three indicator groups: spiders (Araneae), carabid beetles (Coleoptera: Carabidae), and ants (Hymenoptera: Formicidae).

7.3.3 The use of sequence count data

All data analysis was carried out in *R* v. 2.15.2 (R Core Team, 2012). To test the effect of PCR and sequencing bias on beta-diversity analyses, read-number biases were simulated in data subsets

that included only the Control, Turf Strip, and Heath sites. The control table contained abundance data from the STD dataset.

Interspecific bias

In the first simulation, I randomly sampled a number from a uniform distribution ranging from 1-100 inclusive and multiplied all the abundances within a species (i.e. across all MIDs) by that number. This was repeated for each species to simulate the situation where some taxa are amplified more successfully than others, either due to better primer matching or to simple stochasticity. For each version of the dataset (with and without bias), a quantitative Jaccard distance matrix and a two-dimensional NMDS ordination were produced using *R* package *vegan*'s (v. 2.0-7; Oksanen *et al*., 2013) *vegdist()* and *metaMDS()* functions, respectively. These were compared between datasets using Mantel (Spearman's) and Procrustes tests, each with 999 permutations of the data.

Next, I tested whether the bias would affect the results of beta diversity analyses conducted in *R* package *mvabund* (v. 3.7.0; Wang *et al.*, 2012). I carried out multivariate negative binomial likelihood ratio (LR) tests for compositional differences between the three treatment levels represented in the data subset (Control, Turf Strip, Heath), and compared the LR coefficients and *p*-values that were returned.

Intraspecific bias

Results from this analysis showed that taxon-specific amplification bias *per se* has little effect on the results of beta diversity analyses (see Results section for full details). However, amplification success could also be influenced by competition between templates in multiplex PCR reactions, meaning that the amplification of some OTUs might be suppressed in the presence of certain others. This would lead to variation in amplification success *within* species (i.e. across MIDs) as well as *between* species. I investigated whether this was the case for one of the more abundant indicator taxa, the ant *Lasius niger,* by correlating the number of individuals per sample in the STD dataset against read proportion (read count divided by total reads) per MID in the COI-ref dataset, using Spearman's rank correlation (*R* function *cor.test()*). I used read count data from the COI-ref dataset because a reference-based method should more accurately identify reads that come from a particular species.

Because the correlation between *L. niger* read proportions and abundances was weak, a second simulated OTU table was created by multiplying every read number in the first simulated OTU

table with a random multiplier between 1 and 10. Distance matrices and and NMDS ordinations were again compared using Mantel and Procrustes tests.

Read count vs. presence-absence data

Finally, to test whether read count data added useful information to beta-diversity analyses, I used a Mantel test to compare quantitative Jaccard distance matrices from the COI-ref and STD datasets (again, just including Control, Turf Strip, and Heath sites). This was subsequently repeated with the metabarcode data transformed to presence-absence using *vegan* function *decostand()*, and results were compared. The reference-based COI dataset was used here because it only contained taxa that were present in the STD dataset, unlike the COI *de novo* dataset.

Based on the results of these analyses, read count data in subsequent analyses were not transformed to presence-absence data, as they were in previous chapters.

7.3.4 Recovering patterns of occurrence for individual ant species

Inspection of the raw data

The ant subsets of the various datasets contained few species (N ≤ 16), which facilitated closer inspection of the raw data in terms of exploring the recovery of individual species in the metabarcode datasets. I checked whether the most common species in the STD dataset were also those that were most common in the COI-ref and 18S-ref datasets (using the reference-based metabarcode datasets for ease of matching species across datasets).

Identifying specialists

R package *mvabund* was used to identify habitat specialists in the ant subsets of the STD, COI-ref, and 18S-ref datasets. *mvabund* fits GLMs to each species in a dataset to test whether it responds significantly to the test variable, and returns species-specific test coefficients and *p*-values in addition to the overall test results. These species-specific results can be used to identify species that are significantly associated with particular environmental conditions. Heath and trackway specialists were identified using the species-specific LR coefficients and *p*-values from a negative binomial LR test comparing Heath sites with Trackway sites. In previous chapters, specialists were identified based on *p*-values that were not adjusted for multiple tests because the very large numbers of OTUs in the datasets meant that the correction factor tended to be very large, rendering all responses non-significant. However, since the ant data subsets contained few species, correction for multiple tests did not result in all responses becoming non-significant. Therefore, specialists were identified based on adjusted *p*-values, with adjusted $p \leq 0.05$ indicating a significant association, and the direction of the association (Heath or Trackway)

determined based on the LR coefficients. Results from the three datasets were compared in terms of the extent to which they identified the same associations between species and habitats.

7.3.5 The effect of disturbance treatments – beta diversity

The following analyses were performed first for the full OTU tables of each dataset, and then for each of the three taxonomic subsets (spiders, carabid beetles and ants).

Distance-based analysis

Quantitative Jaccard distance matrices were created for the STD dataset and each of the four metabarcode datasets (COI *de novo*, 18S *de novo*, COI-ref, and 18S-ref). The distance matrix for each of the metabarcode datasets was compared with that of the STD dataset using Mantel tests with Spearman's correlation and 999 permutations of the data. Next, two-dimensional quantitative Jaccard NMDS ordinations were created for each dataset and compared using Procrustes tests. Ordinations were plotted for all five full datasets (but not for taxonomic subsets), with points coloured and grouped according to treatment level.

In most ordinations, Heath sites were clearly separated from Trackway (treatment and control) sites, and it was considered that this would probably drive high levels of overall correlation between datasets. Therefore, the Heath sites were subsequently excluded from each dataset, and the tests were repeated in order to establish whether there was still correlation between datasets for the more densely clustered Trackway sites.

mvabund analyses

Multivariate negative binomial regressions were conducted in *mvabund* to test for differences in species composition among treatments. *mvabund* is preferred to traditional distance-based significance testing methods (e.g. PERMANOVA) for two reasons. First, as mentioned in previous chapters, *mvabund* is less likely than distance-based methods to confound dispersion and location effects because it takes into account the appropriate mean-variance relationship for the dataset in question. Second, *mvabund* is less influenced by high variance OTUs than are other methods (Warton *et al*., 2012). This second advantage was irrelevant in previous chapters where presence-absence data were used, but it becomes pertinent here since read count data are not transformed to presence-absence. The negative binomial error distribution was used for *mvabund* analyses because it is appropriate for count data, which tends to be over-dispersed (Wang *et al*., 2012; Warton *et al*., 2012).

Three separate *mvabund* analyses were performed for each dataset, each with pit.trap resampling and 999 bootstrap iterations. First, I tested for a difference in species composition between Heath sites and Trackway sites, using LR tests. Next, I wanted to use pairwise comparisons between each treatment and the control to test which of the disturbance treatments had caused a significant change in community composition to occur. The authors of *mvabund* advised that the *summary.manyglm()* function should be used for this type of analysis, and that Wald tests should be performed instead of the usual LR tests (Y. Wang, *pers. comm*.). Before carrying out this analysis, I first tested for an overall difference among treatments (including Control sites but excluding Heath sites) using multivariate Wald tests and the usual *anova.manyglm* function. For datasets where a significant difference among treatments was detected, I proceeded to test for pairwise differences between treatments and the control, as described above. *p*-values were subsequently corrected for multiple tests using the *p.adjust()* function in *R*'s base package with Benjamini and Hochberg's (1995) correction method (*method="fdr"* in *R*). In the event that no difference among treatments was detected, pairwise tests were not performed. The primary purpose of these tests was to determine the extent to which the various metabarcode datasets returned the same results as the STD dataset.

7.3.6 The effect of disturbance treatments – alpha diversity

For the COI *de novo*, 18S *de novo*, and STD datasets, total species richness was estimated using the Chao2 incidence-coverage method (Chao, 1987; Gotelli and Colwell 2011), which was implemented in *R* via *vegan*'s *specpool()* function. Estimated total species richnesses of the COI and 18S datasets were each compared with that of the STD dataset using a manual Welch's *t*-test based on the estimates and their standard errors. Species richness was expected to be higher in the metabarcode datasets than in the STD dataset, since the metabarcode datasets included all Arthropoda (spiders, carabids and ants, plus 'residue') while the STD dataset contained only spiders, carabids, and ants.

Finally, I tested whether the metabarcode datasets were able to recover alpha diversity information in terms of detecting which treatments resulted in the highest species richness of arthropods. For each of the COI *de novo*, 18S *de novo*, and STD datasets, the *specpool()* function was used to derive the observed number of species across all the sites assigned to each treatment level (Control, six disturbance treatments, and Heath). This function also returned four different estimates of the total number of species per treatment, each of which was generated by a different incidence-coverage method. The four methods were Chao2 (used above), first order jackknife ('Jackknife1'), second order jackknife ('Jackknife2'), and bootstrap (Smith and van Belle,

1984; Chao, 1987; Palmer, 1990; Colwell and Coddington, 1994), each of which uses a different algorithm to estimate the number of 'unseen' species based on the number of low-incidence species in the dataset. Tests for Spearman's correlation of alpha diversity estimates across the eight treatments were carried out between each of the metabarcode datasets and the STD dataset, first for observed species richness and then for each of the four estimates of total species richness.

