Population Genetics and Ecology of the Sugar Beet Leaf Miners

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Thesis Abstract

The genus *Pegomya* (Diptera: Anthomyiidae) contains a number of agriculturally important species. The sugar beet leaf miners are some of these species of significance as they periodically infest sugar beet crops, causing damage to the crop canopy which results in reduced root growth and therefore yield. Historically the sugar beet leaf miners have been regarded as a minor pest, generally cause minor damage in sugar beet crops in the UK. However, in 2015 and 2016 UK sugar beet growers witnessed a surge in leaf miner populations present in their sugar beet crops and this pest seemingly spread across the sugar beet growing area causing substantial damage to the crop canopy. The reasons behind this surge in population numbers was unknown but it was thought that the pending loss of neonicotinoids, and loss of many marketable pesticides used in the control of leaf miner, may have had an influence. One of the major issues when initially researching sugar beet leaf miners is that the taxonomic standing of the group has been historically very complex. Many of the resources available on the species said to be sugar beet leaf miners are vastly outdated and disconnected, meaning that the current knowledge of sugar beet leaf miners has significant limitations. In this thesis I investigate the identity of the sugar beet leaf miners, by a detailed investigation of existing literature and by using genetic characterisation to identify species groups from specimens collected in the field and their associated ecological data. I found that there are two genetically distinct groups from specimens collected in the UK and abroad, and from a range of hosts. These two genetically distinct groups are likely to be separate species and are seemingly distinguished by host plant range, with one group predominately found on sugar beet and sea beet, and the other on Swiss chard and spinach. I also investigated the

phylogenetics of the whole genus in order to understand where the sugar beet leaf miners fit within the wider relationships of other *Pegomya* species. Findings showed that there was some level of species groupings and groupings of species with similar larval feeding behaviours but that the overall placement of these groups was more uncertain. These findings relied partly on the successful determination of mitochondrial DNA sequences from historical specimens, loaned from museum collections. I report findings on culturing attempts made to rear sugar beet leaf miners and my conceptual plans for the cultures if they had been successful, including investigating the effects on yield and host plant preference. I conclude with a chapter describing the genetic characterisation of parasitoids associated with sugar beet leaf miner. This revealed that there are two species of braconid parasitoids associated with *Pegomya* samples collected in the Netherlands, and two different, and uncharacterised braconid species reared from UK samples. Overall, the work described in this thesis advances our understanding and characterisation of sugar beet leaf miner by uncovering that it comprises cryptic, genetically distinct, groups, placing it within its wider phylogenetic context and by characterising some of its major parasitoids.

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CHAPTER 1

General Introduction

Sea Beet fieldwork along the Norfolk coast during COVID 2020 (Photo by Kris Sales)

1.1 The Importance of studying Agricultural Systems

Agriculture is one of the cornerstones of our society, providing sources of food and fiber globally (Rechigl & Rechigl, 2016). Amongst the challenges facing agriculture, insect pests are of particular importance. Insects can cause direct damage to crops either through feeding upon them, or indirectly through the transmission of plant diseases such as viruses, which can cause major strains on crop production (Smith & Chuang, 2014; Reale *et al*., 2014; Pannuti *et al.,* 2015; Traugott *et al.,* 2015; Uesugi *et al*., 2016; Agathokleous *et al*., 2017). Estimating the exact economic losses caused by insect pests can be complex, due to the wide array of interacting factors involved. The species of crop, the environment that the crop is planted in, the technology used in the growing/harvesting process and the social demographic of the growers are all factors that can affect the performance of an insect pest and thus the potential loss caused by insect damage (Oliveira *et al*., 2014). However, what is clear is that the economic cost of agricultural pests is very significant indeed (*Case Studies* – pages 16-18). For example, the diamondback moth (*Plutella xylostella*) is estimated to cost the agricultural industry $4 – 5 billion per annum in management costs alone (Zalucki *et al.,* 2012). In Brazil, insect pests are estimated to cause losses of $17.7 billion per annum, a figure derived from the (average) annual damage to crop production (7.7%) caused by insects, which is equal to the loss of 25 million tons of food, biofuel and fibre per year (Oliveira *et al*., 2014).

Insect pests can inflict direct damage on plants in many different ways, and this varies enormously among pest species. For example, aphid species pierce the plants with their stylet and feed on the phloem of the plant, causing direct damage or transmission of

disease (Smith & Chuang, 2014). Many Lepidopteran or Coleopteran pests have larvae (and/or adult life stages) that directly chew on plant foliage (Pannuti *et al.,* 2015; Traugott *et al.,* 2015; Agathokleous *et al*., 2017). Plant-galling insects, leaf miners and leaf rollers, can actively alter plant morphology through changes they initiate via feeding and/or reproduction (Reale *et al*., 2014; Uesugi *et al*., 2016). Scale insects (Hemiptera: Coccoidea) can attack different areas of their hosts, including the leaves, branches and roots. They can cause direct damage to their hosts through feeding on the phloem, by consuming the parenchyma cells, indirectly through virus transmission or because the build-up of honeydew attracts pathogens (Mansour *et al.*, 2016). In many cases, insect pests are the carriers of viruses and fungal pathogens. Plant viruses cause huge losses to the global economy, and can be vectored by aphids, thrips, whitefly, mites, mirid bugs, mealybugs, beetles and leaf-mining flies (Alkhedir *et al.*, 2015). Because of the wide variety of ways in which insect pests can damage crops, detailed studies of the life-history and ecology of individual pest species are required in order to predict potential crop losses and develop methods of control. By studying the ecology and biology of insect pests, management strategies can be put in place to reduce the economic impact of the damage they cause (Cook *et al*., 2007).

Key features contributing to the success of insect pests include the ability to rapidly produce offspring, often with a number of generations that overlap throughout the year, high levels of dispersal with the potential to colonise a wide range of host plants, organisms or habitats, and few natural enemies (Klick *et al*., 2016). Parthenogenesis of some pests such as aphids can also facilitate rapid establishment, enabling individuals to produce the next generation without need of a mate, and therefore allowing individuals

to focus their energy on the exploitation of new habitats and resources (Wan & Yang, 2016). Co-evolutionary relationships between pests and their host plants can lead to specialist relationships, and many insect pests also show adaptations to utilise these specific host-plant relationships (Peguero *et al*., 2017). However, there are also insect pests that are capable of undergoing host shifts, which can enable them to rapidly expand their distribution range (Bernays & Graham, 1988; Jaenike, 1990). Many crop pests, for example, are also found on wild crop relatives, and host shifts between wild and domestic species and strains are important for understanding crop pest dynamics (Kamala *et al.*, 2016; Syfert, 2017). Agricultural landscapes consisting of monoculture crops with low genetic diversity are particularly susceptible to outbreaks of insect pests, especially if the insect pest shows rapid adaptation to such crops (Pelissie *et al*., 2018). When an invasive pest establishes in such a landscape it is often removed from a situation where it had previously been supressed by host plant resistance and native enemies. Therefore, many of these rapidly expanding insect pest populations are subjects to fewer constraints than apply to those found in their natural geographic and host ranges (Leung *et al.,* 2002; Duan *et al.*, 2015).

Understanding the factors that affect the distribution and spread of pest insects is essential to managing them. The distribution of pest insects depends on a large number of interacting biotic and abiotic factors, from climate and geography, to the distribution of host plants, predators and parasitoids (Hamby *et al*., 2016; Kumar and Kumar, 2015; Bestete *et al.*, 2017; Lewis *et al.*, 2017; Darwell *et al*., 2017). Insects are very responsive to changes in temperature, and can undergo “range shifts” in response to changes in the climate (War *et al.*, 2016). As a result, many areas of the world are expected to face novel

pest threats as a result of climate change, which makes the climatic responses of insects an important area of study (Crespo – Pérez *et al*., 2015). Anthropogenic factors, including intercontinental shipping, tourism and travel, can also be major factors in determining pest species distributions (Dittrich-Schröder *et al.*, 2014; van Wilgen *et al*., 2014). Because the factors that affect pest species distributions are numerous, complex and often associated with each other in a complex series of interactions, it is necessary to study ecology and life history across the taxonomic distribution of insect pests. Such study is, however, often difficult, and requires methods that are fast, reliable and easy to repeat (Kress *et al.*, 2014).

1.2 Case Studies

The Harlequin Ladybird

The Harlequin Ladybird (*Harmonia axyridis*) is one of the best examples of the how the spread of a highly adaptive invasive insect species negatively impacts its surrounding environment. This species is not considered to be a typical agricultural insect pest, as it does not attack crops. However, it does negatively affect biodiversity in countries where it is now established (Roy & Wanjberg, 2008). The first record of the Harlequin ladybird in the UK came from the arrival of a single male in 2004 (Majerus *et al*., 2006). It is not just the UK that has been infested by this species, which is originally native to central and eastern Asia, but many others - as of 2013 *H.axyridis* was recorded as being established in almost 40 countries (Belyakova and Reznik, 2013). The aphidophagous nature of *H.axyridis* meant that it was an attractive candidate for biological control, being sold commercially across the world from 1982 and first bought and used in Belgium in 1997

(Majerus *et al*., 2006). It is capable of surviving across a range of climatic conditions and habitats and is classed as a highly generalist predator with a widely variable diet that has little competition from native predators in the areas that it has invaded (Kenis *et al*., 2017). *H.axyridis* has also been recorded as having stronger anti-bacterial activity that other coccinellids, providing the eggs and larvae with a heightened defence system against predation (Verheggen *et al.,* 2017). Although being sold as a commercial product, the spread of this species into many areas was not through deliberate release as a biological control agent. In its natural range of central and south Asia, the Harlequin ladybird is capable of dispersing across long distances, as the species naturally migrates long distances to overwintering sites (Eilenberg & Hokkanen, 2006). It now poses a threat to native biodiversity through outcompeting and displacing many other species, in particular many native coccinellid and aphidophagous species (Roy *et al.*, 2012).

The Diamondback Moth

The Diamondback moth (*Plutella xyllostella*) is a Lepidopteran pest of cruciferous plants (includes rapeseed, cauliflower, broccoli and cabbage) which are grown across the globe in both temperate and tropical climates (Talekar & Shelton, 1993). Compared to many other insects, the diamondback moth (DBM) has evolved to specialise on the glucosinolates (mustard oils) that their Brassica hosts produce as part of their defence against generalist insects (Hermansson, 2016). The larvae feed on the leaves of the crop, and in some cases can cause complete defoliation (Fernandez-Triana *et al.,* 2018). Females are oligophagous and capable of laying eggs on a wide range of host plants placing DBM amongst the most devastating of the insect pests associated with cruciferous crops (Li *et al.*, 2016). It is thought that the DBM has the largest distribution

range of all Lepidopteran species (Gowri & Manimegalai, 2017) and in India has been reported as causing losses in vegetable crop of up to 90% (Dolma *et al.*, 2018). Females can lay up to 200 eggs per plant, and the number of eggs can vary between *Brassica* hosts and non-*Brassica* host plants (Munir *et al.*, 2015). The main challenge facing management of the DBM is pesticide resistance, as it has showed resistance to nearly all of the insecticides that were used in its management up until the 1980s (Juric *et al.*, 2017). Other than pesticides, DBM populations can be controlled via predator and/or parasitoid attack. In Michigan, a larval parasitoid *Diadegma insulare* (Hymenoptera: Ichneumonidae) controlled populations of DBM to relatively low population levels (Idris & Grafius, 1996). Currently, the diamondback moth is classed as the second most pesticide resistant arthropod globally, having formed immunity across each of the different classes of insecticides (Ninsin, 2015).

1.3 Molecular Tools in Biological Studies

Molecular methods and genetic tools are fast becoming an invaluable asset in almost all fields of ecology (Hadrys *et al*., 1992; Moritz, 1994; Criscione *et al*., 2005; Ekblom & Galindo, 2011). Molecular methods can be especially useful in studies where the organisms are small, hard to identify, are found in high abundance, have a poorly resolved taxonomic history and where there is possible cryptic diversity within the community (Hebert *et al*., 2004; Andújar *et al*., 2015). Recent advances in polymerase chain reaction (PCR) methods and nucleotide sequencing allow scientists to study the role of species within their ecosystems, as well as species diversity, identification and relationships (Campos-Herrera *et al.*, 2015). The integration of molecular tools with traditional

ecological and taxonomic methods has enabled ecologists to investigate the factors that influence biodiversity and ecological interactions (Alvarez *et al*., 2014; von der Heyden *et al.*, 2014). Molecular tools can be used to understand ecological processes, from speciation and phylogeography through to population dynamics and parentage analyses (von der Heyden *et al*., 2014). Molecular sampling techniques can also be less costly and less technically challenging than some traditional identification methods, as they may not require the laborious task of wide range sampling and trapping of individuals or of the identification of individuals in the field (O 'Meara *et al.*, 2014).

1.3.1 Genetic Variation in Insect Pests

Initially after the invasion of a new area, a pest population may experience a loss of genetic variation within the population, or a genetic bottleneck (Dlugosch *et al*., 2015). An array of different factors can affect the amount of genetic variation within an invading population, including the number of founders, the number of introduction events and levels of subsequent migration, the genetic system (haploid, diploid etc.), and the method of reproduction (Puzey & Vallegjo-Marín, 2014; Ferrero *et al*., 2015). A reduction in genetic variation within a founding population generally leads to reduced fitness levels due to inbreeding and genetic drift, with an increased probability of extinction (Labonne *et al.*, 2016; Hufbauer, 2017). This presents a problem – if pests and invasive species have reduced levels of genetic diversity, why are they so successful? Evidence suggests that when compared to native species, some non-native invaders (particularly in plants) actually have greater levels of genetic diversity present within introduced population compared to the founding population (Vandepitte *et al.,* 2014). These high levels of

genetic diversity may suggest that founding populations of pests are generally large, or that there are high levels of immigration into introduced populations (Puzey & Vallego-Marín, 2014). However, the consequences of pest invasion on levels of genetic variation and fitness are yet to be fully resolved.