7.4 Results

7.4.1 Detection of OTUs and assignment of taxonomy

Sanger reference sequences

Sanger sequencing of the morphologically identified reference specimens resulted in COI sequences for 90% of spider species, 85% of carabid species, and 87% of ant species. 18S sequences were obtained for 88% of spider species, 91% of carabid species, and 87% of ant species (Table 7.3). Sequencing failures were attributed to DNA degradation and cross-contamination issues.

Table 7.3: Number of species/OTUs in each dataset assigned to each of the indictor groups (Araneae, Carabidae, and Formicidae) plus the number of residue OTUs in the COI and 18S *de novo* datasets. 'Sanger' refers to the number of indicator species for which reference sequences were obtained.

	STD	Sanger COI	Sanger 18S	COI-ref	18S-ref	COI *de novo*	18S *de novo*
Araneae	59	53	52	40	40	49	5
Carabidae	55	47	50	15	33	9	1
Formicidae	15	13	13	9	10	11	2
Other Arthropoda	0	0	0	0	0	264	68
Total	**129**	**103**	**115**	**64**	**83**	**361**	**76**

COI de novo dataset

Sequencing coverage was low, with just 71,661 raw sequence reads across the 68 samples. Quality control and initial clustering at 99% reduced this to 10,013 reads, and *de novo* clustering in CROP at 97% similarity returned a total of 810 OTUs. Of these, 361 were assigned by SAP to the Arthropoda and were represented by > 1 sequence read. These were retained for downstream analysis and formed the COI *de novo* dataset. Overall, 96% of arthropod OTUs were identified to order level, and 32% were identified to species. Multiple OTUs were assigned to each of the Formicidae, Carabidae, and Araneae, which were the indicator groups that made up the STD dataset (Table 7.3), and a subset of the COI *de novo* dataset was created for each of these groups. Residue (i.e. non-indicator) groups included the Myriapoda (36 OTUs), Collembola (22 OTUs), Isopoda (19 OTUs), Diptera (47 OTUs), and Orthoptera (73 OTUs; discussed in Section 7.5.3, below). The only vertebrate species detected was the Eurasian Jay (*Garrulus glandarius*).

COI-ref dataset

Reference-based OTU-picking in UCLUST detected only 62% of the indicator species in the Sanger reference library, including just 32% of carabids (Table 7.3). The number of species detected in each taxonomic subset was similar to the number detected by *de novo* OTU-picking.

18S de novo dataset

For 18S, pyrosequencing returned 92,079 raw sequence reads, which was reduced to 5119 following quality control and initial clustering at 99%. *De novo* OTU-picking in CROP returned a total of 486 OTUs, but BLASTing against the Silva 108 reference database assigned only 76 non-single-read OTUs to the Arthropoda. These formed the 18S *de novo* dataset. As above, a subset of this dataset was created for each of the Araneae, Carabidae, and Formicidae, although few OTUs were assigned to each (Table 7.3). Other arthropod groups represented in the 18S Arthropoda dataset included the Myriapoda (16 OTUs), Diptera (7 OTUs), Isopoda (1 OTU), Collembola (3 OTUs), and Orthoptera (3 OTUs).

18S-ref dataset

Reference-based OTU-picking detected 81% of species in the Sanger reference library. For each taxonomic subset, the 18S-ref dataset included at least as many species as the COI-ref dataset and many more than were detected by *de novo* OTU-picking (Table 7.3).

7.4.2 The use of sequence count data

Here, I explore whether raw sequence count data should be used for beta-diversity analyses by considering the effects of amplification/sequencing bias on distance-based ordination and *mvabund* analyses.

Interspecific bias

When abundances within each species were multiplied by a random number between 1 and 100 to simulate interspecific bias, the Jaccard NMDS ordination was identical to that based on the raw abundance data (Procrustes test: R^2=1.0, p=0.001; Figure 7.2). Mantel tests found that the Jaccard distance matrices from the two datasets were also very strongly correlated (r=0.95, p=0.001).

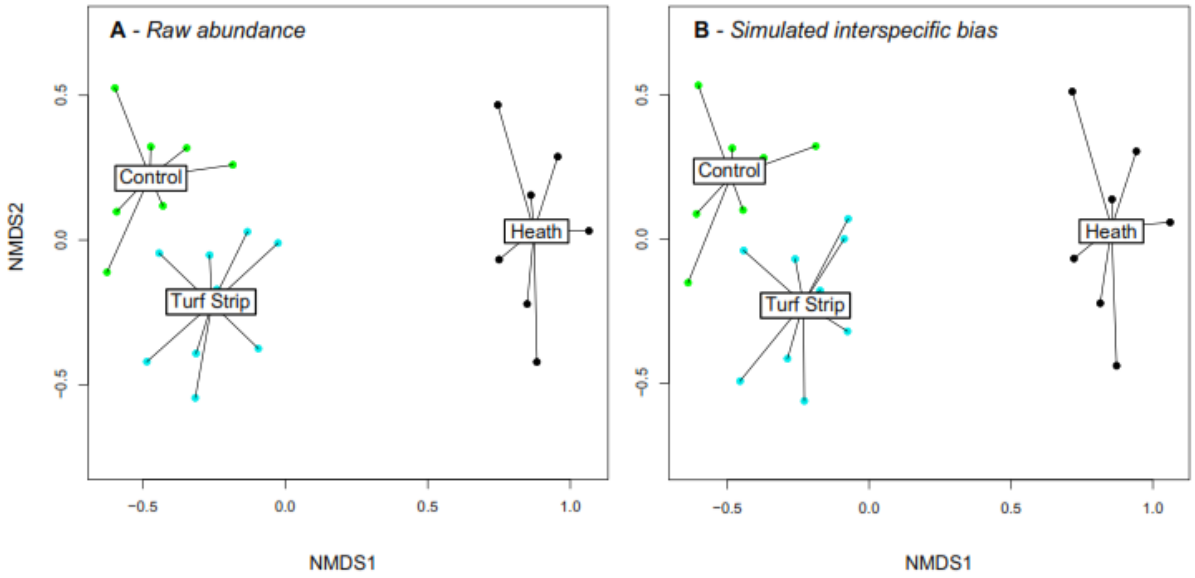

Figure 7.2: Quantitative Jaccard NMDS ordinations of Control, Turf Strip, and Heath sites based on (A) raw abundance data and (B) abundance data multiplied within OTU by a random number between 1 and 100.

Negative binomial LR tests for the effect of treatment level (Control, Turf Strip, Heath) in *mvabund* found that the LR value was marginally reduced when interspecific bias was present, but the significance of the treatment effect was unchanged (LR_{raw}=813.8, p=0.001; LR_{biased}=742.0, p=0.001).

These results suggest that taxon-specific amplification bias *per se* should have minimal effect on the results of the beta diversity analyses used in this study.

Intraspecific bias

Nonetheless, it remains possible that variation in bias factor could occur *within* OTUs due to (1) stochasticity and (2) competition among templates in the multiplex PCR reaction leading to the suppression of some OTUs in the presence of others. In this case, the multiplication factor will not be consistent across all samples for a given OTU. I tested for correlation between sequence read proportion (COI) and species abundance (STD) within one well-represented ant species, *L. niger*. Correlation was significant, but weak (Spearman's ρ=0.66, p<0.001; Figure 7.3), which suggests that some variation in amplification and sequencing success does indeed occur *within* as well as *between* OTUs.

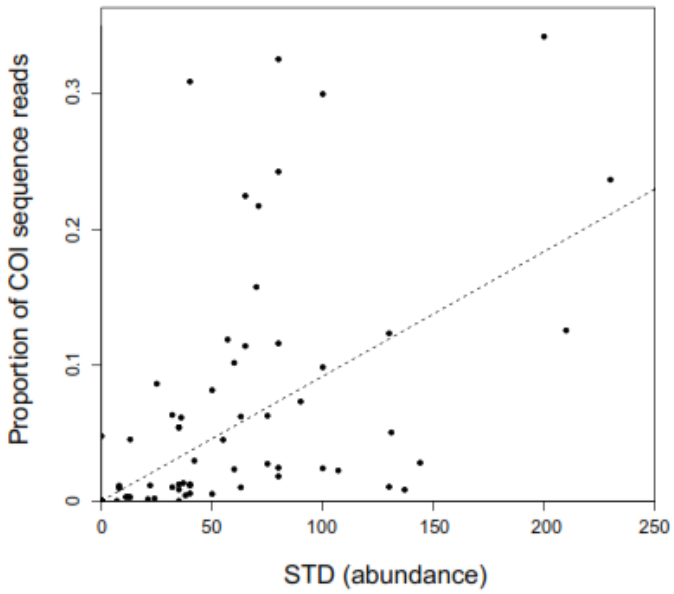

Figure 7.3: Relationship between number of *L. niger* individuals recorded in the STD dataset and the proportion of sequence reads assigned to *L. niger* in the COI-ref dataset, with regression line.

When this variation was incorporated into the simulated dataset by multiplying every sequence count value by a random number between 1 and 10, Mantel correlation between this and the original dataset was slightly reduced (Mantel r = 0.92, p=0.001), but ordinations remained virtually identical (Procrustes R^2=0.99, p=0.001), suggesting that the rank order of dissimilarities was not affected. In *mvabund*, the LR value was further reduced by the introduction of intraspecific variation, but the result was still highly significant (LR=713.8, p=0.001).

Read count vs. presence-absence data

Using sequence count data substantially improved Mantel correlation between the distance matrices of the COI-ref and STD datasets, compared with using presence-absence data (Mantel test, MBC vs. STD, presence-absence data: r=0.59, p=0.001; read count data: r=0.73, p=0.001). This suggests that read count data contain useful information regarding the relative abundance of OTUs, at least cancelling out whatever error arises from amplification and sequencing bias. Therefore, I allow the sequence count information to contribute to analyses in this chapter and do not transform metabarcode data to presence-absence.

The main purpose of the analyses below is (1) to establish the extent to which ecological information in the STD dataset is recovered by metabarcoding and (2) to compare the performance of the COI and 18S barcode amplicons in describing arthropod communities.

7.4.3 Recovering patterns of occurrence for individual ant species

Inspection of the raw data

Although the 18S-ref dataset appeared to out-perform its COI equivalent in terms of the number of indicator species recovered (Table 7.3), closer inspection of the OTU tables revealed some striking inconsistencies between the 18S-ref and STD ant subsets. For instance, in the STD dataset, the ant species *L. niger* was recorded in 100% of Trackway sites (mean=85 individuals per site) but was not recorded in any Heath sites. The same pattern is seen in the COI-ref dataset where *L. niger* occurs in 97% of Trackway sites (mean=50 sequence reads per site) and in none of the Heath sites. In the 18S-ref dataset, however, *L. niger* occurs in 39% of Trackway sites and 57% of Heath sites, and is represented by no more than two sequence reads in any site. As a second example, the ant species *L. umbra* occurs in only two sites in the STD dataset (sites D7 and FH9; one and three individuals, respectively), in just one site in the COI-ref dataset (site FH9; one sequence read), but in all 68 sites in the 18S-ref dataset, where it is represented by relatively high numbers of sequence reads (mean=63.5 sequence reads per site). This suggests that the quality of the 18S-ref dataset is low.

Identifying specialists

Heath and Trackway specialists were identified from the ant subsets of the STD, COI-ref, and 18S-ref datasets using the species-specific adjusted *p*-values and LR coefficients from an *mvabund* analysis comparing the ant communities of Heath and Trackway sites (Table 7.5). In the COI-ref dataset, two ant species, *L. niger* (*p*=0.001) and *Myrmica ruginodis* (*p*=0.03) were identified as Trackway specialists, and one species, *L. psammophilus* (*p*=0.007), was identified as a Heath specialist. The same associations were found in the STD dataset (*L. niger*: *p*=0.001 (Trackway); *M. ruginodis*: *p*=0.001 (Trackway); *L. psammophilus*: *p*=0.05 (Heath)), as well as three additional Trackway specialists: *Formica fusca* (*p*=0.001), *M. scabrinodis* (*p*=0.001), and *M. sabuleti* (*p*=0.02), only the last of which is represented in the COI-ref dataset at all. The 18S-ref dataset did detect *F. fusca* and identify it as a Trackway specialist, but it failed to identify any other specialists.

7.4.4 The effect of disturbance treatments – beta diversity

COI datasets

The COI *de novo* and COI-ref datasets were both at least as powerful as the STD dataset in detecting significant effects of the disturbance treatments (Table 7.5; Table 7.6), both for the full datasets and for two of the three taxonomic subsets. The exception is that both COI datasets performed poorly for the carabid subset, which is probably the result of a high level of drop out in this group (Table 7.3), arising from a combination of DNA degradation, low sequencing coverage, and the fact that the large body size of carabids meant that only legs were included in the bulk sample for DNA extraction.

Even when heath sites were excluded, COI Jaccard distance matrices and NMDS ordinations were significantly correlated with those of the STD dataset (again, with the exception of the carabids) (Table 7.4).

Table 7.4: For each metabarcode dataset, results of Procrustes and Mantel tests comparing Jaccard NMDS ordinations and distance matrices with those of the equivalent subset of the STD dataset. Each test is based on 999 data permutations and the Mantel tests use Spearman's correlation. NA indicates that tests were not performed due to lack of data.

		Including Heath				Excluding Heath			
		Procrustes		Mantel		Procrustes		Mantel	
		R^2	*P*	*r*	*P*	R^2	*P*	*r*	*p*
Arthropoda (full dataset)	COI *de novo*	**0.67**	**0.001****	**0.57**	**0.001****	**0.57**	**0.001****	**0.36**	**0.001****
	18S *de novo*	**0.51**	**0.001****	**0.26**	**0.001****	**0.37**	**0.001****	0.07	0.083
	COI-ref	**0.48**	**0.001****	**0.47**	**0.001****	**0.38**	**0.001****	**0.23**	**0.002****
	18S-ref	**0.42**	**0.001****	**0.33**	**0.001****	**0.33**	**0.004****	**0.23**	**0.002****
Araneae	COI *de novo*	**0.46**	**0.001****	**0.33**	**0.001****	**0.27**	**0.03****	**0.25**	**0.001****
	18S *de novo*	NA	NA	NA	NA	NA	NA	NA	NA
	COI-ref	**0.51**	**0.001****	**0.4**	**0.001****	**0.31**	**0.013***	**0.32**	**0.001****
	18S-ref	0.22	0.059	0.06	0.156	0.2	0.153	0.04	0.287
Carabidae	COI *de novo*	NA	NA	NA	NA	NA	NA	NA	NA
	18S *de novo*	NA	NA	NA	NA	NA	NA	NA	NA
	COI-ref	NA	NA	NA	NA	NA	NA	NA	NA
	18S-ref	**0.34**	**0.002****	**0.22**	**0.001****	**0.35**	**0.004****	**0.18**	**0.002****
Formicidae	COI *de novo*	**0.31**	**0.005****	**0.19**	**0.004****	**0.25**	**0.033****	**0.18**	**0.004****
	18S *de novo*	NA	NA	NA	NA	NA	NA	NA	NA
	COI-ref	**0.62**	**0.001****	**0.56**	**0.001****	**0.32**	**0.004****	**0.22**	**0.001****
	18S-ref	**0.27**	**0.01***	**0.36**	**0.001****	**0.27**	**0.017***	**0.22**	**0.002****

Table 7.5: For each dataset, the results of negative binomial *mvabund* analyses comparing (1) the community composition of Heath sites with Trackway sites, using LR tests, and (2) among treatments in the Trackway sites (including Control), using Wald tests. This second test asks if any treatment level is different from any other (including control), and where a significant effect is detected, pairwise tests were then conducted (Table 7.6). All *mvabund* tests were performed with pit.trap resampling and 999 bootstrap iterations. N/A indicates that the analysis was not performed due to lack of data.

		Heath vs Trackway		Treatments	
	Dataset	LR	*p*	Wald	*p*
Arthropoda	STD	**647**	**0.001****	**24.71**	**0.001****
	COI *de novo*	**640.8**	**0.001****	**25.54**	**0.001****
	18S *de novo*	**8016**	**0.001****	**18.41**	**0.002****
	COI-ref	**3366**	**0.001****	**13.54**	**0.007****
	18S-ref	**1901**	**0.001****	**19.64**	**0.007****
Araneae	STD	**337.8**	**0.001****	**18.11**	**0.001****
	COI *de novo*	**78.41**	**0.001****	**10.71**	**0.047***
	18S *de novo*	N/A	N/A	N/A	N/A
	COI-ref	**81**	**0.001****	**10.87**	**0.018***
	18S-ref	34.9	0.631	**10.87**	**0.017***
Carabidae	STD	**109.6**	**0.001****	**13.33**	**0.027***
	COI *de novo*	N/A	N/A	N/A	N/A
	18S *de novo*	N/A	N/A	N/A	N/A
	COI-ref	N/A	N/A	N/A	N/A
	18S-ref	42.89	0.079	9.29	0.178
Formicidae	STD	**199.3**	**0.001****	**10.71**	**0.007****
	COI *de novo*	**50.03**	**0.001****	**9.059**	**0.011***
	18S *de novo*	N/A	N/A	N/A	N/A
	COI-ref	**61.05**	**0.001****	**7.32**	**0.027***
	18S-ref	**78.7**	**0.001****	7.34	0.246

In Figure 7.4, the COI *de novo* dataset separates the different treatments in ordination space to a greater extent than do the COI-ref and STD datasets, which is not surprising given that it is based on the responses of a wider range of taxonomic groups. However, both COI ordinations show the same key patterns as the STD dataset. Briefly, these are:

1. The centroid furthest from the target (Heath) centroid is that of the control, while the treatment centroids are intermediate to the control and the target, indicating that the arthropod communities at treatment sites have changed to become more similar to those of the target sites.
2. The centroids of the most severe treatments (Agricultural plough, Forest plough, and Turf Strip) are among those that have moved furthest from the control, while that of Swipe

(the least severe treatment) remains closest to the control centroid, suggesting that the Swipe treatment has had little effect on arthropod communities;

3. With the exception of Forest Plough in the COI-ref dataset, all treatment centroids remain closer to the control than to the target centroids (Figure 7.4), indicating that no treatment has yet resulted in an arthropod community that is fully representative of the heath habitat.