By directly studying genetic variation in pests and invasive species it is also possible to make general inferences about evolutionary and ecological processes (Ferrero *et al*., 2015). Molecular tools can be applied to pests and invasive species in order to accurately identify the presence of a species within an area, and to investigate patterns of historical and contemporary dispersal (Darling, 2015). Molecular markers can also be used to determine the genetic variation within an invading pest population, to identify the vectors of introduction and spread, and to estimate how many introduction events have occurred (Blanchet, 2012; Lawton *et al.*, 2018). Approaches from population genetics and techniques such as molecular barcoding are increasingly being applied to investigations of the migration routes of invading pests, as well as to determine the origin of pest outbreaks (Rius *et al.*, 2015).

1.3.2 Molecular Markers and their Applications

Molecular methods have revolutionised our understanding of evolutionary biology. In addition to addressing scientific questions about genetic variation and evolution, molecular markers are often used to address a range of applied problems. These include informing conservation and breeding programmes worldwide (Hess *et al.*, 2014), studying invasive and ecologically important species (Vandepitte *et al*., 2014), and promoting

fisheries management (Abdul-Muneer, 2014). The rate at which molecular methods and tools have been adapted and deployed has been extraordinary. The decision to use a molecular marker and type of marker depends upon how reliable it is, if it is cost effective, and how accessible it is to the user (Mueller & Wolfenbarger, 1999 ; Kuta *et al*., 2015; Xiao *et al*., 2017). In this section, I will describe some of the most common molecular tools and approaches used throughout ecological studies.

Restriction fragment length polymorphism (RFLP) approaches involve the amplification of a fragment of DNA through PCR and the digestion of the PCR product, or amplicon, by restriction enzymes (Bukowski *et al.*, 2014; Watanabe *et al.*, 2015). More recently, RFLP has been used for identifying microbial species, including those which have not yet been cultured or identified (Bukowski *et al*., 2014; Castro-Carrera *et al.*, 2014). Furthermore, RFLP is still used for detecting field strains of Bovine viral diarrhoea virus (BVDV), as it was found to be affordable, reliable and fast (Kuta *et al.*, 2015). A similar approach to RFLP is amplified fragment length polymorphisms (AFLP) screening. AFLP is more robust and reliable than is RFLP at detecting genetic diversity within a species (Mueller & Wolfenbarger, 1999), and as a result has been commonly used in the study of endangered species.

Polymorphic amplified DNA (RAPD) techniques require no prior sequencing of DNA, instead utilising a single primer of around 10 nucleotides in length which is amplified through a PCR reaction to detect genetic variation at the nuclear level (Kumar & Gurusubramanian, 2011). In theory it is able to amplify very discrete fragments of DNA (Hadrys *et al*., 1992). RAPD markers have been used in studies determining the genetic

uniformity of transgenic crops in order to assess their safety (Mikkelson *et al.,* 1996; Whitton *et al*., 1997), and in studies investigating genetic diversity (Ebrahimi *et al*., 2015; Toan *et al*., 2017). However, there are some disadvantages when using RAPD techniques due to concerns about the efficiency and reproducibility of the technique (Ali *et al*., 2004).

Microsatellites (also known as simple sequence repeats/SSRs or short tandem repeats/STRs), are short repeat sequences of up to around ten base pairs in length (Hadi *et al.,* 2014). Microsatellites mutate via the addition or deletion of repeat units and are among the most rapidly changing regions of the genome (Fan & Chu, 2007). As such, microsatellites have been used within forensic case studies since the 1990s, particularly for the identification of missing persons and paternity testing (Parson *et al.*, 2016). They are the most common choice of forensic marker for this discipline (Scheible *et al*., 2014). Microsatellite markers are also used frequently throughout population genetic analyses (Sun *et al.*, 2017), and have been used to study the structure of populations, genetic variability (Albertin *et al.*, 2014), parentage and kinship (Weinman & Solomon, 2015). These markers can be found throughout the genomes of all eukaryotes and only require a small amount of DNA for analysis (Morgante & Olivieri, 1993).

SNPs are commonly used DNA-based markers which can be found throughout the genome, and have great potential for typing using high-throughput sequencing (Khazaei *et al*., 2014; Spanic *et al.*, 2016). They can be identified as single differences in nucleotide composition amongst individuals of the same species (Zbawicka *et al.*, 2014). As a result they have proven to be highly successful in a wide range of areas, including the molecular breeding and propagation of crops and their genetic studies (Khazaei *et al*., 2014; Mora

et al., 2015). In comparison to microsatellites, SNPs can be commonly found in many species genomes, and more frequently, so genotyping is considerably cheaper due to the compatibility of SNPs with higher throughput methods (Morin *et al*., 2004; Hiremath *et al*., 2012).

Sanger sequencing can be used to directly identify the sequence of nucleotides within a gene or genetic region amplified by PCR (Diekstra *et al*., 2015). Data produced from Sanger sequencing generally contains up to around 1000bp of sequence, usually from a single gene (Moorthie *et al*., 2011). Due to the robustness and high accuracy of this method it quickly became one of the most commonly used sequencing methods used for DNA detection (Heather & Chain, 2016), and was used to sequence the first human genome (Venter *et al*., 2001). Sanger sequencing methods have been used in the studies of the phylogenetics of almost every group of organisms and have been instrumental in the study of systematics (Miyamoto & Cracraft, 1991; Straub *et al*., 2012; Chun & Rainey, 2014). Sanger sequencing has a relatively low error rate and is still useful when data from only one or a few genes is required (Moorthie *et al*., 2011).

1.3.3 DNA Barcoding and Phylogeny

DNA barcoding is the sequencing of short, standardised sections of DNA in order to assess which species are present in a sample (Valentini *et al.*, 2009; Ruhsam *et al.*, 2015). This method involves creating a DNA barcode library of the species of interest (if one is not already available), then pairing up unidentified samples against this library (Savolainen *et al*., 2005). The position within the genome of DNA barcode must be standardised for this

method to be successful across laboratories (Li *et al.*, 2015). For organisms within the animal kingdom, the use of fragments (648 base pairs in length for standard DNA barcoding) from the Cytochrome *c* Oxidase 1 (CO1) mitochondrial gene, has been successful in the application of DNA barcodes (Coissac *et al*., 2016). DNA barcoding is useful in the identification of species that have already been described, and in gathering information on their presence and distribution (Wilson *et al.*, 2014). In the context of pest species, this approach has high potential. For example, material from an infested crop can be screened and pests identified without the need for intensive expert morphological identifications. Indeed, DNA barcoding has allowed ecologists to address a range of questions which had previously been difficult to tackle (Allendorf *et al*., 2010; Joly *et al*., 2015; Figure 1.1). These problems include understanding of the diet of predators and herbivores (Kajtoch *et al*., 2015), and identifying the parasites of a wide range of species (Besansky *et al.,* 2003).

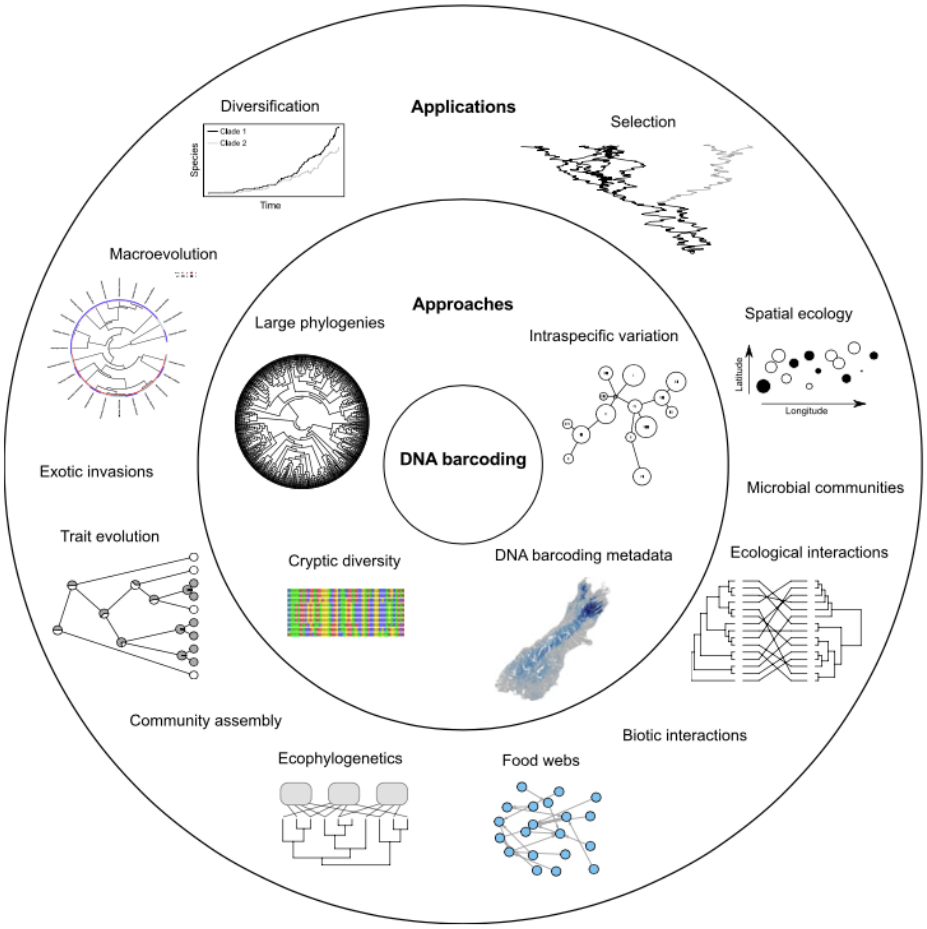

Figure 1.1 Applications of DNA barcoding in ecology, with reference to areas in which DNA barcoding is most applicable (figure reproduced from Joly *et al.,* 2015).

Metabarcoding is the identification of multiple species from mixed species samples or environmental DNA. This approach has been most commonly applied to microbial community analysis but is increasingly being applied to larger fauna (Cristescu, 2014). As with DNA barcoding, the most commonly used barcode for metabarcoding with animal samples is the mitochondrial CO1 gene (Deagle *et al.*, 2014). Metabarcoding approaches are becoming increasingly popular, as when combined with NGS approaches they show potential to sequence entire ecological communities (Taberlet *et al.,* 2012). As a result, these approaches are increasingly being used in biodiversity monitoring programmes (Pawlowski & Holzmann, 2014), and in the identification of pests and diseases (Yu *et al*.,

2012; Evans *et al*., 2016). Metabarcoding provides a promising alternative in the analyses of larger sample sizes of microscopic and macroscopic communities without the need of taxonomic expertise and large amounts of time (Vasselon *et al*., 2017). Although metabarcoding is useful for identifying the species present within a community, over- or under-amplification of target species can occur due to bias in primer binding (Geisen *et al.*, 2015).

Next generation sequencing (NGS) is capable of producing large amounts of genetic data for almost any species and is both efficient and cost effective (Hess *et al.*, 2014; Xiao *et al.*, 2017). Some of the advantages of this new technology include being able to sequence historic samples, poorly preserved samples, greater sample sizes than other sequencing technologies, and its ability to sequence vast numbers of genetic markers or individuals at one time (Pauls *et al.*, 2014). NGS can be used in DNA barcoding and metabarcoding, to identify large numbers of individuals, populations and species from environmental or pooled samples (Schuster, 2007; Futschik & Schlotterer, 2010; Ekblom & Galindo, 2011; Anderson *et al.,* 2014). As such, NGS has enormous potential for the study of insect pests.

In this review I have so far discussed some of the molecular approaches used in studying insect pests, and how their applications can vary and be used to answer ecological questions. The use of molecular markers in biological studies have proven useful in the investigation of a number of different research areas and across a wide range of organisms. In particular, their use in tackling problematic agricultural pests is what has inspired a lot of the work in this thesis with regards to how they may be used to tackle the sugar beet leaf miner pest complex. Below I introduce the leaf miners – which are the

topic of investigation for my PhD, and focus on their biology and ecology, as well leaf miner associated parasitoids. I discuss some of the roles leaf mining species play within the agricultural and horticultural industry, and examine the ways in which molecular tools have already been used to help study leaf miners and parasitoids in an agricultural setting. A detailed review of the sugar beet leaf miners and related species follows in Chapter 2.

1.4 Understanding the Evolution and Ecological Importance of Leaf Mining Behaviour

Leaf mining behaviour has evolved a number of times during the diversification of insect-plant relationships (Auerbach *et al*., 1995; Whiteman *et al*., 2012), with relationships between insects and plants dating back to the start of the Devonian period (415-400 Ma) when terrestrial ecosystems first began to take shape (Lopez-Vaamonde *et al*., 2006). As a result of this evolutionary phenomena, leaf mining behaviour can be found in four of the insect orders; the Lepidoptera (Table 1.1), the Diptera (Figure 1.3), the Coleoptera (Figure 1.1) and the Hymenoptera (Figure 1.2) (Hespenheide, 1991; Auerbach *et al*., 1995; Connor & Taverner, 1997). Leaf miners, along with all other herbivorous insects are amongst the most diverse of the arthropods. Estimates vary between phytophagous insects dominating 50% of all insect life (Whiteman *et al.,* 2012) and excluding microorganisms, to 50% of all known species (Lopez-Vaamonde *et al*., 2006). Lepidoptera have the highest number of leaf mining species within the insects, and they span across 34 of the Lepidopteran families, the most speciose family being the Gracillariidae (Auerbach *et al*., 1995). The Diptera also have a number of leaf mining families, particularly in the Agromyziidae which is the second most speciose family next to any of the Lepidoptera (Hespenheide, 1991) and the Ephydridae. In the Coleoptera, the family

Chrysomelidae have the largest number of leaf mining species, whereas in the Hymenoptera there are only around 100 leaf mining species across the whole order, the majority being found within the family Tenthredinidae (Auerbach *et al*., 1995).