Thus, overall, the same conclusions are drawn from all three datasets (STD, COI *de novo*, COI-ref): of the methods trialled here, aggressive soil disturbance is the most effective way of creating a heath-like habitat on forest trackways, although even the best corridor habitat remains unsuitable for some heathland specialists.

18S datasets

18S was also able to detect significant effects of the most severe treatments using *de novo* OTU picking (Table 7.6), and interpretation of the 18S *de novo* ordination (Figure 7.4C) yields similar conclusions to interpretation of the STD and COI *de novo* ordinations. However, no effect of any of the less severe treatments was detected, and results were generally less strongly correlated with the STD dataset than were their COI equivalents (Table 7.4).

There were insufficient data to analyse the effect of treatments on the taxonomic subsets of the 18S *de novo* dataset, and reference-based OTU-picking, which yielded more species per group (Table 7.3), detected significant treatment effects only for spider communities (Table 7.6). However, the 18S-ref dataset was the only metabarcode dataset that detected sufficient carabid OTUs for analysis. This data subset was weakly but significantly correlated with the carabid subset of the STD dataset (Table 7.4).

In summary, the COI metabarcode data performed well in recovering beta diversity patterns, while 18S data were less powerful.

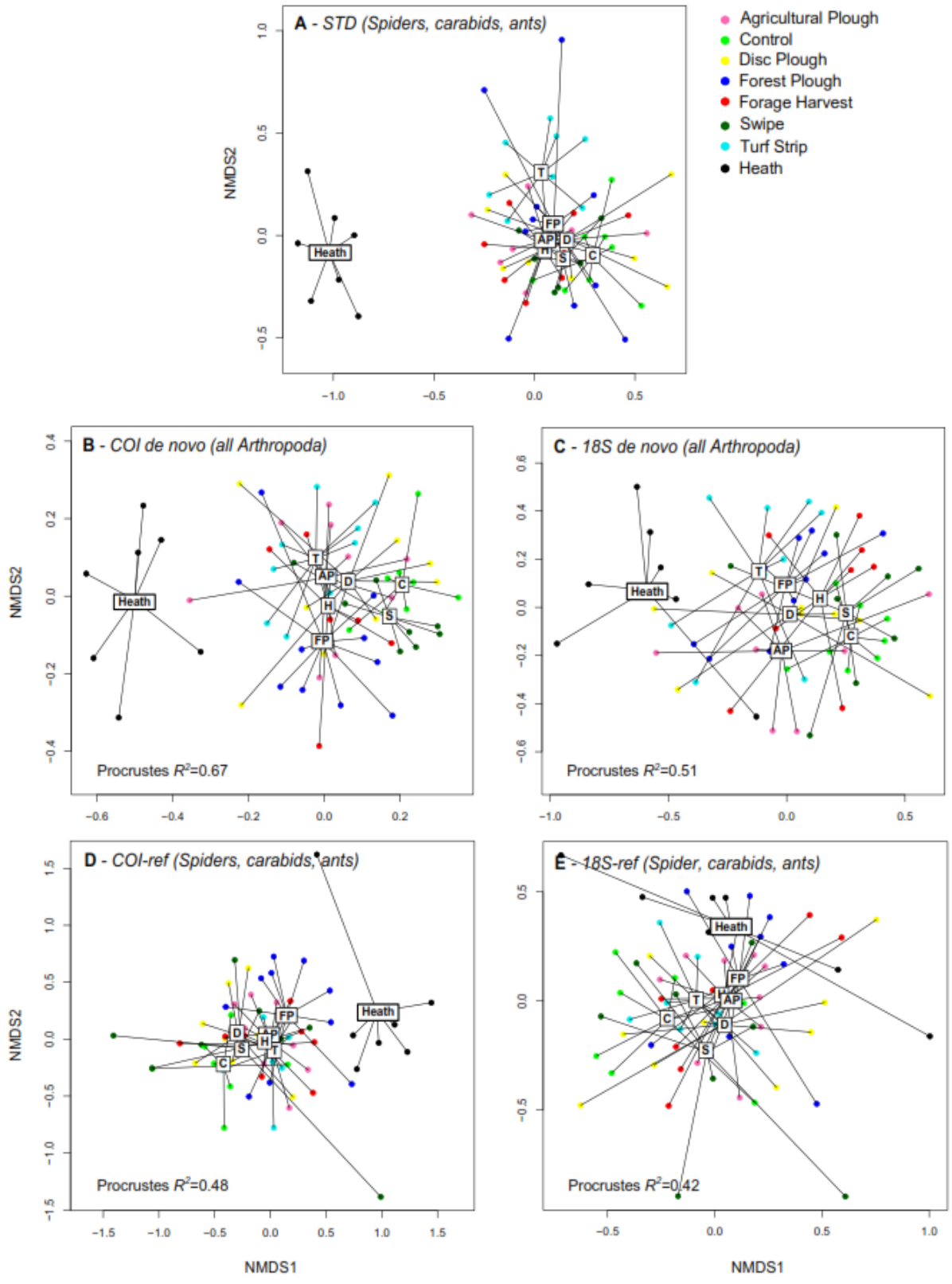

Figure 7.4: Quantitative Jaccard NMDS ordinations for (A) the STD dataset (spiders, carabids and ants), (B) the COI *de novo* dataset (all arthropods), (C) the 18S *de novo* dataset (all arthropods), (D) the COI-ref dataset (spiders, carabids and ants), and (E) the 18S-ref dataset (spiders, carabids and ants). Procrustes R^2 values describe the correlation between each metabarcode dataset and the STD dataset when heath sites are included. Points are coloured and grouped by treatment (Control (C), Agricultural Plough (AP), Disc Plough (D), Forest Plough (FP), Forage Harvest (H), Swipe (S), Turf Strip (T), and Heath).

Table 7.6: For each dataset, results of negative binomial Wald tests in *mvabund* comparing communities from each of the six treatment levels with those from the control sites. "N/A" indicates that the analysis was not performed due to insufficient data and "ns" indicates that the analysis was not performed because the Wald test in Table 7.5 found no difference among treatments for that dataset. Original *p*-values are reported, but significance is determined following correction for six tests using *p*.adjust*(method="fdr")* in *R*'s base package. * $p < 0.05$, ** $p < 0.01$.

	Treatment	STD		COI *de novo*		COI-ref		18S *de novo*		18S-ref	
		Wald	*P*	Wald	*P*	Wald	*p*	Wald	*p*	Wald	*p*
All Arthropods	Swipe	7.62	0.162	**9.233**	**0.012***	**5.4**	**0.002****	5.4	0.555	6.333	0.048
	Forage harvest	**9.06**	**0.004****	**12.047**	**0.001****	**6.28**	**0.001****	6.69	0.088	7.57	0.022
	Disc plough	**8.62**	**0.021***	**10.48**	**0.001****	**5.62**	**0.003****	7.11	0.052	5.75	0.161
	Forest plough	**9.35**	**0.007***	**11.36**	**0.001****	**5.6**	**0.009****	**7.64**	**0.008***	7.69	0.025
	Agricultural plough	**11.18**	**0.001****	**11.88**	**0.001****	**7.74**	**0.001****	**7.624**	**0.02***	**8.48**	**0.008***
	Turf strip	**12.04**	**0.001****	**13.541**	**0.001****	**7.73**	**0.001****	**7.89**	**0.003***	6.99	0.048
Araneae	Swipe	5.56	0.225	**4.74**	**0.006****	**5.2**	**0.004****	N/A	N/A	**5.2**	**0.004****
	Forage harvest	**7.3**	**0.009***	**6.01**	**0.001****	**5.88**	**0.001****	N/A	N/A	**5.88**	**0.002****
	Disc plough	6.11	0.071	3.68	0.085	**4.53**	**0.006****	N/A	N/A	**4.53**	**0.007****
	Forest plough	**7.59**	**0.006***	**5.31**	**0.013***	**4.84**	**0.007****	N/A	N/A	**4.84**	**0.011***
	Agricultural plough	**7.94**	**0.004***	**6.28**	**0.001****	**6.61**	**0.001****	N/A	N/A	**6.61**	**0.001****
	Turf strip	**9.96**	**0.001****	**6.21**	**0.002****	**5.94**	**0.001****	N/A	N/A	**5.94**	**0.003****
Carabidae	Swipe	3.84	0.208	N/A	N/A	0.13	0.881	N/A	N/A	ns	ns
	Forage harvest	4.45	0.08	N/A	N/A	0.76	0.561	N/A	N/A	ns	ns
	Disc plough	4.63	0.094	N/A	N/A	0.48	0.563	N/A	N/A	ns	ns
	Forest plough	4.87	0.059	N/A	N/A	1.16	0.397	N/A	N/A	ns	ns
	Agricultural plough	**7.12**	**0.002***	N/A	N/A	0.58	0.589	N/A	N/A	ns	ns
	Turf strip	**5.24**	**0.009***	N/A	N/A	1.42	0.17	N/A	N/A	ns	ns
Formicidae	Swipe	3.52	0.262	2.49	0.253	2.08	0.214	N/A	N/A	ns	ns
	Forage harvest	3.03	0.431	2.84	0.121	0.77	0.766	N/A	N/A	ns	ns
	Disc plough	4.12	0.072	3.31	0.073	2.41	0.098	N/A	N/A	ns	ns
	Forest plough	2.80	0.437	2.65	0.13	1.53	0.42	N/A	N/A	ns	ns
	Agricultural plough	**4.19**	**0.033***	2.77	0.153	2.31	0.168	N/A	N/A	ns	ns
	Turf strip	**4.45**	**0.011***	**6.11**	**0.002****	**3.91**	**0.007****	N/A	N/A	ns	ns