It is generally understood that the evolution of phytophagous insects has been accompanied by the ability and need to adapt to a more specialised set of host plants, therefore narrowing down the host range of the insect (Whiteman *et al*., 2012). But whether the plants or the insects evolved first had always been much debated. Plant chemical defences are likely to have affected past host range shifts in herbivorous insects, with theories suggesting direct links between the colonisation of novel hosts with different chemical defences and the increased diversification in insect herbivores (Winkler *et al*., 2009). Most importantly however was the evolution of the angiosperms. This new, more resilient host provided herbivorous insects with abundant resource, allowing increased herbivory and therefore diversification of the herbivorous insects (Labandeira *et al*., 1994). In the Lepidoptera, leaf mining is most likely to be a trait that evolved early on in their lineage as the leaf mining behaviour can be observed in the most primitive of the Lepidopteran families. In the Coleoptera leaf mining is found within six families and most likely evolved a number of times. Chrysomelidae, the family with the most numerous amount of leaf mining species, is likely to have evolved this behaviour from ancestors that fed externally on the leaves of their host plants. And although the Hymenoptera have the fewest leaf miners of all the insect orders, it is suggested that they may have undergone up to six evolutionary changes during their course of their history and similarly to the Coleoptera, the leaf mining behaviour is most likely derived from an ancestral external feeding herbivore (Connor and Traverner, 2000).

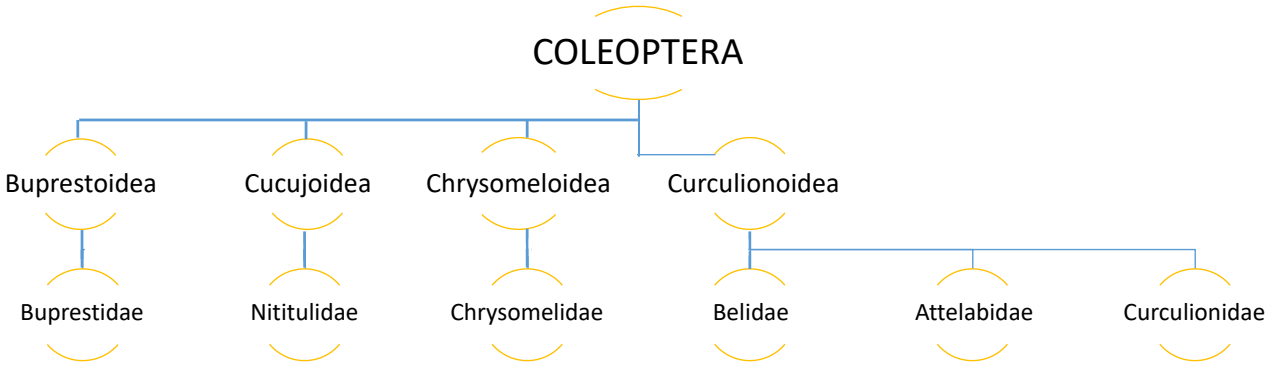

Figure 1.2 Leaf mining families within the Order Coleoptera, sub-order Polyphaga (adapted from Connor and Taverner, 2000).

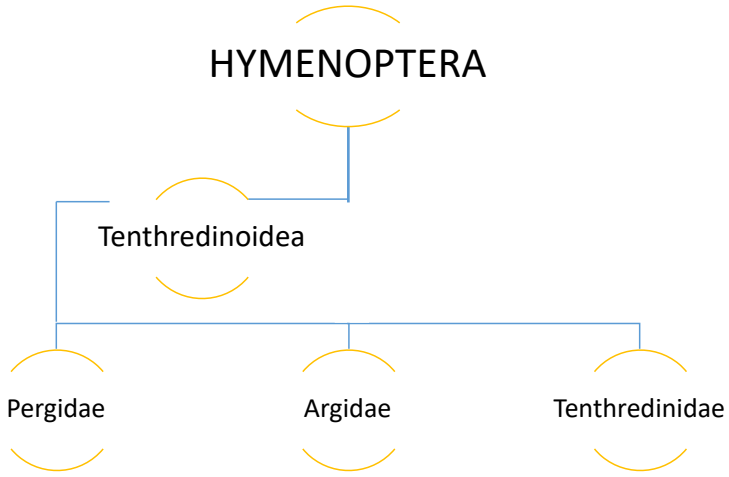

Figure 1.3 Leaf mining families within the Order Hymenoptera, sub-order Symphyta (adapted from Connor and Taverner, 2000).

Table 1.1 Leaf mining families within the order Lepidoptera (adapted from Connor and Taverner, 2000).

Suborder	Superfamily	Family
Heterobathmiina	Heterbathmioidea	Heterobathmiidae
Dacnonypha	Eriocranioidea	Eriocraniidae, Acanthopteroctetidae
Monotrysia	Nepticuloidea	Nepticulidae, Opostegidae
	Tischerioidea	Tischeriidae
	Palaephatoidea	Palaephatidae
	Incurvarioidea	Incurvariidae, Prodoxidae, Adelidae, Heliozelidae
Ditrysia	Gracillaroidea	Gracillariidae, Bucculatricidae, Douglasiidae, Roeserstammidae
	Gelechioidea	Oecophoridae, Elachistidae, Coleophoridae, Momphidae, Cosmopterygidae, Scythrididae, Gelechiidae
	Copromorphoidea	Carposinidae, Epermeniidae, Glyphipterigidae
	Yponomeutoidea	Acrolepiidae, Argyrestiidae, Yponomeutidae, Heliodinidae, Ochsenheimeriidae, Lyonetiidae
	Tortricoidea	Tortricidae
	Pyraloidea	Pyralidae
	Pterophoroidea	Pterophoridae

Leaf mining larvae feed on the plant tissue within the leaves of their host plant. Some species feed upon specific layers of plant tissue whereas others may feed throughout tissue layers. It is only the larval stage that display this feeding behaviour in the insects (Connor & Taverner, 1997). In many species, identification is possible based on the shape and patterns of their mines as leaf mining can create very distinctive patterns and chambers within the leaf of the host plant (Salvo & Valladares, 2007). Dependent upon the species of leaf miner, some larvae will complete their whole larval development within the leaf, or some may only spend the first part of the larval development feeding

within the leaf (Rott & Godfray, 2000). This length of time spent feeding within the leaf can be classed as facultative miners, or species which only spend part of their development inside the leaf, and obligate miners, species which spend the whole of their larval development consuming leaf tissue (Mustafa *et al.,* 2014). In some species larvae which spend their whole development time inside the leaf may then continue their development and pupate within the mine itself (Ayabe & Hijii, 2016). Leaf mining behaviour is thought to have many advantages for the developing insect larvae. Being enveloped inside the leaf of a host plant lowers the rate of predation and protects the larvae from environmental conditions that insects living on the surface of the plant encounter, such as UV radiation and protection against desiccation. It also protects against pathogen attack. However, there are some disadvantages to the leaf mining behaviour too in that the larvae are generally less active or mobile which results in a higher rate of parasitism (Connor and Taverner, 1997). This is reflected in the diversity and high number of known parasitoids associated with leaf mining communities (Edwards & LaSalle, 2004; Eber, 2004).

Leaf miners constitute some of the most economically important pests of ornamental and vegetable crops across the world (Mustafa *et al*., 2014; Sridhar *et al*., 2014; Gao *et al.,* 2017; Sooda *et al*., 2017). Pests exist within each of the insect orders. In Diptera, over 100 species of leaf miners are classed as pests to the agricultural industry (Salvo & Valladares, 2007). This unique mode of life and method of herbivory means that the larvae reduce the photosynthetic capacity of the host plant. When this happens in agricultural or ornamental crop plants it results in a reduction in crop yield, and therefore the crop is less profitable or unfit for sale (Johnson *et al.,* 1983; Bjorksten *et al*., 2005). If

particularly large numbers of a leaf miner species persist on a host plant, this behaviour can also lead to plant death (Bjorksten *et al*., 2005). Control of leaf miners in agriculture can be more difficult than controlling other pests as the leaf miner in encased within the leaf and therefore protected for part of or all of their larval development (Shareef *et al*., 2016). This means that translaminar acting pesticides are required in order for the control of the pest species to be effective (e.g. Wei *et al*., 2015). Many species of leaf miner pests will often not pupate within the host plant, but will migrate elsewhere or pupate within the soil, making even the pupal stage of many species problematic for growers (Bjorksten *et al*., 2005). Leaf mining pests may also develop rapid immunity to pesticides in areas where chemical treatment has been overused which can trigger population outbreaks (Johnson, 1993). Secondary resistance can also be an issue. This is where a minor or less problematic pest, such as some leaf miners, become resistant to pesticides that are being applied to other more problematic pests (Weintraub *et al*., 2017).

1.4.1 Dipteran Leaf Miners

The Diptera have many leaf mining families, the most studied group being the Agromyzidae, but leaf mining larvae can also be found in the Ephydridae, Drosophildae and the Anthomyiidae (Hespenheide, 1991). In total however there are nine leaf mining fly families Figure 1.3). Unlike the Coleoptera and the Hymenoptera, the leaf mining trait found in Diptera is derived from a variety of different modes of ancestral feeding behaviours (Table 1.2).

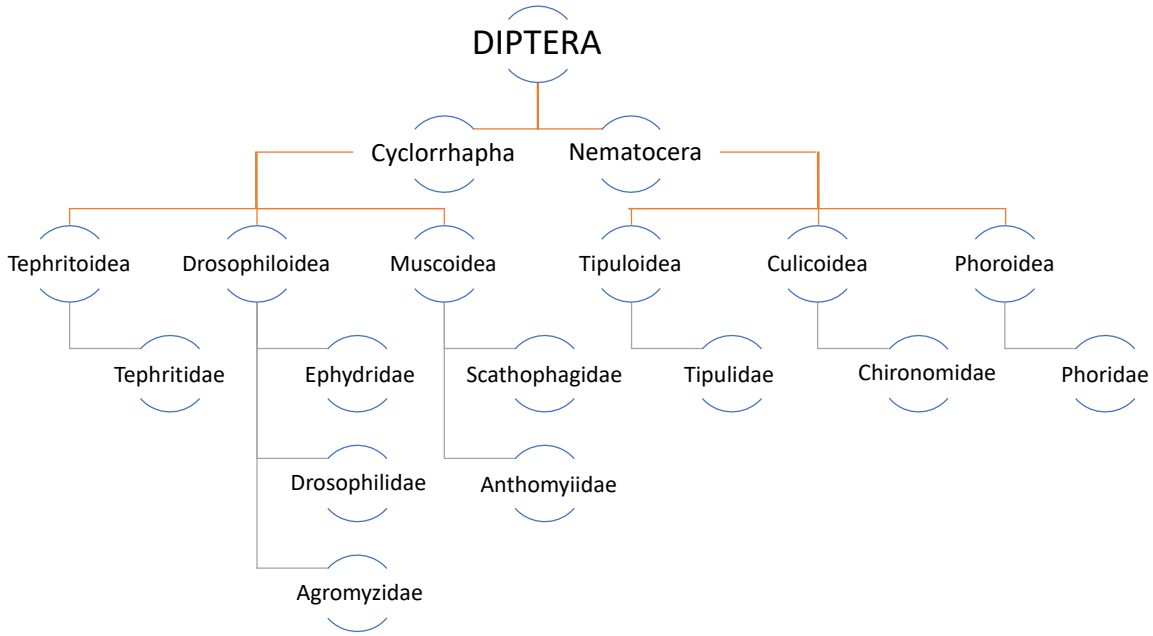

Figure 1.4 Leaf mining families within the order Diptera (adapted from Connor and Taverner, 2000).

The number of leaf mining species within the Diptera is fairly large compared to some of the other leaf mining orders but still the most documented of all those species are the pest species. The *Liriomyza* (Family: Agromyzidae) are the most notable for this as they contain some of the most prolific of the dipteran pests (24 of economic value, 330+ species worldwide)(Gao *et al.*, 2017). Species within the genus *Liriomyza* are generally termed the serpentine leaf miners due to their polyphagous natures, an uncommon trait within the Agromyzidae, and difficulty in identifying between species (Parrella & Keil, 1984). Many of the *Liriomyza* species are actually monophagous, but the pest species often attack a large range of hosts (Gao *et al.*, 2017). *Liriomyza huidobrensis* is a highly polyphagous species known to attack a range of different host crops outside of its main potato host, including peas, beans, beet and spinach (Maharjan *et al.*, 2014). It has been

suggested that its actual number of host plants ranges in the hundreds, including many flowering crops, fruits and vegetables (Weintraub *et al*., 2017).

Table 1.2 Ancestral modes of life associated with modern day leaf mining families within the Diptera (adapted from Connor and Taverner, 2000).