7.4.5 The effect of disturbance treatments – alpha diversity

Estimated total species richness of the COI *de novo* dataset was significantly greater than that of the STD dataset (STD: observed species = 129, Chao2 = 146.6 ± 9.6 (s.e.); COI: observed OTUs = 361, Chao2 = 419.6 ± 15.3; Welch's *t*-test: $t_{df=120.8}$=15.11, p<0.001), which is unsurprising given that the metabarcode datasets include all sampled arthropods while the STD dataset includes only spiders, carabids, and ants. However, the 18S *de novo* dataset had significantly lower species richness than the STD dataset (18S: observed OTUs = 76, Chao2 = 78.89 ± 2.69; Welch's t-test: $t_{df=77.4}$=6.79, p<0.001) despite comprising a wider range of taxonomic groups.

Significant Spearman's correlation was detected between the COI and STD datasets across the eight treatment levels, both for raw observed species richness and for three of four species richness estimators (Jackknife1, Jackknife2, and Bootstrap; Table 7.7). Both datasets show that Heath has low species richness, while the Forest Plough treatment has the highest (Figure 7.5).

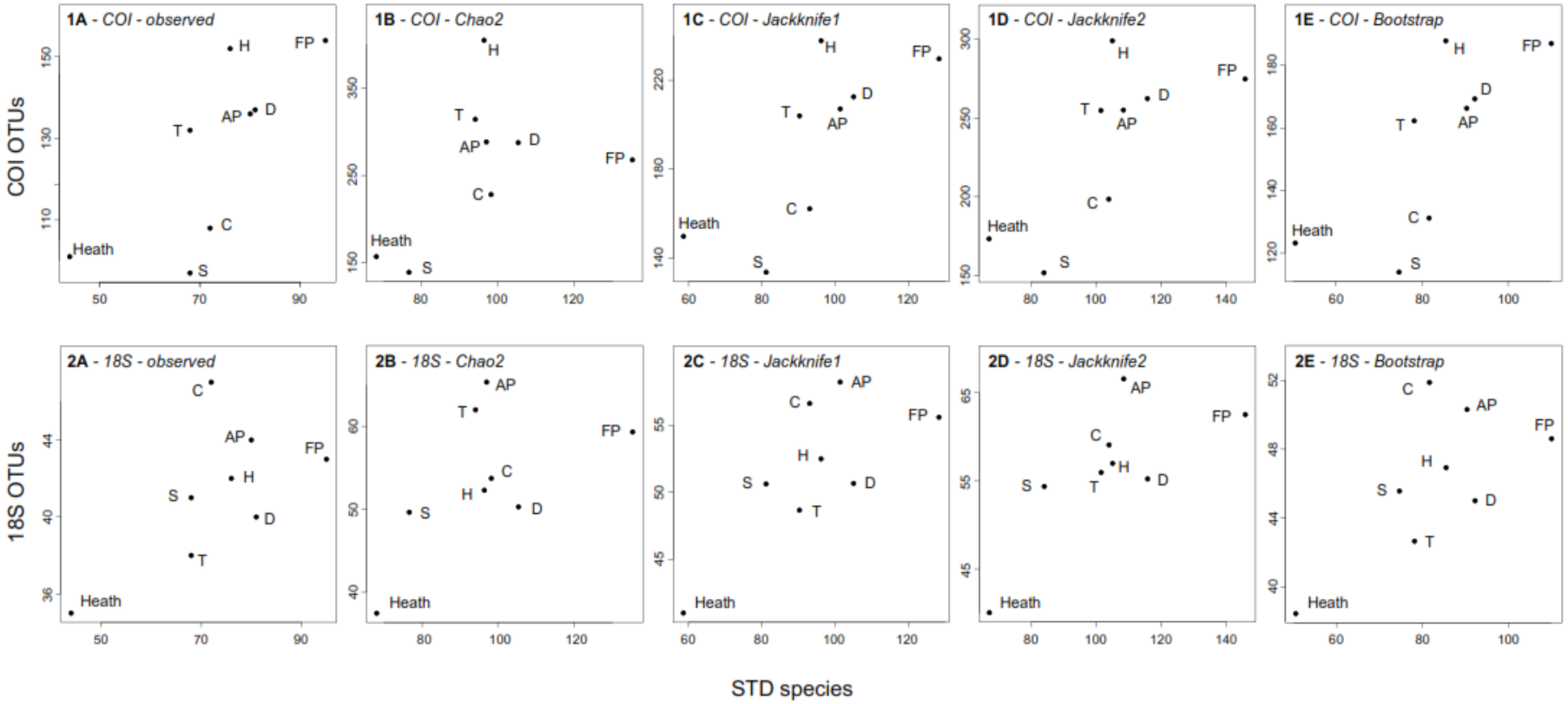

Figure 7.5: Relationship between metabarcode ((1) COI and (2) 18S) and STD species richnesses of the eight treatment levels. Plots are shown for (A) observed species richness, and estimated total species richness based on four different incidence-coverage methods: (B) Chao2, (C) Jackknife1, (D) Jackknife2, and (E) Bootstrap. Points are labelled according to treatment (Control (C), Agricultural Plough (AP), Disc Plough (D), Forest Plough (FP), Forage Harvest (H), Swipe (S), Turf Strip (T), and Heath).

Although Figure 7.5 appears to suggest a similar positive relationship between the species richnesses of the 18S and STD datasets, tests found no significant correlation in terms of either observed or estimated species richness (Table 7.7).

Table 7.7: Results of Spearman's correlation tests between number of arthropod OTUs (COI/18S) and number of spider, carabid, and ant species (STD). Results shown for (1) observed species richness and (2) four estimates of total species richness of each of the eight treatment levels (generated using *vegan*'s *specpool* function).

	CO1 vs STD		18S vs STD	
	rho	*p*	rho	*p*
Observed	**0.85**	**0.007***	0.53	0.18
Chao2	0.58	0.238	0.45	0.27
Jackknife1	**0.81**	**0.022***	0.67	0.083
Jackknife2	**0.81**	**0.022***	0.69	0.069
Bootstrap	**0.81**	**0.022***	0.5	0.216

In summary, metabarcode data can successfully identify which treatments result in the greatest species richness. Once again, COI outperformed 18S.

7.5 Discussion

7.5.1 The use of read counts as a proxy measure for species abundances

The abundance or biomass of individuals is a key metric in community ecology and represents one of the main challenges for the metabarcoding community to address. Taxon-specific bias, which occurs mainly during PCR, when some taxa amplify more readily than others, means that there is no straightforward link between the biomass of a species and the number of sequence reads that it generates. Furthermore, this study found evidence to suggest that the relationship also varies within species across different samples (Figure 7.3), which is probably due to a combination of factors, including competition within multiplex PCR reactions and stochastic elements arising from factors such as DNA degradation and low sequencing coverage (Hajibabaei *et al*., 2011). This means that caution should be exercised in making inferences about relative abundance or biomass based on sequence count data, and the conservative approach adopted in most studies that span a wide range of taxonomic groups is to consider only presence and absence on a sample-by-sample basis (e.g. Yu *et al*., 2012; Ji *et al*., 2013; Bradford *et al*., 2013; and previous chapters in this thesis).

Nevertheless, it is probable that some genuine information is retained in sequence count data. The question is whether, overall, the "good" information outweighs the "bad" information such that use of sequence count data in community analyses yields results that are closer to the truth than is achieved by relying on presence-absence alone. By comparison with a comprehensive STD dataset that includes species counts, I found that the use of sequence count data improved the ability of metabarcoding to detect 'true' beta-diversity patterns compared with the use of presence-absence data. This suggests that the retained "good" information at the very least compensates for the error associated with PCR and sequencing.

7.5.2 Issues arising from the storage and handling of specimens

Sampling in this project was not conducted with genetic analysis in mind, which means that the way in which samples were stored and handled could have led to low quality genetic data.