Family	Ancestral Feeding Habit
Agromyzidae	Internal feeding on the root, stems and bark of a host plant
Tipulidae	Saprophagous
Scatophagidae	Coprophagous

1.4.2 Leaf Miner Pests

A key feature of many herbivorous pests is their lack of host specificity. Many non-pest insect herbivores, including leaf miners, are associated with one or a small number of host plants, whereas pest species are often more polyphagous (Parrella & Keil, 1984; Mayhew, 2001). The tomato leaf mining moth, *Tuta absoluta*, is an oligophagous pest, known to attack its primary host of tomato crops, as well as a variety of secondary hosts within the family Solanaceae, and is a highly invasive pest (Sridhar *et al*., 2014). There are many hypotheses as to why some leaf mining species are polyphagous, while the majority are monophagous. In general specialisation is thought to be favoured, due to i) allowing increases in the nutritional uptake and quality in the insect's diet (Bernays & Cornelius, 1989), ii) avoiding costs associated with seeking out and evaluating the quality of the potential host plant (Janz, 2003), and iii) reduced pressure from natural enemies and effects of host plant quality (Richards *et al*., 2015; Moreira *et al*., 2015). Generalism is

likely to evolve when either, i) resource availability is high, and/or ii) mixing foods may improve nutritional uptake by the herbivore and reduce costs associated with host plant defence (Bernays & Minkenberg 1997).

Pest leaf miners cause massive losses to crop production worldwide. Some examples include the dipteran leaf miner known to attack potato crops, *L.huidobrensis*, or the potato leaf mining fly, which has caused losses in yield of up to 50% on potato crops in Peru (Maharjan & Jung, 2016), while the leaf mining moth of tomato plants, *T.absoluta* (Lepidoptera: Gelechiidae) has been recorded to cause yield losses of 80-100% in areas that it has newly invaded (Rostami *et al*., 2017). Adult leaf miners can also cause damage to the plant through feeding, for example the Oil Palm leaf miner, *Coelaenomenodera elaeidis* (Coleoptera: Chrysomelidae), creates longitudinal trenches on the lower surface of oil palm leaflets (Thomas *et al*., 2015). The larvae of leaf mining pests are also known to facilitate plant diseases and pathogens, such as the association between *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) and citrus canker outbreak in citrus orchards (Mustafa *et al*., 2014).

1.4.3 Factors that affect Leaf Miner Distribution

A particularly concerning aspect of global warming is the effect it may have on pest species. Fluctuations in temperature are considered the most important abiotic factor in determining insect population dynamics (Bale *et al*., 2002; Maharjan & Jung, 2016). The developmental stages of insects are highly influenced by temperature, with many insects having a narrow range of thermal tolerance. As a result, temperature is often used as a

major predictor in mathematical models for predicting the abundance and activity of insect pests (Damos & Savopoulou-Soultani, 2012). Other factors in association with climate change that can affect the relationship between host plant, insect herbivore and natural enemies is the increasing level of CO2 in the atmosphere (Jiang *et al*., 2016). Elevated CO2 can have negative effects on leaf miner survival and population density, and this effect appears to interact with temperature (Johns & Hughes, 2002). The relationship between leaf miner density and CO2 may also vary among host plants, with differential pest survival rates on different host species (Gherlanda *et al*., 2015).

Distribution and range of host plants play a significant role in leaf miner and all insect distributions. Transportation and worldwide trade of crops and plants have aided the rapid spread of many of these pests, enabling them to rapidly colonise new areas (Work *et al*., 2005). The tomato leaf mining moth, *T.absoluta*, has recently spread from its native range across South Africa, to Europe, Asia and the Mediterranean, as a result of a range increase in its host plants (Kamali *et al*., 2018). The horse chestnut leaf miner, *Cameraria ohridella*, is a common leaf mining pest of horse chestnut and sycamore trees (Kopacka & Zemek, 2017) and has been classed within the 100 most invasive species in Europe (Barta, 2018). Due to increased abundance and planting of these tree species within urban, parkland and woodland areas over centuries, and dispersal by wind and transportation, the horse chestnut leaf miner has been able to migrate along areas where these species are planted and infest many tree populations worldwide (Walas *et al*., 2018). Similarly, leaf miners associated with birch trees (*Betula* spp.), have spread throughout the introduced range of this tree, with 44 reported species of leaf miner attacking birch in Siberia alone (Kirichenko *et al*., 2017).

1.4.4 The Leaf Miner – Parasitoid Complex

Leaf miner parasitoids are an extremely species rich and diverse group (Edwards & LaSalle, 2004) with more species than any other group of parasitoids of herbivorous insects (Eber, 2004). Studies suggest that leaf miner parasitoids occur at much lower densities in fragmented environments, e.g. agricultural landscapes (Inclán *et al*., 2014). Intolerance to pesticides can also affect the ability of parasitoids to find their host, their ability to reproduce and their overall life span, reducing the number of species present in these landscapes (Mgocheki & Addison, 2009). This can result in an increase in leaf miner densities – for example, *Liriomyza* outbreaks have been positively correlated with a reduction in natural enemies during periods of pesticide usage (Trumble, 1985). Conversely, parasitoids have been documented as playing a key role in the subduing of leaf mining pest populations in agricultural areas where pesticides are sparingly used (Chen *et al*., 2003).

The study and conservation of naturally occurring native populations of parasitoids has been encouraged for the control of pest leaf miners, rather than introducing non-native parasitoids that may have a more generalist habit (Edwards & LaSalle, 2004). Parasitoids of the Agromyzidae are suggested to be highly generalist species that can attack a wide range of agromyzid fly hosts (Murphy & LaSalle, 1999). Within the braconids, a subfamily of solitary koinobiont parasitoids known as the Opiinae are recorded as attacking a range of Agromyzid and Anthomyiid hosts (Zikic *et al*., 2013). However, host preferences, or in some cases, host limitations, may also exist within habitats that agromyzid parasitoids use (Murphy & LaSalle, 1999). A parasitoid's host choice may be limited to a specific

herbivorous host living within a specific habitat or even on a specific host plant (Askew & Shaw, 1979; Johnson & Hara, 1987; Salvo and Valladares, 2007). This is a potential problem when there are monoculture crops within the agricultural landscape, as with newly established populations of leaf miner, the parasitism success rate can be relatively low (Gilbert *et al*., 2003).

1.4.5 Molecular Approaches to Leaf Miner and Parasitoid studies

Many leaf mining groups are difficult to identify morphologically. Generally, the understanding of these species is low, with little information on their biology and which when combined with complex taxonomic histories, can result in misidentification. There has been a noticeable reduction in the number of experts capable of identifying "difficult" insect groups in recent years, so the development and use of molecular tools for the identification of complex taxonomic groups has been invaluable. Molecular tools enable the rapid identification of a large number of individuals and enable non-specialists to collect specimens and identify them accurately down to the species level.

An example of the use of molecular tools to identify and gain new insight into leaf mining pests comes from the genus *Liriomyza* (Agromyzidae), which contains around 24 reported pest species (Gao *et al*., 2017; Rodriguez – Castaneda *et al*., 2017; Sooda *et al.,* 2017). Adult *Liriomyza* are difficult to identify to species using morphological features alone, and their larvae are more difficult to identify still (Amin *et al*., 2014). Additionally, pest species within this genus have large host ranges, raising more challenges to identification (Weintraub & Horowitz, 1995). Misidentification in the field was one of the

main reasons one of the major pests in this genus, *Liriomyza trifolii,* became a problem (Parrella & Keil, 1984). Molecular research into variation in *Liriomyza* has revealed new genetic strains and even cryptic species using sequence information on just a small number of genes (Parish *et al*., 2017). DNA can even be obtained from recently excavated leaf mines (Derocles *et al.,* 2015; Mlynerek *et al*., 2016), although this approach has a relatively low success rate (around 19%), and genetic material sampled from larvae is clearly preferable (Mlynerek *et al*., 2016). Regardless, these studies highlight the benefit of molecular tools for accurate pest identification.

Molecular tools can moreover be used to study trophic interactions within an ecosystem, and as result it is now possible to identify food webs and species interactions (Lefort *et al*., 2017). In particular, host – parasitoid networks are of interest to many crop scientists because of the information that can be gained on the potential role of parasitoids as biological control agents (Avalos *et al*., 2016). Until recently, molecular studies have focused largely on the identification of host insect – primary parasitoid relationships, but it is now becoming clear that molecular approaches are allowing the identification of secondary parasitoids/hyperparasitoids (Ye *et al*., 2017a; Ye *et al.,* 2017b). Hyperparasitoids belong to the fourth trophic level within a food web (Buitenhuis *et al*., 2017) and their presence often influences the level of success of parasitism of primary parasitoids (Sithole *et al*., 2017). Hyperparasitoids become increasingly difficult to identify from morphological characteristics (Ye *et al.,* 2017a), and molecular markers are now being employed to understand the relationships between hosts, parasitoids and hyper-parasitoids (van Nouhuys, 2016; Kitson *et al*., 2018).

1.5 Conclusions

Even amongst the more well-known and prolific insect pest species affecting world crops, there are knowledge gaps in what is known about their biology and ecology. Basic information on identification, distribution, host plant range and behaviours are lacking for many species. Molecular techniques have advanced significantly in recent years, enabling ecologists to use molecular approaches alongside traditional ecological methods to answer questions that had previously been hard to approach. Molecular tools have enabled the identification of morphologically similar and closely related pest species, enabling biologists to accurately identify morphologically challenging pest species. The progress of molecular identification has now allowed scientists to answer questions on pest species biology, ecology, distribution and spread. Further research has also been conducted on host-pathogen associations and pesticide resistance.

Leaf mining insects are often major pests, and interesting model organisms for evolutionary and ecological study. They have unique larval feeding behaviours, complex population dynamics, and highly varied parasitoid communities. However, there is a general lack of knowledge of the biology and ecology of many leaf miners. Studies so far have been mostly limited to the major leaf mining pests, such as those in the genus *Liriomyza*. However, even in some pests, such as the sugar beet leaf miners, very little is known about their taxonomy and ecology. Specifically, we lack basic information about leaf miner host specificity, the factors which limit or increase leaf mining insect distributions and the parasitoid complex associated with many leaf mining insects. Surprisingly few leaf mining insects have been reared in laboratory cultures, despite their

short generation times and their hardy nature. Studying leaf miner life histories both in the wild and under controlled conditions is likely to result in significant advances in the ecology and life history of these species. Combining such detailed ecological study with molecular tools is likely to be a promising way forward.

1.6 Thesis Aims

The aim of this PhD was to provide new insights into the taxonomy, ecology and life history of the sugar beet leaf miner complex. A combination of morphological and molecular techniques was used to do to unravel taxonomic history. I then studied the life history, ecology and distribution of the leaf miners. This included an investigation into the leaf miners' host plant range and associated parasitoids. Understanding these aspects of the leaf miners' biology and ecology will help to develop sustainable and more effective management strategies for the control of this pest in the future.

1.7 References

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CHAPTER 2

Sugar Beet and Its Leaf Miners

Left: *Pegomya hyoscyami*. Right: *Pegomya betae*. From the collection at Liverpool World Museum, 2017 (Photos by Siobhan Hillman)

2.1 Abstract

Sugar beet leaf miners are sporadic dipteran pests that infest sugar beet. The larvae of the sugar beet leaf miners consume the parenchyma of the leaf resulting in loss of crop canopy, and therefore potential reduction in yield. Due to difficulty in identifying these pests the exact species that attacks sugar beet remains somewhat unclear. In this review of the sugar beet leaf miners I investigate how past literature has defined what I refer to throughout this thesis as the sugar beet leaf miner complex. I specifically target past literature that is associated with dipteran leaf miners of sugar beet and related hosts (Swiss chard, beetroot, spinach), as well as those species defined by Michelson (1980) as part of the *P.hyoscyami* complex*; P.hyoscyami, P.betae, P.cunicularia* and *P.exilis*. I will give a brief overview of some of the complicated taxonomic history of the species in association to the current understanding of their taxonomic placements. This includes species similar to the sugar beet leaf miners, as well as those that may have historically been referred to as a sugar beet leaf miner but aren't found within the Anthomyiidae or *Pegomya* genus. I also undertake a short review of the reported host plants of the sugar beet leaf miners, as well as what species have been documented as naturally enemies of these species. I found that the past literature often referred to two species as a 'sugar beet leaf miner', these were usually *P.hysocyami* or *P.betae*. Past literature has often defined the two based on colouration or geographic range, and usually refers to one or the other as a beet leaf miner. Mention of the remaining two species from Michelson's review of the complex, *P.cunicuarlia* and *P.exilis*, have very few records in the available literature, and these are usually in species checklists. The accompanying material for both is usually a description of the morphology of the species, rather than details on their

biology or ecology. The species *P.mixta*, a synonym for *P.cunicularia*, crops up throughout the literature to present day as the true sugar beet leaf miner, though what facts determine this distinction from other species remains unclear. Likewise, I identified two non-Anthomyiid flies which have been referred to in the past as sugar beet leaf miners within published literature. A common theme with these two species, as well as with the use of *P.mixta* and some of the acclaimed host plants of the sugar beet leaf miners, is the lack of information supporting these claims. I conclude therefore that much of the literature of 'sugar beet leaf miners' should not be taken as definite fact unless the information backing up any claims on taxonomy, biology or ecology is determined as reliable.