DNA degradation

Generally, in order for DNA to be well preserved, samples should be either frozen or stored in absolute ethanol or another suitable reagent such as RNAlater® solution or DESS (Creer *et al*., 2010). In the case of this study, for the two years prior to DNA extraction, the pitfall trap samples

were stored with specimens densely packed in dilute (70%) ethanol, which was not changed at any point. Therefore, substantial DNA degradation is likely to have occurred.

The COI barcode region is relatively long (658 bp) compared with those that are designed specifically for degraded DNA (e.g. Epp *et al*., 2012; Taberlet *et al*., 2012), and the 18S amplicon used here is even longer (830 bp). This means that DNA degradation is likely to lead to low sequence counts and, potentially, substantial levels of drop-out. This is indeed observed in the data, with particularly high levels of drop-out among the carabid beetles (Table 7.3).

Cross-contamination

It is also possible that the handling of samples during processing and morphological identification might have led to cross-contamination. However, there is nothing in the results to suggest that this was a problem, and, in fact, patterns of occurrence for individual ant species in the COI-ref dataset very closely matched those in the STD dataset. Of particular note is *L. niger*, which is widespread and abundant in all trackway samples but absent from all heath sites. If cross-contamination had occurred, it is likely that some *L. niger* sequences would have been recorded in the heath samples in the metabarcode dataset. However, I found that the occurrence of *L. niger* closely matched its occurrence in the STD dataset, being present in 59 of 60 trackway samples in the metabarcode dataset and absent in all heath samples. Together with the fact that the two datasets identified the same species as habitat specialists, this strongly suggests that cross-contamination did not have a major impact on results.

7.5.3 Comparing the performance of COI and 18S metabarcode data

18S metabarcode data

The results of this study support the findings of Tang *et al*. (2012) that 18S underestimates diversity. Compared with the COI and STD datasets, very few 18S arthropod OTUs were detected via *de novo* OTU picking with BLAST-assigned taxonomy (18S *de novo* dataset; Table 7.5). A significant factor in this is undoubtedly the slower mutation rate of 18S compared with COI (Chang and James, 2011), which makes 18S more suitable for the study of higher level diversity (Hillis and Dixon, 1991) and limits its ability to separate closely-related species. However, the effect is probably exacerbated by the drop-out of OTUs as a result of DNA degradation. This would be expected to affect 18S data more than COI data because of the longer amplicon length, as mentioned above.

At first sight, the fact that reference-based OTU-picking detected more species in each indicator group than did *de-novo* OTU-picking (Table 7.3) suggests that the taxonomic assignment step may

have underperformed in the 18S *de novo* pipeline. However, the quality of the data in the 18S-ref dataset was poor: species often did not show the same patterns of occurrence as in the STD dataset, and ordinations were generally less strongly correlated with the STD dataset than were the equivalent COI ordinations (Table 7.4).

Overall, my findings support the suggestions of other authors (e.g. Creer *et al*., 2010; Derycke *et al*., 2010; Tang *et al*., 2012; Leray *et al*., 2013) that the use of 18S may not be appropriate for the study of species-level diversity, despite its current wide-spread use in studies of meiofaunal communities (e.g. Chariton *et al*., 2010; Creer *et al*., 2010; Fonseca *et al*., 2010; Bik *et al*., 2012b; Bradford *et al*., 2013).

COI metabarcode data

COI has been reported to overestimate diversity compared with both 18S and morphological estimation (Machida and Tsuda, 2010; Tang *et al*., 2012). I found no evidence to suggest that this was the case within any of the three indicator groups when sequences were clustered at 97% similarity in CROP (Hao *et al*., 2011); for all three taxonomic subsets, there were fewer *de-novo* COI OTUs than there were species in the STD dataset, and numbers were consistent with those given by reference-based OTU-picking (Table 7.3).

However, one non-indicator group, the Orthoptera, was dramatically over-split by COI, being assigned 73 OTUs despite the fact that only 28 species have been recorded in the UK since 2009 (BRC, 2013). A potential explanation is that co-amplification of nuclear mitochondrial pseudogenes (numts) may have occurred. This is where primers inadvertently amplify copies of sections of the mitochondrial genome that have become assimilated into the nuclear genome. Because they are free from selection, numts mutate rapidly, often becoming sufficiently divergent from the original mitochondrial sequence as to be identified by DNA barcoding as separate species. Orthoptera are known to have a particularly high frequency of numts (Bensasson *et al*., 2000; 2001), which has been shown to lead to overestimation of their species diversity in barcoding studies (Song *et al*., 2008; Moulton *et al*., 2010). Indeed, Song *et al*. (2008) found that diversity was overestimated by a factor of 4.25 (4 species identified as 17), which fits well with the level of overestimation observed here. My results agree with previous studies (e.g. Bensasson *et al*., 2001) in suggesting that the Orthoptera are unusual among arthropods in respect of the frequency of numts.

The detection and removal of numts is bioinformatically challenging, especially when they lack indels or atypical substitutions that can differentiate them from target mitochondrial DNA

(Ramirez-Gonzales *et al*., 2012). The bioinformatics pipeline used in this thesis does not contain any step for detecting and removing numts. An alternative pipeline, PyroClean (Ramirez-Gonzales *et al*., 2012; designed specifically for use with protein-coding amplicons), is able to remove numts identified on the basis of indels and atypical substitutions. For those that are not identifiable in this way, the authors suggest removal on the basis of low frequency relative to a related sequence in the same MID pool, but this risks the loss of genuine low frequency target sequences.

Nevertheless, COI generally performed well in terms of recovering community information: ordinations and distance matrices were significantly correlated with those of the STD dataset (Table 7.4); species detected by reference-based OTU-picking showed very similar patterns of occurrence in the COI-ref dataset compared with in the STD dataset; the same habitat specialists were identified; and alpha diversity estimates were significantly correlated with those of the STD dataset, which was not the case for 18S (Figure 7.5).

7.5.4 Metabarcoding in the context of ecological restoration

In this study, COI metabarcode data has been shown to yield very similar biodiversity patterns to the STD data and to generate the same overall conclusions about the effectiveness of the various disturbance treatments in creating a habitat that allows early-successional heath specialists to percolate along forest trackways. In short, the most effective treatments are the most aggressive ones (Forest Plough, Agricultural Plough, and Turf Strip), which expose more soil. However, as of two years since implementation, no treatment has yet resulted in a ground arthropod community that is fully representative of the target heath habitat, and at least one group (the ants) has shown minimal response to restoration measures (Figure 7.4).

Time lags are a common feature of restoration projects, with major implications for the success of biodiversity offsetting as an approach to conservation (Bekessey *et al*., 2010; McKenney and Kiesecker, 2010; Drechsler and Hartig, 2011). The fact that habitats and communities often take many years to fully develop means that monitoring will usually need to be sustained for substantial periods of time, and this makes it even more important that it can be carried out rapidly and cost-effectively without relying upon the availability of taxonomic expertise. As an illustration of the limitation of traditional approaches in this context, S. Pedley collected a third year's samples from the forest trackways, but the specimens are not being identified because Pedley has graduated, leaving no clear way to sustain monitoring, which is the foundation for any kind of adaptive management of these corridors. In contrast, samples could be collected for

metabarcoding in a matter of a few weeks by Forestry Commission personnel and sent to an academic or commercial laboratory for processing.

The level of agreement between the COI metabarcode and STD datasets serves as validation for both approaches: it indicates that the COI metabarcode data are robust and that conclusions drawn from such data can be believed; it also shows that combined data from the three indicator groups used here (spiders, carabid beetles, and ants) yield results that are representative of the response of ground arthropods generally. However, in practice, the STD approach of Pedley *et al*. (2013a), in which three arthropod groups are considered, is unlikely to be widely adopted by restoration practitioners because the time, cost, and requirement for taxonomic expertise all increase with every group added. As shown both here and in Pedley *et al*. (2013a), considering one group in isolation (a common approach in practice) does not yield a representative picture of the wider biodiversity response and may generate misleading conclusions about the overall success or failure of a project.

Metabarcoding is many times faster than traditional morphological approaches (Ji *et al*., 2013), and the fact that taxonomic expertise is not required crucially allows consideration of groups that are not usually considered in restoration projects due to being morphologically intractable. Such groups, including terrestrial arthropods and soil fauna, are often of functional importance in an ecosystem, which means that their condition is likely to affect the long-term viability of a restored or created habitat (Lytzau Forup *et al*., 2008; Majeur, 2009; Ohsowski *et al*., 2012). Moreover, if trap samples are pooled within site, it is possible to include many groups of arthropods sampled using multiple trapping methods for no more cost than would apply to a single group. This is because processing costs increase with the number of samples, rather than with the number of specimens or taxonomic groups they contain. Although still only covering arthropod diversity, this clearly allows for a much better understanding of the biotic responses to restoration measures than do traditional approaches.

For extra cost, additional complementary metabarcoding approaches can add further insight: DNA from the soil can reveal community patterns of plants (Yoccoz *et al*., 2012), fungi (Jumponnen *et al*., 2010; Blaalid *et al*., 2013) and soil fauna (Bienert *et al*., 2012; Epp *et al*., 2012); eDNA from water bodies can reveal those of aquatic fauna (Fonseca *et al*., 2010; Thomsen *et al*., 2012a; 2012b); and iDNA from invertebrate parasites can provide information about terrestrial vertebrate communities (Schnell *et al*., 2012; Bohmann *et al*., 2013; Calvignac-Spencer *et al*., 2013). In most projects, it will probably not be practical to employ all of these approaches, but for

particularly high profile or sensitive projects, they collectively offer the opportunity to measure directly something approaching total eukaryotic biodiversity (Tautz *et al*., 2010).

Through enabling the consideration of a wider variety of taxonomic groups, a metabarcoding approach would enable restoration issues to be flagged that might otherwise go unnoticed because they pertain to groups that would not have been selected as indicators. Thus, by increasing the potential for adaptive management, it becomes more likely that a successful outcome will be achieved.

7.6 References

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Chapter 8: Applications and limitations of metabarcoding

8.1 Biodiversity as a response variable

In order to prioritise conservation investment and make informed decisions about environmental management, there is a pressing need to be able to treat biodiversity as a response variable that is measureable directly and repeatedly across time and space. With the development of meta-barcoding technology, this has become possible for the first time.

The previous three chapters have demonstrated the applicability of a metabarcoding approach to a range of environmental management challenges that are characterised by a lack of ecological data to inform decision-making. In these areas and many others, metabarcoding can revolutionise our capacity to monitor and manage biodiversity by enabling species richness and community composition to be compared quickly across sampling locations/dates to detect biodiversity changes across space and time. Although not demonstrated in this thesis, metabarcode data can also be used to estimate phylogenetic diversity (Bryant *et al*., 2012; Yu *et al*., 2012), which can be useful in making value judgements (Bottrill *et al*., 2008). If applied in conjunction with sound experimental design and robust statistical analysis, these measures make it possible to characterise the biodiversity response to environmental change, implement programmes of adaptive management, identify locations or habitats with highly distinctive biological communities, and determine which management and conservation strategies are most effective in protecting or enhancing biodiversity.

Results from Chapter 7 suggest that COI is an appropriate marker for arthropod metabarcoding studies (whereas 18S is not) and that, when sequences are clustered at 97% similarity in CROP (Hao *et al*., 2011), OTU diversity is closely aligned with that of morphological species (Table 7.3). In chapters 5 and 6, sequences were clustered instead at 98% similarity, which is likely to have resulted in inflated species richness estimates, at least among some groups. However, a recent study has shown that beta diversity patterns are insensitive to the level of clustering (Baselga *et al*., 2013), and this can also be expected to be true of alpha diversity patterns (i.e. richness of haplotypes or sub-specific clusters likely correlates with species level richness).Therefore, valid conclusions about community responses can be drawn even when OTUs are not truly representative of morphological species, although the potential for drawing conclusions about the responses of particular species will be diminished.

A recurring result in Chapters 5-7 is that, although individual taxonomic groups vary considerably in their responses to environmental variables, when several orders are considered together, they seem to give beta diversity patterns that can be considered representative of the response of arthropods generally. That is, any combination of groups seems to give the same or similar conclusions about the compositional differentiation of samples. This was seen in Chapter 5, where pitfall trap and Malaise trap samples showed the same patterns of differentiation among forest sites; in Chapter 6, there was correlation between soil arthropods and those sampled using Malaise traps; and in Chapter 7, combined analysis of taxa from three indicator groups yielded the same conclusions as the more taxonomically comprehensive metabarcode dataset regarding the effectiveness of different heath restoration treatments. This suggests that broad compositional indicators can be used reliably for environmental biomonitoring purposes, so long as they comprise multiple orders. In practice, the required breadth means that this kind of indicator will not be practical to implement at large scales if there is reliance on morphological identification of specimens, and with a molecular approach, greater resolution can be obtained by including all sampled taxa.

While focusing on arthropods to inform management can still be considered use of a surrogate of total animal biodiversity, it is a surrogate of substantial taxonomic breadth and diversity, containing taxa across all trophic levels that perform a wide range of ecological functions (Cardoso *et al*., 2011). Therefore, arthropod diversity is much better able to indicate fine-scale or incremental environmental changes than are currently-employed (and usually untested) surrogates. Where desired, something approaching total animal, plant, and fungal diversity can be measured by combining the whole-organism approach with analysis of DNA sourced from the environment (e.g. soil and water; Fonseca *et al*., 2010; Yoccoz *et al*., 2012; Epp *et al*., 2012; Thomsen *et al*., 2012a; 2012b;) and from invertebrate parasites (Schnell *et al*., 2012; Bohmann *et al*., 2013; Calvignac-Spencer *et al*., 2013).

8.2 Applications in the UK

8.2.1 Government programmes

EU funding for rural development is set to be reduced in 2015, sparking an urgent debate concerning efficient use of available funds in the UK (DEFRA, 2013). If money is not to be wasted on programs that have little or no impact on biodiversity, it is vital that actions are linked to outcomes. One of the major allocations of funds has been to agri-environment schemes via farmer subsidies, but there has been little effort to validate the effectiveness of these schemes for

groups other than farmland birds (Chapter 6). Therefore, this represents one of the most pressing issues to be addressed. Metabarcoding can provide direct data concerning the biodiversity response to agri-environment measures, while also offering the opportunity to test the usefulness of coarse variables as biodiversity surrogates and indicators. There are also specific issues concerning agricultural ecosystem services, particularly the effect of different farming models on pollinator communities (DEFRA, 2013). To facilitate metabarcoding studies of pollinator diversity, work is ongoing with Conservation Grade Producers Ltd, researchers from the University of Reading, and the Beijing Genomics Institute to sequence the mitochondrial genomes of all 272 native UK bee species.

For many applications, there is a need for large-scale base-line monitoring of biodiversity. One such application is the detection and monitoring of pests and non-native species, which have an estimated economic impact of £1.7 billion annually in the UK (Williams *et al*., 2010); another is the detection of changes (e.g. range expansions/ contractions) associated with climate change; a third is to provide reference data for the purposes of habitat restoration and biodiversity offsetting. Although metabarcoding makes it possible to carry out large-scale base-line monitoring of invertebrates, sampling and funding are still likely to present problems. One idea that I am keen to pursue is to link large-scale biodiversity monitoring in the UK with an educational project to bring biodiversity research into secondary schools. Under this scheme, co-ordinated insect trapping would be carried out by schools in habitats local to them. Students would then have the opportunity to study the specimens and learn about their ecological functions before sending them for metabarcoding over the summer. Because of the distribution of schools, it would be possible to gather data from a wide variety of habitats nationwide, and the school curriculum is ideally suited for implementing the same programme year after year. The national dataset would be made publicly available for use by environmental managers and researchers, and each school's data returned to the school along with analysis tools and simple bioinformatics scripts so that students can gain experience of handling real scientific datasets.

8.2.2 Private sector

Many private companies generate ecological impacts that require assessment and mitigation (BBRC, 2013a; IFC, 2013), but impact assessments are currently associated with a high degree of uncertainty (Geneletti *et al*., 2003), and the effectiveness of mitigation measures tends to be poor (Drayson and Thompson, 2013). Metabarcoding can reduce uncertainty in impact assessment by directly comparing pre- and post-impact communities and providing crucial data concerning the success of efforts to restore or recreate damaged habitats (Chapter 7). This would lead to

increased accountability on the part of companies and consultancies, as well as providing businesses with stronger evidence in support of their environmental credentials, which can bring benefits in terms of customer and investor relations and ranking in indices such as FTSE4Good and the Business in the Environment (BiE) Index of Corporate Environmental Engagement (BBRC, 2013b).

Assessment and mitigation of impacts on biodiversity is a key requirement for obtaining funding in the resource extraction sector (IFC, 2012), and baseline surveys can rank among the most significant project costs for companies (L. Bland, SRK Consulting (UK) Ltd, *pers. comm*.). However, surveys are so time-consuming that data are rarely obtained in time to influence project implementation, rendering them little more than expensive tick-box exercises, and their scope is heavily influenced by the availability of local experts who can identify different animal and plant groups. A major benefit of metabarcoding is that data can be obtained within a useful timeframe for influencing actions, including the identification of 'critical habitat' areas (sites with high biological conservation value, where additional regulations apply) and the implementation of adaptive management, both of which are nominally required by the International Finance Corporation (IFC, 2012). Detection of vertebrates via DNA from blood meals or environmental samples is likely to be a key tool in this sector because current regulations have a strong focus on species listed by the IUCN as 'endangered' or 'critically endangered' (IFC, 2012), and these are predominantly vertebrates. From the company perspective, a metabarcoding approach is likely to lead to financial savings because sampling can be carried out by non-experts, meaning that those staff already employed to make collections for chemical analysis can also carry out the biodiversity sampling.