2.2 Introduction

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is an important crop for sugar production globally and has been grown commercially since the 18th century (Izzatullayeva *et al*., 2014). It became increasingly popular in the twentieth century and is now grown in around 50 countries (Draycott, 2008), with around one quarter of the sugar sold provided by the sugar beet industry (the remaining three quarters coming from sugar cane; Draycott, 2008). Sugar beet is grown as part of a 3-year rotation with cereals such as winter wheat and winter barley (Marlander *et al*., 2003), but can also be grown in rotations with maize, beans, potatoes and oilseed rape (Marlander *et al*., 2003; Koga, 2008). Although sugar beet is predominately grown in the northern hemisphere within temperate climates, recent expansion of the cultivation of sugar beet during winter months in tropical zones has been made possible due to advances in agricultural technology and genetic knowledge (Sharma *et al*., 2017).

Table 2.1 Overview of the taxonomic status of the genus *Beta* (adapted from Biancardi *et al.,* 2010).

Genus + species	Sub-species	Common Names
Beta vulgaris	*vulgaris*	Leaf beets Garden beets Fodder beets Sugar beets
Beta vulgaris	*maritima*	Sea beet

Sugar beet has a pest complex ranging across vertebrates (such as some bird species), nematodes, insects and mites (Dunning, 1974). Cultivars that are derived from *B.vulgaris*, such as beetroot, may also share the same pest complexes (Cooke & Dewar, 1992). Two

major pests of sugar beet are the black bean aphid (*Aphis fabae*) and the peach potato aphid (*Myzus persicae*) (Albittar *et al*., 2016; Golizadeh *et al.*, 2016). These aphids cause direct damage to the plant through their feeding behaviour which aids the transmission of viruses, such as the Beet Yellow Virus (BYV) which can cause yields reduction of up to 50% (Kaya & Yilmaz, 2016). Damages caused by these aphids have been estimated at around a loss of 2 million tonnes of sugar beet in Europe (Albittar *et al.,* 2016). Another major pest of the global sugar beet industry is the root cyst nematode (*Heterodera schachtii*), which can be found across Europe, America, and Asia (Madani *et al*., 2005). Root cyst nematode is a soil dwelling species, protected by its cyst for the majority of its lifetime, only emerging as a juvenile in its second stage (J2) to infest the roots of sugar beets (Hussain *et al*., 2017), heavily stunting crop growth and causing yield losses of up to 60% (Stevanato *et al*., 2015). Above ground symptoms include wilting, resulting in the loss of leaves and yellowing, and below ground the growth and expansion of secondary roots occurs, deforming the roots (Reuther *et al*., 2017). Sugar beet also has to compete with weeds. During the early stages of development (around the 6-8 leaf stage) the crop is at its most vulnerable to emerging weeds, which, if not maintained can cause reductions in crop yield between 26-100% (May, 2003).

The aim of this systematic review is to outline the importance of the leaf mining behaviour, with regard to its economic importance and its importance especially with regards to sugar beet. I aim to provide an overview of the Anthomyiidae family as well as the genus *Pegomya*, and to critically discuss and review the species associated with the sugar beet leaf miner complex, particularly with reference to their morphology and life history. The taxonomic history of these species will also be discussed, with reference to

each of the component members that make up this complex and how they may be distinguished from one another.

2.3 Taxonomy, Life history and Morphology of *Pegomya*

2.3.1 *Anthomyiidae*

The Anthomyiidae, more commonly referred to as root-maggot flies (Michelson & Baez, 1985), are a large group of understudied calypterate flies with over 1000 species found worldwide, the majority being distributed across Europe and Northern America (Suwa, 1974). The larvae of this diverse group of flies have exploited several different feeding mechanisms, from leaf miners, stem borers and fungal feeders (Ackland *et al*., 2017) to saprophagy, scavenging and feeding on any decaying matter and faeces (Ishijima, 1967). Anthomyiidae are renowned as being a morphologically and taxonomically difficult group to identify and have been described as an 'obscure and unattractive' group (Pont & Ackland, 2009). A common feature of the Anthomyiidae that is used to distinguish them from other closely related families, such as the Muscids, is a feature of the wing morphology. In Anthomyiids a general rule is that the anal vein on the wing is extended and reaches the wing margin (Komzáková & Barták, 2009). Although there is now a key to the British Anthomyiidae (Ackland *et al*., 2017), there are few accounts of the morphology of the larvae and other stages of these species. It is also well known that the most accurate way of identifying any anthomyiid species is from examination of the male terminalia (Komzáková & Barták, 2009). They are often associated with pests due to the feeding behaviours of some larvae and their impact upon our crops. However, they are

also underappreciated pollinators as adults. Diptera are one of the most important insect orders that carry out pollination, with several fly families acting as pollinators throughout their lives (Larson *et al*., 2001). They are also amongst the most primitive of the flower-pollinating insects (Woodcock *et al*., 2014). There are many generalist pollinators within the Anthomyiidae, but they also contain some highly specialised pollinator species too (Larson *et al*., 2001). The genus *Chiastocheta* contains species with seed feeding larvae, but within some species, the adults are also the exclusive pollinators of the host plants which the larvae attack, described by Larson *et al*. (2001) as rivalling even the relationship between the fig and the fig wasp and the yucca plant and yucca moth.

2.3.2 Overview of the genus

The genus *Pegomya* is divided into two sub-genera depending on the feeding habit of the larvae of each species and ovipositor length of the female. The sub-genus *Phoraea* includes species whose larvae feed on fungi and those that are classed as stem borers, whereas the sub- genus *Pegomya* includes species with leaf mining larvae. The genus *Pegomya* is often distinguished from other genera within this family from the distinct yellow colouring of the legs (Ackland *et al*., 2017). Although in some specimens, this yellow colouring can be distinctly darker or lighter, making the trait quite variable in some individuals. It contains some economically important pest species, such as the sugar beet leaf mining complex, but is generally very understudied and relatively little is known about its members.

2.3.3 Sugar Beet Leaf Miner Complex

The complex has undergone many species name changes as well as changes to the genera they have been classed in, so at first glance the literature on what I call the sugar beet leaf miners appears very confused. Sugar beet leaf miner is also referred to by a number of different common names. Cameron (1914) refers to the species *P.hyoscyami* as the *Belladonna* leaf miner, based from his success in rearing the species on this species, i.e. the deadly nightshade plant. Yasumatsu and Sasagawa (1953) refer to *P.hyoscyami* as the spinach leaf miner, which is a more commonly used identifier. A checklist of Anthomyiidae in Saudi Arabia (Al Yousef *et al*., 2015) states the common name of *P.betae* as being the beet leaf miner, also commonly used. Another species. *P.mixta*, is also sometimes referred to as the sugar beet leaf miner by authors based in Egypt, but this appears now to be a synonym for *P.cunicularia* (see UKFlyMines – *Pegomya cunicularia* (Pitkin *et al*., 2018)). It is now acknowledged that Michelson's review of the complex published in 1980 is the most accurate, based on the morphological descriptions provided. His description of the complex includes *Pegomya hyoscyami* and *P.betae*, possibly the most common species recognised as leaf miner of sugar beet, as well as *P.cunicularia* and *P.exilis*. Along with the confusion over taxonomy, there is a severe lack of distribution data and basic ecological information on these flies. At the start of my PhD I requested the records of the four species named above from the relatively newly formed Anthomyiidae Recording Scheme and these came to a total of 36 records; 1 record of *P.exilis*, 4 of *P.hyoscyami*, 13 of *P.cunicularia* and 18 records of *P.betae*.

Table 2.2 Summary of the taxonomic standing of the 'sugar beet leaf miners' by various different authors through time, and how each species has been named.

Species Name	Common Name	Author	Description
P.hyoscyami	*Belladonna* leaf miner	Cameron (1914)	Successfully reared on *Belladonna* host plant.
	Mangold fly	Cameron (1916)	Leaf miner of mangolds.
	Spinach leaf miner	Yasumatsu & Sasagawa (1953), Guyer et al. (1957), Boetel (2005), El-Serwy (2008b)	Leaf miner of spinach.
	Beet fly	Sabbour & Soleiman (2019)	Leaf miner of beet.
P.betae	Beet leaf miner	El-Serwy (2008b), Al Yousef *et al*., 2015	Leaf miner of beet.
	Sugar beet leaf miner	Boetel (2005), Stevens (2014, 2015), White (2016)	Leaf miner of sugar beet.

Historically, the sugar beet leaf miners have only ever been sporadic pests that attack sugar beet and related *Beta vulgaris* sub-species. Large blotch like mines can be seen on host plants where the larvae of this complex have fed (White, 2016). This may result in major canopy losses in sugar beet growing areas where large leaf miner populations are found, leading to the restoration of canopy and potential negative impacts on yield (Stevens, 2014). Although usually sporadic pests, the sugar beet leaf miners experienced a sudden surge in numbers in 2015 and 2016. Increasingly large numbers of the larvae were seen over a wider range of the sugar beet growing areas. It was particularly

problematic for the sugar beet industry during this time due to limited control measures of a pest that is usually not found in such high numbers (Stevens, 2015).

2.3.4 Pegomya hyoscyami and *Pegomya betae*

It is only the more recent publications (e.g. The checklists and Michelson's 1980 review of the complex) that appear to take into account the more reliable and commonly used characteristics for identifying flies. Separation of the two main species, *P.hyoscyami* and *P.betae*, appears to have previously been based on colouration of the antennal segments, with *P.betae* being a "darker form" of *P.hyoscyami*. *P.betae* has been described as having wholly black antennal segments, whereas the second antennal segment of *P.hyoscyami* is described as lighter in colour (reddish) (Cameron, 1914). Separation of the two species has also been entirely based on the fact that one is thought to mine one particular plant family, and one another. Separation of both species by distribution ranges and associated host plant families has been used by past authors, with *P.betae* distributed in the North and attacking members of the Chenopodiaceae, and *P.hyoscyami* distributed in the South and attacking members of the Solanaceae (Michelson, 1980). Separation of the two species has also been based on associations with single host plants, with *P.hyoscyami* sometimes referred to as the spinach leaf miner (Yasumatsu and Sasagawa, 1953; Guyer *et al*., 1957; Boetel, 2005) and *P.betae* as the sugar beet leaf miner (Boetel, 2005).

2.3.5 Pegomya mixta

Steyskal (1970) refers to *P.mixta* as the proper name of the leaf mining fly that attack sugar beets in Egypt and refers to *P.mixta* as a separate, but closely related species to *P.hyoscyami* and *P.betae*, which he states as only have a distribution range north of the Mediterranean. This opinion was formed from the fact that specimens were examined that didn't conform to pervious records of the "dark forms" of *P.hyoscyami* and *P.betae*. Since this paper was published, other more recent studies on the species still refer to *P.mixta* as the (sugar) beet leaf miner (El-Serwy, 2008a; 2008b; El-Rawy & Shalaby, 2011; Abo El-Ftooh *et al*., 2012; Sabbour *et al.*, 2020). El-Serwy (2008b) refers to *P.mixta* as the sugar beet leaf miner, *P.betae* as the beet leaf miner, and *P.hyoscyami* as the spinach leaf miner. In this publication both the beet leaf miner and spinach leaf miner are said to now be considered as a single species (*P.hyoscyami*) and that *P.mixta* is one of eight *P.hyoscyami* species complexes. However, the same author (El-Serwy, 2008a), refers to *P.mixta* as the beet leaf miner. Interestingly, a study on the use of chitosan and nanchitosan in the control of beet fly by Sabbour and Soleiman (2019) refers to the species as *P.hyoscyami*, but in 2020 the same authors refer to the sugar beet fly as *P.mixta* (Sabbour *et al*., 2020). There are fewer accounts of this species being mentioned outside of Egypt. Suwa (1974) classifies *P.mixta* as one of the three distinctive species found in Japan, alongside *P.hyoscyami* and *P.betae*. This appears to have been rectified as in a recent publication by Suwa (2013), in which *P.mixta* is quite clearly a synonym for *P.cunicularia*. Steyskal (1970) appears to have judged the identification of specimens on colouration, a highly variable trait in many Diptera, and in insects in general. This appears to be a common occurrence in past identifications and may have led to inaccurate

identifications of fly specimens. Exactly how these species are being defined is also unclear and the Latin and common names are potentially being used interchangeably.

2.3.6 Pegomya cunicularia and *Pegomya exilis*

Amongst all the species referred to as the sugar beet leaf miners, the least information is available on *P.cunicularia* and *P.exilis*. Michelson (1980) refers to the two as being part of the sugar beet leaf miner complex, and this is one of the few accounts of both species in the literature. In the checklist of the Anthomyiidae of Korea II by Kwon & Suh (1982), *P.cunicularia* is referred to as part of a *Pegomya* complex. It is recorded in a recent publication by Suwa (2013) as being found in Japan, Korea, China, Europe and Northern Africa. Indeed, a survey of Dokdo Island, South Korea (Park *et al.*, 2017), recorded the species there. More recently, a publication by Edmunds (2022) states that *P.cunicularia* is generally regarded as an uncommon coastal species in the UK. The specimen in question was reared from perpetual spinach. A publication on new species recorded from the Czech Republic and Slovakia (Komzáková & Michelson, 2015) references *P.exilis* amongst the species collected from a malaise trap in the Bavarian Forest in 2003, but as this specimen was caught in the trap there were no host plant data. The paper does reference other countries in which *P.exilis* is known from though, including; Belgium, Denmark, Finland, France, Germany, Great Britain, Greece, Italy, Norway, Spain, Sweden, Switzerland, Near East, East Palearctic and Nearctic regions.