International standards help businesses to address global challenges through the provision of clear guidelines, processes, and quality norms. For instance, ISO 14064 provides detailed guidance for the implementation and third-party verification of greenhouse gas inventories (ISO, 2010). Biodiversity is conspicuous by its absence from international standards, despite being widely recognised as one of the most pressing global challenges (Secretariat of the CBD, 2010). A barrier to the development of comprehensive standards for biodiversity has been the difficulty of standardising approaches across highly variable environments when a vast array of different indicator groups can be selected for use (Lindenmayer and Likens, 2011). Metabarcoding represents an important step towards standardisation because (1) it can be employed equally in any terrestrial landscape, (2) it does not rely on local expertise for making identifications, and (3)

it does not require the selection of indicators. Moreover, unlike traditional approaches, it is auditable and third-party verifiable (Ji *et al*., 2013).

8.3 Limitations to current methodology

There are two key limitations that must be addressed if metabarcoding is to be incorporated into standards for biodiversity assessment. These are (1) estimation of abundance and (2) assignment of taxonomy. I consider each in turn.

8.3.2 Estimation of abundance

Our ability to make inferences about species abundance, which is a key aspect of community ecology, is limited by biases that arise during DNA extraction, PCR, and sequencing (Coissac *et al*., 2012; Deagle *et al*., 2013; Ross *et al*., 2013). Interspecific amplification bias is a particular problem when dealing with bulk samples that contain a wide range of taxonomic groups, and so studies based on such samples have tended to consider only presence-absence data (e.g. Yu *et al*., 2012; Ji *et al*., 2013). Results presented in Chapter 7 of this thesis suggest that (1) using read count data does not lead to misinterpretation of ecological patterns, and (2) within species, there is some correlation across samples between read count and abundance. However, there is still likely to be a considerable amount of error, which means that read counts should be interpreted with caution. To obtain information about relative interspecific abundance, a practical approach would be to sample using multiple traps in each location, process them as separate MIDs, and use the proportion of traps in which each species occurs as an index of abundance. This was demonstrated in the pooled dataset in Chapter 5, where each species was scored from 0 to 8 for each forest site according to the number of Malaise trap samples in which it was detected (although note that there was temporal separation of the eight samples in this case). The extra information comes at the price of increased costs associated with processing a greater number of MIDs.

Reducing bias to allow the recovery of abundance or biomass information has become a major focus of current efforts to develop new methodologies, with PCR-free NGS approaches much discussed (Shokralla *et al*., 2012; Taberlet *et al*., 2012a). Zhou *et al*. (2013) recently demonstrated an approach whereby mitochondrial DNA is enriched following extraction, and shotgun sequencing is applied with ultra-deep coverage using the Illumina HiSeq 2000 platform. This approach succeeded in detecting 97% of species contained in a fairly small bulk insect sample (73 individuals belonging to 37 species), and the study found that sequence volume was correlated with biomass. However, for several reasons, caution should still be exercised in assuming that

biomass can be reliably estimated using this method. First, bias can occur as a result of processes other than PCR; for instance, DNA may be more readily extracted from some groups of arthropods than from others, and Zhou *et al*. (2013) considered only a small number of groups in their study. Second, this method has not been tested on large bulk samples that are typical of those obtained via Malaise trap sampling, and so it remains unknown whether sufficient sequencing depth could be achieved for the recovery of the large numbers of species (potentially hundreds) contained in this type of sample. Moreover, the approach remains expensive (approx. $20 per species per sample; Zhou *et al*., 2013), which means that it is not yet a realistic option for most large-scale biodiversity studies. Another approach that is considered promising is the 'sequence capture' method, which uses oligonucleotide probes to sequence target regions across the genome, without requiring an initial PCR step (Shokralla *et al*., 2012; Taberlet *et al*., 2012a). However, this is yet to be demonstrated on bulk arthropod samples.

Thus, accurate estimation of abundance using metabarcoding remains some way off and represents a limitation of which it is important to be aware. Nevertheless, Ji *et al*. (2013) and studies in this thesis have shown that presence-absence data can be highly informative when based on the responses of very large numbers of species. In combination with repeated measures and careful use of read count data, much ecological information can be obtained. Where it is important to estimate the abundance or population size of a particular species, it remains better to target that species individually.

8.3.3 Assignment of taxonomy

Our ability to assign taxonomy to OTUs is limited by incomplete reference databases and imperfect taxonomic assignment software. SAP (Munch *et al*., 2008), the programme used in Yu *et al*. (2012), Ji *et al*. (2013), and in the preceding chapters in this thesis, was chosen because it provides identifications at a variety of taxonomic levels, along with associated confidence estimates. However, SAP is highly conservative, which means that while there is a low probability of making false identifications, the majority of OTUs are not identified to species level. Indeed, of the three studies presented here, the highest proportion of OTUs identified to species was 32% (Chapter 7) and the lowest just 19% (Chapter 5). Although many analyses can be performed without species-level identifications, names give us access to knowledge of species traits and functions that has been accumulated over hundreds of years. Where a species is found to respond to an environmental variable or to have strong habitat preferences, it is important to be able to tap into that accumulated knowledge to ask whether the species is endangered or of conservation concern; whether it is a pest, disease vector or an invasive species, the distribution or population

size of which we want to control; or whether it provides valuable ecosystem services. Thus, species-level identifications can influence value judgements and management responses to biodiversity studies. In addition, they can help to align metabarcoding studies with those using traditional approaches, which are often species-focused. Therefore, there is a need for improved taxonomic assignment methods.

Zhang *et al*, (2012) developed a method that uses fuzzy-set-theory to assign OTUs to species and showed that this performs much better than SAP in correctly identifying query sequences to species and identifying when the query sequence is a singleton (i.e. there is no conspecific in the reference database). This apparently promising tool is currently being tested for use with metabarcode datasets, which are typically both messier than those with which the method was originally tested and inclusive of a wider range of taxonomic groups. The fuzzy-set-theory approach crucially provides confidence estimates for identifications, but it does not assign OTUs to higher taxonomic levels. In future, a two-part approach to species identification could be used, with OTUs first identified to ordinal level in SAP and then identified to species where possible using the fuzzy-set-theory method of Zhang *et al*. (2012). Alternatively, the RDP Classifier method (Wang *et al*., 2007) may be able to improve on SAP. This is a Bayesian method that was developed for assigning taxonomy to 16S rRNA bacterial sequences but can be retrained for other types of genetic data. It is now possible to retrain and implement the RDP Classifier in the QIIME environment (Caporaso *et al*., 2010; http://qiime.org/ tutorials/retraining_rdp.html).

Where there is interest in a particular species or set of species (e.g. endangered species, pests, or a group that has previously been used as an indicator), reference-based OTU-picking can be employed to detect the presence of those species in a bulk sample (Bik *et al*., 2012), as demonstrated in Chapter 7. This approach is of course reliant on possession of a reference library for the focal species, but such a library can usually be generated by Sanger sequencing of morphologically identified specimens if sequences are not already held in a curated global reference database such as BOLD Systems (Ratnasingham and Hebert, 2007).

8.4 Evolving beyond COI

One of the key principles of DNA barcoding is the use of a standardised DNA marker for species identification (in animals, COI; Hebert *et al*., 2003), and this has been successful for a wide variety of applications, including metabarcoding of bulk invertebrate collections. However, due to its length, COI is less useful for metabarcoding approaches that target degraded DNA, including DNA sourced from the environment or from invertebrate parasites, for which studies usually focus on

alternative, shorter markers (Riaz *et al*., 2011; Taberlet *et al*., 2012a; 2012b). Lack of reference sequences for these alternative markers seriously limits the ability of such studies to make species-level identifications. This is important because metabarcoding of water eDNA and invertebrate blood meals promises to be a key tool for assessing vertebrate diversity in challenging landscapes, including the detection of flagship species and those of conservation concern (e.g. Saola Working Group, 2013). In this context, reliable species identifications are crucial.

In 2012, the Canadian Barcoding of Life (CBOL) organisation received $3 m from the Google Foundation to barcode 2,000 endangered species and 8,000 of their close relatives. Unfortunately, since efforts will focus entirely on COI, this vital reference dataset may be of limited use for projects that make use of blood meals or environmental DNA for species detection. For species of economic or conservation importance, where high confidence identifications are important, it would be preferable to generate references covering much wider portions of the genome. Moreover, as methods develop more generally towards PCR-free shotgun sequencing or sequence capture approaches that generate genome-wide data, it would be ideal to be able to make use of all generated sequence data for informing identification. It is important that the barcoding community remains willing to adapt; while standardisation is clearly desirable where possible, a refusal to expand the concept of barcoding to make use of the data that technology can provide will render barcoding outdated even as it emerges (Taylor and Harris, 2008).

8.5 Final remarks

454 metabarcoding of bulk arthropod collections has been extensively validated against standard biodiversity surveys, and its use has been demonstrated in a range of environmental management scenarios. It is now time to open a dialogue with policy makers and environmental managers to address the incorporation of this technique into biodiversity policies and standards, in order to link actions with outcomes and to increase accountability for impacts. While there are undoubtedly many questions that remain best answered using standard morphological approaches, metabarcoding expands the horizons of biodiversity research by enabling the assessment of diversity patterns at a scale and depth that is simply impossible using morphological identification of specimens. Used appropriately, this tool has the potential to contribute greatly to efforts to address biodiversity loss, both in the UK and globally.

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