2.3.7 Life History

Life history traits of the sugar beet leaf miner complex are fairly uniform amongst authors. Species within the sugar beet leaf mining complex are multivoltine and it is generally thought that there are up to three generations per year for these *Pegomya* species. Much of the life history data is patchy or severely out of date, and methods for studying life history data have changed and become more accurate. Hence, much work is needed to develop a full understanding of the life history of these species.

Adult flies emerge from overwintering pupae between April and May, during this time they will mate and lay the next generation of leaf miner on the underside of the host plants leaves. These larvae hatch within around five days of being laid and burrow into the leaf to feed on the leaf tissue. To complete larval growth takes around two weeks, at the end of which the larvae are ready to pupate. This generation then emerge as adults around June/July after pupating in the soil, completing the same life cycle as the previous generation. A third generation then emerges again in August (White, 2016). Yasumatsu and Sasagawa (1953) observed that the third generation of *P.hyoscyami* (referred to by the authors as the spinach leaf miner) emerged in October/November. This is the only reference to a generation emerging at this time of year within the literature. It is possible that the generations are highly responsive to their environment and therefore are able to stagger emergence times, resulting in a later emerging generation from earlier in the year, or produce an extra generation cycle when the elements are favourable.

2.3.8 Host Plants

There are a number of common host plants that appear frequently amongst the literature. The two most frequently referred to plant families are the Chenopodiaceae and the Solanaceae. Yasumatsu and Sasagawa (1953) comment that the spinach leaf miner, *P.hyoscyami*, has been recorded as mining members of the Polygonaceae, Compositae, Caryopyllaceae and Rosaceae. Suwa (1974) and Kwon and Suh (1982) list some of the most commonly featured host plants of the species complex, that are often referred to by many of the authors listed in this chapter. These being; *Atriplex subcordata, Beta vulgaris, Chenopodium album, C.ficifolium, C.glaucum* and *Spinacia oleracea*. UK fly mines (Pitkin *et al*., 2018a; 2018b; 2019a; 2019b) contains detailed host plant information on two of the *P.hyoscyami* complex (as defined by Michelson, 1980) species (Table 2.2). Cases of rearing the species *P.hyoscyami* on deadly nightshade, *Atropa belladonna*, have also been noted. Cameron (1914) successfully reared the leaf miner on this host plant, however, it is worth noting that this was within a lab environment and not actually observed in the field. Cameron (1914) also notes that in previous studies (conducted by another author) it was observed that the larvae of *P.hyoscyami* may complete their development on other substances, such as decaying leaves and animal manure, in the absence of a suitable host plant.

Table 2.3 Summary table of reported host plants (UK only) of *Pegomya* species (*P.hyoscyami* complex as defined by Michelson, 1980) reported on the UK Fly Mines website (Pitkin *et al*., 2018a; 2018b; 2019a; 2019b).

Pegomya Species	Plant Family	Host Plant Species
Pegomya hyoscyami	Carophyllaceae	*Silene maritima, S.vulgaris*
	Chenopodiaceae	*Atriplex glabriuscula, Beta vulgaris, Chenopodium album, C.polyspermum, C.urbicum, Spinacea oleracea*
	Solanaceae	*Hyoscyamus niger, Solanum dulcamara*
Pegomya betae	Chenopodiaceae	*Atriplex* sp*., Beta vulgaris*
	Polygonaceae	*Polygonum* sp.
Pegomya cunicularia	Unknown	Unknown
Pegomya exilis	Unknown	Unknown

2.3.9 Other leaf mining Pegomya

Of the 48 known species of UK *Pegomya*, 19 are known for their leaf mining habit (Table 2.4 - Ackland *et al*., 2017). It is generally understood amongst entomologists that study Anthomyiidae, and in particular *Pegomya*, that rearing through any juvenile specimens to adulthood is a necessity in the identification of these species as it is nearly impossible to identify species based on differences in their mines, particularly blotch mines (Edmunds, R., personal communications, 2021)

Table 2.4 Known leaf mining flies within the genus *Pegomya* (Anthomyiidae) and their known host plant groups (adapted from Ackland *et al*., 2017).

	Species	Host Plant
1	*P. betae*	*Beta*
2	*P. bicolor*	Polygonaceae
3	*P. conformis*	*Chenopodium*
4	*P. cunicularia*	*Beta* (often coastal)
5	*P. depressiventris*	*Solidago*
6	*P. dulcamarae*	*Solanum dulcamarae*
7	*P. exilis*	*Beta*
8	*P. flavifrons*	*Carophyllaceae*
9	*P. haemorrhoum*	*Rumex*
10	*P. holosteae*	*Stellaria*
11	*P. hyoscyami*	*Beta*
12	*P. interrupterlla*	*Chenopodium*
13	*P. laticornis*	*Arctium*
14	*P. nigrisquama*	*Solidago, Aster*
15	*P. seitenstettensis*	*Oxalis*
16	*P. setaria*	*Polygonum*
17	*P. solennis*	*Rumex*
18	*P. steini*	Thistles
19	*P. vanduzeei (P. versicolor)*	*Rumex*

2.4 Other leaf miners of Sugar Beet

2.4.1 Amauromyza flavifrons

Amauromyza flavifrons is a leaf mining fly found within the family Agromyzidae, a group well known for many of their distinctive leaf mining species. Some authors have classed this species as a species that can mine sugar beet. *A.flavifrons* is a leaf miner that attacks host plants found primarily within the Caryophyllaceae (eg. Carnations/white campion)

but has also been reared from plants within the genus *Beta*, mainly sugar beet, and spinach, *Spinacia oleracea* (Chenopodiaceae) (Scheffer, 1999a). However, *A.flavifrons* has rarely been seen in the field attacking these hosts that occur outside their usual host range (Scheffer, 1999a, 1999b). This appears to be due to some aspects of host avoidance and acceptance within populations of *A.flavifrons.* Flies that were reared near populations of sugar beet actively avoid using this host and populations reared away from sugar beet accepted sugar beet as a 'novel' host plant. This is because mortality rate of *A.flavifrons* larvae and pupae is much higher when reared on these unusual hosts compared to their accepted hosts within the Caryophyllaceae (Uesugi, 2008). It seems that this species is often mistaken with leaf mining *Pegomya* species. *Pegomya* blotch mines cannot be distinguished between, and although many leaf mining Agromyzidae have distinctly recognizable mines (often deduced by a number of physiological traits and patterns), *A.flavifrons* also have a fairly indistinguishable mine that can often be compared to many of the *Pegomya* mines. There are some characteristics that can separate *A.flavifrons* mines from *Pegomya* ones, with the most recognisable one being the obvious difference between the larvae of the species. Agromyzidae leaf mining larvae are notably smaller and different morphologically to the leaf mining larvae of the Anthomyiidae. Other characteristics can be identified from the leaf mines created by the larvae and include; a) presence of egg cases – *Pegomya* larvae are quite mobile so egg cases may not always be present at the start of the mine, b) the mines of *Pegomya* may not be full depth and the dispersion of frass within *Pegomya* mines may not follow a distinct pattern unlike the larvae of *Amauromyza* (Warrington, B., personal communications, 2022). Despite past literature stating that *A.flavifrons* has been observed mining sugar beet, the Agromyzidae Recording Scheme, which has >700

records of this species, have no records of this species mining *Beta* spp. (Warrington, B., personal communications, 2022).

2.4.2 Psilopa leucostoma

Psilopa leucostoma, a fly within the family Ephydridae (the shore flies) has occasionally been noted, with records of its larvae mining host plants associated with those of the *Pegomya* sugar beet leaf mining complex. The shore flies, or brine flies, are widely distributed across the globe, with around 1700 species spanning 115 genera making them one of the largest of the acalypterate fly families. Within the shore flies there are four genera which have leaf mining larvae: *Hydrellia, Psilopa, Clanoneurum* and *Lemnaphila*. In Foote's (1995) review of shore flies, *Psilopa* are referred to as mining plants within the Chenopodiaceae. The first record of this fly as a pest of sugar beet was made by Landis *et al.* (1967) who reported that the first observation of *P.leucostoma* on sugar beet was made in Umapine, Oregon, in 1962. The mines differ greatly from those made by *Pegomya*, with records stating that the mines are serpentine, much like those made by Agromyzidae leaf miners. Tamaki (1975) suggests that this species has been prevalent in these areas for some time, and that is likely that the serpentine mines of *P.leucostoma* had been mistaken for those made by the first generation of the sugar beet leaf miner (referred to here as *P.betae*). However, the mines of the leaf mining *Pegomya* associated with *Beta* are generally thought to be 'blotch-like' (White, 2016), so it seems unlikely that they could have been mistaken as a species with serpentine leaf miners. During 1965, *P.leucostoma* was observed as being distributed across not only the sugar beet growing areas of Oregon, but also across Washington, Idaho and parts of Northern Utah and it

was suggested that this species of fly had become adapted to sugar beets grown in irrigated salt/alkaline soils (Landis *et al*., 1967). Tamaki *et al*. (1975) continue referring to this species on sugar beet, stating that it has been prevalent, being sparsely but widely distributed, in the United States for many years. It was thought that saltbush (*Atriplex patula* var. *hastata*) was acting as a source, or reservoir, for the late emerging *P.leucostoma* which emerge later in the season, around August, allowing large infestations of this fly to attack sugar beet fields. It has also been observed that this species has been reared from *Chenopodium album* (Lambs quarter/Fat Hen) before (Landis *et al*., 1967; Tamaki *et al*., 1975; Foote, 1995), which is a recorded host plant of the *Pegomya* sugar beet leaf mining complex. Another author, von Kramer (1961, 1962) also refers to *P.leucostoma* as mining sugar beet. Landis *et al*. (1967) predicted that this species of fly would become a much more serious pest of sugar beets than the sugar beet leaf miner (named here as *P.hyoscyami*) in future years due to its effects on the plants later in the growing season, however, records of this are practically non-existent.

2.5 Hymenoptera

Cameron (1914) recorded three species of hymenopteran parasitoid that emerged when attempting to culture *P.hyoscyami.* These three species were braconids wasps, with two of these species being identified as part of the genus *Opius*. One of these species was identified to species level as *O.nitidulator*. The third parasitoid was identified by Cameron as being a hyperparasitoid of presumably one of the two *Opius* species he identified, a proctotrypid parasitoid species. It is to be noted that the culture used during this research was very small, with a total number of 8 larvae.

Other accounts of parasitoids of the genus *Pegomya* are far and few between, with a few general checklists of parasitoids of the Anthomyiidae family and little information on parasitoids attacking the species within this complex. Žikić *et al*. (2013) compiled a list of parasitoids within the subfamily Opiinae, a group of solitary koinobiont parasitoids (approximately 1-1.5mm in size) spanning over 33 genera and 1863 species worldwide. This subfamily is known to attack members of the Agromyzidae, Anthomyiidae and the larvae of the Tephritidae. Two species within this list were identified as parasitoids of *Pegomya* species. *Apodesmia irregualris* is a known parasitoid of *P.solennis* and *Opius pallipes* is a known parasitoid of *P.solennis* and *P.bicolor*, as well as another anthomyiid species, *Delia echinata*. Another genus within the Opiinae, *Diachasma*, has been recorded as having parasitoids associated with Anthomyiidae hosts. Particularly *Diachasma fulgidum*, a parasitoid distributed across the West Palearctic, which has been recorded as parasitizing leaf-mining flies within the genus *Pegomya*, along with two other species within the same genus, very similar in morphology to *D.fulgidum*. (Shirley *et al*., 2014). Presumably one of the other two species, not named by Shirley *et al*. (2014), is *D.hispanicum* which Jimenez *et al.* (1992) records as parasitizing *P.cunicularia* on *Beta vulgaris.*

Marchiori *et al*. (2013) surveyed species of parasitoids from economically important groups of flies and identified *Pachycrepoideus vindemmiae* (Hymenoptera: Pteromalidae) as a solitary parasitoid of a number of fly species within the Anthomyiidae and other fly families. This species falls within the family Pteromalidae, a large and highly diverse chalcid family containing around 3000 species (Marchiori *et al*., 2013). The Natural

History Museum's Universal Chalcidoidea Database (Noyes, 2019) only lists five Anthomyiidae species as primary hosts (*Delia antiqua, Hylemya species* and *Phorbia brassicae*) of *P.vindemmiae* but it is likely that there are still unrecorded host species for this genus of parasitoids. Perez – Hinarejos and Beia (2008) record *Spalangia cameroni* as attacking a tephritid host, *Ceratitis capitata*. This species is also known to attack other dipteran hosts, including those from the Anthomyiidae. Both *P.vindemmiae* and *S.cameroni* have been used as biological control agents of the house fly, *Musca domestica* and the stable fly, *Stomoxys calcitrans* (Perez - Hinarejos and Beitia, 2008; Marchiori *et al*., 2013).

Table 2.5 Parasitoids of *Pegomya* species (*P.hyoscyami* complex as defined by Michelson, 1980) reported on the UK Fly Mines website (Pitkin *et al*., 2018a; 2018b; 2019a; 2019b).

Pegomya Species	Parasitoid SuperFamily	Parasitoid Species
Pegomya hyoscyami	Chalcidoidea	*Cyrtogaster clavicornis, C.vulgaris, Trichomalopsis hemiptera, T.evanescens, T.minutum, T.semblidis*
	Ichneumonoidea	*Dacnusa pubescens, Phaenocarpa ruficeps, Microgaster polita, Apodesmia rufipes, Biosteres carbonarius, B.spinaciae, B.sylvaticus, B.wesmaelii, Diachasma fulgidum, Eurytenes silenis, Phaedrotoma munda, P.variegata, Utetes fulvicollis, U.testaceus, Rhysipolis meditator, Phygadeuon detestator, P.pegomyiae*
Pegomya betae	Chalcidoidea	*Trichomalopsis hemiptera, T.evanescens*
	Ichneumonoidea	*Biosteres spinaciae, B.wesmaelii, Phaedrotoma munda, Utetes fulvicollis, Phygadeuon elegans, P.pegomyiae, P.rotundipennis, P.trichops*
Pegomya cunicularia	Unknown	Unknown
Pegomya exilis	Unknown	Unknown

2.6 Coleoptera

Parasitoids also exist within other insect orders, such as the Coleoptera. Within the Staphylinidae family (the rove beetles), species within the genus *Aleochara* have been recorded as parasitoids of cyclorrapheous flies. Species within this genus are known to be exclusively pupal parasitoids, burrowing into the pupae of their host species and consuming the developing larvae inside. Dependent upon the species, some species within the *Aleochara* will leave the pupae after developing through a set number of instars whilst feeding on their hosts and some will pupate themselves within the remains of the host pupae after consumption (Maus *et al*., 1998). There are five species in this review by Maus *et al*. (1998) known to attack one of the *Pegomya* leaf miners. *A.curtula, A. laevigata, A. intricate, A.bilineata* and *A.bipustulata* have been recorded as parasitoids of the pupae of *Pegomya betae*. This review of parasitoids species is quite extensive, and species within the genus *Delia* have more recorded numbers of parasitoids than other Anthomyiidae. It is possible that with the taxonomic confusion of the *Pegomya* leaf mining complex that there are more species/differences within the *Aleochara* that are parasitoids of this group.

2.7 *Conclusions and Future Directions for Research*

Even though the sugar beet leaf miners compromise species which have been referred to as pests within the sugar beet and spinach growing industries for almost a century, relatively little is actually known about their biology and ecology. There are several major gaps in the known host range of these species and too few records on their distribution, which is surprising when considering that these species are classed as pests. The

understanding of the sugar beet leaf miner behaviours and the factors that influence their population growth and dynamics is also quite poor. With regards to known parasitoid species that attack them, considering that leaf miner parasitoids are amongst the most diverse and speciose of all parasitoid communities, it is also surprising there are so many gaps in the historic records.

Overall, in this review I have synthesised a diverse literature on the sugar beet leaf miners. While several of the sources can be viewed as being reliable, there are equally as many that are likely not. The current knowledge of the taxonomy of sugar beet leaf miners seems to be widely accepted amongst many authors, with some remaining confusion as to what actually defines a sugar beet leaf miner. Though the use of a plethora of common names is widely acknowledged to be interchangeably used amongst various species, defining species based on host plant association, like with the sugar beet leaf miners, can be problematic and potentially challenging in the future management of this pest if this results in taxonomic error. The genetic characterisation of the sugar beet leaf miners in the next chapter should hopefully contribute towards solving some of the remaining confusion surrounding this.

2.8 References

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CHAPTER 3

Genetic characterisation of dipteran leaf miners of sugar beet and related taxa

Leaf mines on sea beet – Havergate Island, Suffolk, 2020. (Photos by Siobhan Hillman)

3.1 Contributions

Collection of leaf samples was done through a combination of fieldwork conducted by myself with some help from former John Innes Entomology Team member Susannah Gill who helped in 2019 and Dr. Kris Sales in 2020, the BBRO network and by the public. Dr. Tony Irwin identified the adult *Pegomya* specimens in this chapter and I conducted the molecular work myself.

3.2 Abstract

Sugar beet is an economically important crop, providing a substantial proportion of the sugar sold in Britain. Leaf mining larvae of flies (*Pegomya* spp) have historically been a minor pest known to infest the sugar beet crop. In some recent years, however, sugar beet growers have witnessed increasingly severe outbreaks of leaf mining flies, with few control options available and new restrictions on neonicotinoid pesticides. A significant barrier to developing sustainable controls on leaf miners of sugar beet is an almost complete lack of data on the taxonomy, ecology and life history of this species or group of species. Here I characterise leaf miners of sugar beet at a molecular level, using samples obtained from across the UK and abroad, and from a range of host plants including sugar beet and its close relatives. Sequence analysis of the mitochondrial cytochrome oxidase I gene strongly indicates that there are two divergent groups of leaf miner. I found a clear distinction between the host plants associated with each genetic group, with one found predominantly on sugar beet, and the other on spinach and swiss chard. I also found a significant difference in the geographical distribution of the two groups, but this may also be attributable to variation in host plant sampling. The genetically distinct groups of *Pegomya* may represent cryptic, separate species, although further work is required to confirm this. I discuss my findings in the context of sustainable pest control.

3.3 Introduction

Insect pests cause large amounts of economic damage to the agricultural industry every year (Mazzi & Dorn, 2012; Oliveira *et al*., 2014). Herbivorous insects cause damage through their feeding behaviour (Rao *et al*., 2000; Walling, 2008, Oliveira *et al.*, 2014), and some species are vectors of various plant pathogens (Ng & Perry, 2004; Safari *et al*., 2019). Chemical control has been the dominant means for regulating insect pest outbreaks within agriculture (Hedlund *et al*., 2020), but with national and global bans on pesticides such as noenicitinoids (Dewar, 2017; Jactel *et al*., 2019; Mc Namara *et al*., 2020), and with growing resistance of some pests to different chemical controls (Forgash, 1984; Weddle *et al*., 2009; Midingoyi *et al*., 2018), the agricultural industry is looking to alternative and sustainable methods. For example, Integrated Pest Management Strategies (IPM) are alternatively implemented to fight outbreaks of pests in a more sustainable way (Furlan & Kreutzweiser, 2015; Stenberg, 2017; Plumecocq *et al*., 2018; Dara, 2019; Rose *et al*., 2019). Ecological factors such as climate change, changes in agricultural practices and the interactions of pest species with host plants, can also influence the migration and growth of insect pest populations (Ezcurra *et al*., 1978; Porter *et al*., 1991; Mazzi & Dorn, 2012; Corrêa *et al*., 2019; Grünig *et al.*, 2020). In order to implement more economically and ecologically sustainable management practices within agriculture a deeper understanding of the biology and ecology of the pest species themselves is required.

Before even considering control methods for insect pests, we must be able to identify them from one another, and from other, beneficial species. Identification of pest insects

is often heavily dependent on traditional taxonomic methods, which aim to categorise taxa using distinct external morphological characteristics (Cano *et al.*, 2004; Ko *et al.*, 2013) or internal anatomical features (Uceli *et al.*, 2011; Virgilio *et al.*, 2012). Taxonomists may also incorporate behavioural or ecological information into what defines a species (de Queiroz, 1992; Ward *et al.*, 2009). There are, however, some limitations with identifying specimens through traditional taxonomic methods. Over recent decades there has been a substantial decline in the number of trained taxonomists (new and established) creating a generational knowledge gap (Thompson & Newmaster, 2014; Engel *et al.*, 2021), along with incomplete or inadequately updated taxonomic material and keys that do not necessarily encompass all the aspects required for species identification (Barratt *et al.*, 2003; Williams *et al.*, 2012; Fremdt *et al.*, 2012). In addition, the identification of some groups based on morphology alone can be particularly difficult. The identifiable characteristics can be ambiguous, highly variable or insufficient for complete species identification (Huang *et al.*, 2007; Ferri *et al.*, 2009) and may even be impossible in some cases (Floyd *et al.*, 2002; Huang *et al.*, 2007; Hajibabaei *et al.*, 2011; Kesanakurti *et al.*, 2011). The cost, both monetary and in time, to train new taxonomists (Tautz *et al.*, 2003), as well as reductions in funding mean that traditional taxonomy is struggling to maintain its pace with more modern-day approaches (Godfray, 2002; Guerra-García *et al.*, 2008). This is problematic as species identification plays a fundamental role in the research of many other wider biological subject areas (Böttger-Schnack & Machida, 2011; Lehmann *et al.*, 2017).

The evolution of molecular methods has greatly added to current biological knowledge, especially with regards to species characterisation and identification (Frézal & Leblois,

2008; Aslam *et al*., 2017). The sequencing and analysis of genetic data has proven useful in uncovering cryptic diversity within and amongst species (Hebert *et al*., 2004; Smith *et al*., 2006; Havermans *et al*., 2011; Tyagi *et al*., 2017) and in uncovering connections between species life stages that may have been previously unknown or impossible to detect (Floyd *et al*., 2002; Huang *et al*., 2007; Ferri *et al*., 2009; Rivera & Currie, 2009; Ekrem *et al*., 2010; Hajibabaei *et al*., 2011). Genetic data allow relationships among individuals to be characterised even where there are no clear morphological characteristics and avoids many of the biases associated with morphological-based taxonomy (Lefébure *et al*., 2006). Such genetic characterisations provide the first step towards understanding the ecological drivers of geographical distribution, ecology and genetic structure of species (e.g., González-Wevar *et al*., 2011) such as insect pests. Genetic characterisation using a standardised set of primers (Folmer *et al*., 1994) and depositing reference sequences on publicly accessible online platforms such as GenBank (Benson *et al*., 2013), provides a foundation for broader biological applications, such as DNA barcoding, which has substantial potential for biosecurity (Maralit *et al*., 2013; Shokralla *et al*., 2015; Siozios *et al*., 2020).

Sugar Beet crops are periodically infested with leaf mining flies of the family Anthomyiidae (*Pegomya* spp), known as mangold fly or the sugar beet leaf miners (Michelson, 1980). Previously regarded as a minor pest of sugar beet, outbreaks in recent years have become increasingly severe, and there is increasing concern about loss of sugar beet yield due to leaf miner damage (Stevens, 2015). Spinach, another economically important crop, which is closely related to sugar beet, is also infested with leaf miner (Michelson, 1980), but whether different crops are infested with the same or

different species is not yet known. Finally, until recently leaf miner outbreaks have been controlled using seed treatment with neonicotinoid insecticides, and with national and international moratoria on these chemicals, new, sustainable control measures are urgently needed.

Formerly thought to be one species, the taxonomy of the sugar beet leaf miners is in dispute. There have been many alterations in species designation, with the most recent description from morphological data suggesting that four species make up the *Pegomya hyoscyami* complex (Michelson 1980). However, these 4 species are morphologically very similar, there is a high chance for inadvertent error in the identification of the adults through traditional taxonomic methods. Little is also known about the biology and ecology of this species complex. In addition, the true extent of the geographical distribution and host plant preferences of the group are largely unknown. In an agricultural context, it is important to know how many species make up this complex, and whether they differ in their level of damage to sugar beet crops. Rapid identification of the larvae, rather than the adults which are not often encountered by growers in the field, would also be very useful, but is not possible using morphology. The use of genetic tools to characterise this complex therefore has considerable potential for generating scientific and economic impact.

Here, I aim to characterise on a genetic level the sugar beet leaf miner complex, incorporating host plant associations and geographical distribution. I sequenced specimens collected from known host plants that are associated with the species complex, and used phylogenetic analyses to identify broad-scale groups. I then quantified

how genetic groups vary among different host plants, and across geographical regions. Through genetic characterisation I provide a framework for investigating and uncovering any differences in the species biology and ecology of the sugar beet leaf miners. Sequencing specimens of these leaf miners lays the foundation for the effective and rapid identification of leaf miner samples through DNA barcoding. A greater knowledge of the species complex encourages more ecologically sustainable alternatives of control in the field, optimising the management outcome and potentially reducing loss for growers.

3.4 Methods

3.4.1 Sample Collection and Identification

Samples of leaf miner larvae were collected between March and October 2017-2021 (Table 3.1). Sampling was restricted to known and potential host plants of the sugar beet leaf mining complex, which included three cultivars of *Beta vulgaris* subsp. *vulgaris* (sugar beet, Swiss chard and garden beets), a wild relative *B.vulgaris* subsp. *maritima* (sea beet) and *Spinacea oleracea* (spinach), all from the Amaranthaceae family, with some samples from *Chenopodium* spp. Samples obtained from sugar beet were collected through our connection with the British Beet Research Organisation (BBRO) from growers, British Sugar Area Managers and BBRO employees as outbreaks occurred. Some larval and pupal samples from sugar beet were also procured through BBRO connections with Klein Wanzlebener Saatzucht (KWS) in Germany and from the Institute for Research in Sugar Beet (IRS) in the Netherlands. To obtain samples from sea beet, sampling of actively mined leaves and empty leaf mines was carried out across East Anglia, Lincolnshire,

Sussex and Wales. Leaf miner samples were also acquired from gardens and allotments around the UK from advertising the project through a blog, (https://sugarbeetleafminers.wordpress.com/), which I targeted at gardening enthusiasts and advertised on social media. For this, I specifically targeted samples from Swiss chard, beetroot and spinach.

For all samples, leaf mines of *Pegomya* were distinguished as accurately as possible from other leaf mining species, based on descriptions of mine shape (Ellis, 2020). Where possible, leaves with live larvae were reared through to adulthood and identified using a dichotomous key to the identification of British Anthomyiidae; Part 1 (Text) and Part 2 (Figures) (Ackland *et al.*, 2017) which was made available to participants who took part in the Dipterists Forum Anthomyiidae ID course at the FSC Preston Montford in February 2018. All morphological identification of adult field specimens was conducted by Dr. Tony Irwin. Larvae, pupae and empty leaf mines were kept in tubes of 95% ethanol where possible, with some samples provided by associates in unknown liquid storage, and were frozen at -82°C. Adult flies were generally kept as dry specimens at room temperature.

Table 3.1 Location, host plant and collection dates of 135 sequenced leaf miner specimens. Locations are reported at broad geographical scales (UK: county/region, non-UK: country) to protect grower and individual anonymity.

Host Plant	Area	Month Collected	Year Collected	Number of Samples
Beetroot	Norfolk	May	2018	3
		June	2018	2
	Cambridgeshire	July	2020	2
	Leicestershire	July	2020	1
	Dumfries	July	2020	2
	Gloucestershire	July	2020	1
	Warwickshire	July	2020	1
	London	September	2020	2
Chenopodium sp.	Norfolk	July	2018	2
Fat Hen	Norfolk	August	2018	3
Spinach	Norfolk	June	2019	2
	Cambridgeshire	July	2020	3
	Oxfordshire	July	2020	3
	London	September	2020	1
Swiss Chard	Norfolk	June	2018	1
	Kent	July	2020	4
	Oxfordshire	July	2020	1
	Warwickshire	July	2020	4
	London	September	2020	1
Sea Beet	Anglesey	June	2018	3
	Suffolk	July	2018	2
		July	2019	4
		September	2019	7
		August	2020	5
	Lincolnshire	July	2020	1
	Norfolk	July	2020	17
	Sussex	September	2020	17

Sugar Beet	Nottinghamshire	October	2017	1
	The Netherlands	July	2018	6
	Germany	May	2018	2
	Norfolk	July	2019	4
		July	2020	2
		October	2020	6
	Lincolnshire	June	2020	2
		July	2020	3
		August	2020	5
		September	2020	3
	Suffolk	July	2020	1
		October	2020	1
	Nottinghamshire	September	2020	1
	Cambridgeshire	October	2020	3

3.4.2 Molecular Methods

DNA was extracted from specimens using a modified salt extraction technique based on Richardson *et al.* (2001). When samples were empty leaf mines, larvae or pupae, whole specimens were used for extraction. With adult specimens the thorax was initially used, and extractions were later refined to extracting DNA from a single leg, only extracting from the thorax where there were a multitude of adult samples from a single location available. All equipment was UV sterilised in a laminar flow cabinet before use. 2ml Eppendorf's and micropestles were briefly submerged into liquid nitrogen, and adult specimens (as well as some empty leaf mine samples) were then added and homogenised. Tubes were then submerged into liquid nitrogen and homogenised two more times. Any instruments that came into contact when handling the specimens were sterilised using 70% ethanol and flame in order to avoid potential contamination of the DNA between samples. To each specimen 265µl of DigiMix solution (250µl DigiSol + 15µl

Proteinase K) was then added and digested overnight at 55°C, after which 1.5µl RNase was added to each. 300 µl of pre-warmed 4M ammonium acetate was next added to each sample, which was then centrifuged for 20 mins at 13000 rpm. The supernatant was then poured into fresh, UV sterilised 2ml Eppendorf tubes. To separate the DNA from the supernatant, 1ml of ice cold 100% ethanol was added to each sample. Samples were then centrifuged again for 20 mins at 13000 rpm. The supernatant was then carefully poured away, each Eppendorf carefully blotted on tissue paper, leaving a DNA pellet in each tube. To clean the DNA pellet, 500µl of ice cold 70% ethanol was then added to each sample, which was centrifuged again for 20 mins at 13000 rpm. This process was repeated once more. Each sample was then air dried for 1 hour in an incubator at 55°C. Once dry, 50µl of Low TE was added to each sample and incubated at 55°C for one hour. DNA quantity and quality were quantified for each sample using a NANODROP 8000 (Thermo SCIENTIFIC), and high concentration samples were diluted down to 10-50 ng/µl.

I amplified a ~658bp fragment of the mitochondrial cytochrome c oxidase I (COI) gene; a commonly used tool in molecular species identification (Kerr *et al*., 2009; Lohman *et al*., 2009; Robideau *et al.*, 2011; Che *et al*., 2012; Xia *et al*., 2012), using the universal primers LCO1490 (5' GGT CAA ATC ATA AAG ATA TGG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') (Folmer *et al*., 1994). The PCR reaction contained 1µl of DNA template, 0.5µl of each primer (Sigma-Aldrich), 3µl autoclaved H_20 and 5µl PCRBIO Taq Mix Red (PCRBIOSYSTEMS). All PCR reactions were conducted using the DNAEngine TETRAD 2 (MJ Research). The PCR program was as follows; an initial denaturation stage of 95°C for 1 min, followed by 40 cycles of 95°C for 15 secs (denaturation), 64°C for 1 min (annealing), 72°C for 1 min (amplification) and then a final extension at 72°C for 5 mins.

The annealing stage was later changed to a lower temperature of 58°C to work with a new batch of Taq Red used in the master mix. Amplified DNA fragments were run on a 1.5% agarose gel to test whether DNA amplification was accomplished. All products were then purified in preparation for sequencing with the addition of a 10µl clean up reaction mix; 0.1µl Exo I, 0.2µl FastAP (PCRBIOSYSTEMS) and 9.7µl autoclaved H_20, followed by heat treatment of 37°C for 15 mins and then 80°C for 15 mins. 2µl of forward primer was added to the end reaction and sent for sequencing at Eurofin Genomics (Eurofins Scientific, 2018).

3.4.3 Analyses

Chromatograms were visually inspected and failed sequences were removed before alignment was attempted. Successful sequences were each compared to the sequence database available on GenBank (Benson *et al*., 2013), using BLAST (Altschul *et al*., 1990) to confirm broad-scale taxonomic identification of the amplified sequences. *Pegomya* sequences were aligned by eye using AliView v1.26 (Larsson, 2014), and all segregating sites were checked and verified using the accompanying chromatograms. The whole process of sequence alignment was repeated for due diligence and to check the veracity of the alignments. Upon securing the final alignments, sequences were trimmed to a length of <542 bp, with shorter sequences (<542 bp) removed from alignment.

I reconstructed a maximum likelihood tree from the sequence data using the IQ-Tree Web Server (Trifinopoulos *et al*., 2016). I used ModelFinder (Kalyaanamoorthy *et al*., 2017) to select the model of best fit based on Bayesian Information Criterion (Schwarz,

1978) (model of best fit: HKY+F), with 1000 ultrafast bootstrap (Hoang *et al*., 2018) replicates to assess node support. The constructed tree was then edited using figtree (Rambaut, 2010). Branches with bootstrap values of less than 50 were not reported. Pairwise genetic distances between all individuals were calculated in MEGA v7.0.26 (Kumar *et al*., 2016) based on tree reconstruction parameters (Tamura, 1992).

Haplotype networks of all COI sequences were constructed and calculated using the pegas package (Paradis, 2010) in R. Distances between haplotypes were calculated based on the number of differences in nucleotide composition and networks were visualised and coloured based on host plant association. Finally, because I had identified two broad genetic groups in the data (see results), I tested for differences in host plant association and geographic groups between these two groups, using chi-squared tests in RStudio v.1.3.959 (R Core Team (2020).

3.5 Results

135 specimens were successfully sequenced aligned. One of 27 empty leaf mines for which I attempted DNA extraction was also successful and so was also sequenced and included in the final alignment of 135 COI sequences. Seven sequences were confirmed as hymenopteran species from BLAST searches (families Opiinae and Alysiinae, with no species level identification) and removed from the analysis.

Maximum Likelihood (ML) analysis suggested that there were two distinct monophyletic clusters (Figure 3.1; Group A = 99 sequences, Group B = 36 sequences). Both main group

branches had strong bootstrap support (100%) (Figure 3.1). The mean pairwise intragroup distance was 0.001 for group A and 0.002 for Group B, while mean distance between the two groups was (0.014). The mean distance calculated across all sequences was 0.006, with pairwise distances calculated across the unique COI sequences ranging between 0.002 and 0.021 (Figure 3.2). A total of 16 distinct haplotypes were identified from the 135 sequences. The haplotype network (Figure 3.3) corroborates the ML phylogeny, with two distinct haplotype groupings present. Haplotypes in group A (7 haplotypes) formed a predominantly linear pattern, while those in group B (9 haplotypes) formed a star-like pattern.

I observed substantial differences in host plant frequencies between the two groups (Figure 3.4), and this was highly statistically significant (Chi-squared test, $\chi^2 = 88.578$, d.f = 6, $P < 0.001$). Leaf miner sampled from sugar beet and sea beet almost exclusively belonged to group A, while samples from spinach, swiss chard and *Chenopodium* predominantly belonged to group B (Figure 3.3, Figure 3.4). I also observed a significant difference in the frequency of the two genetic groups across counties (Chi-squared test, $\chi^2 = 75.189$ d.f = 15, $P < 0.001$), although this effect may have been driven to a large extent by host plant ID, as sugar beet and sea beet tended to be sampled from different counties to spinach, swiss chard and *Chenopodium* sp. (Table 3.1). Notably, if I consider only samples taken from Norfolk, where samples were obtained from multiple host plants, I still observed a significant difference in the frequency of genetic groups among host plants ($\chi^2 = 28.58$, d.f = 5, $P < 0.001$). Similarly, considering samples only taken from sugar beet sampled across multiple counties and countries, I likewise observed a significant difference in distribution ($\chi^2 = 19.487$, d.f = 6, $P < 0.003$).

Morphological analyses of the adult *Pegomya* specimens were identified as either *P.betae* or *P.cunicularia*. Samples identified as *P.cunicularia* were entirely from UK samples of sea beet and sugar beet, whereas samples identified as *P.betae* were almost exclusively those identified from the Netherlands samples collected from sugar beet. The single specimen I sequenced that was collected and reared through from sugar beet in Nottingham (2017) was identified at *P.betae*. From a site in Suffolk I reared through three adult specimens, two male and one female, all originally identified as *P.cunicularia*. Upon later inspection and comparison to the identifications of female *P.betae* from the Netherlands, the original identification of the female UK sea beet specimen from Suffolk was changed to *P.betae* also. The male specimens remained as *P.cunicularia*. Five identified adults were successfully sequenced and spread between groups A and B, with a mixture of those identified as *P.betae* and *P.cunicularia* in both (Figure 3.1).

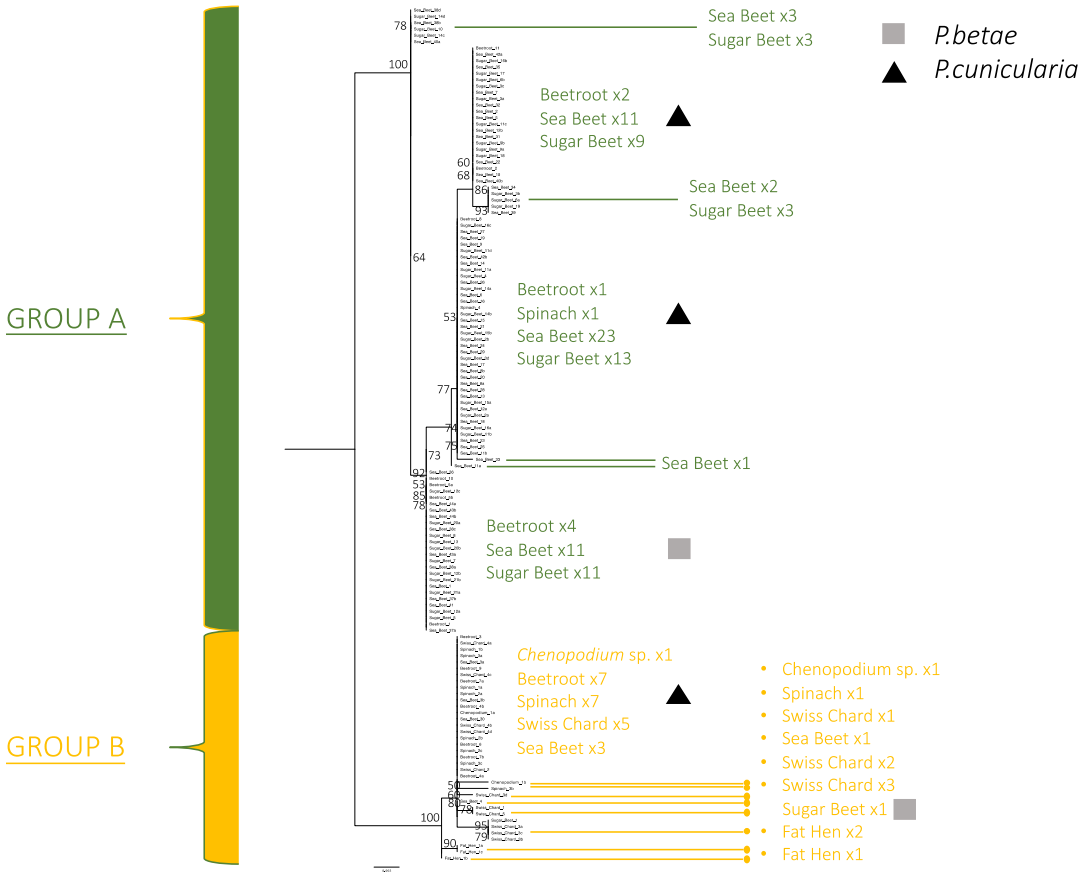

Figure 3.1 Maximum Likelihood tree of 135 Cytochrome C Oxidase Subunit I (COI) sequences containing *Pegomya* sequences from the UK and EU constructed using the model of best fit HKY+F with 1000 ultrafast bootstrap values. Sequences have been divided into two groups, A and B, based on the two distinct branches within the tree. Host plant information for each sequence is coloured based on sequencing grouping. Branches with a grey square includes the sequence from an adult specimen identified as *P.betae*, those with a black triangle includes a sequence from an adult specimen identified as *P.cunicularia*.

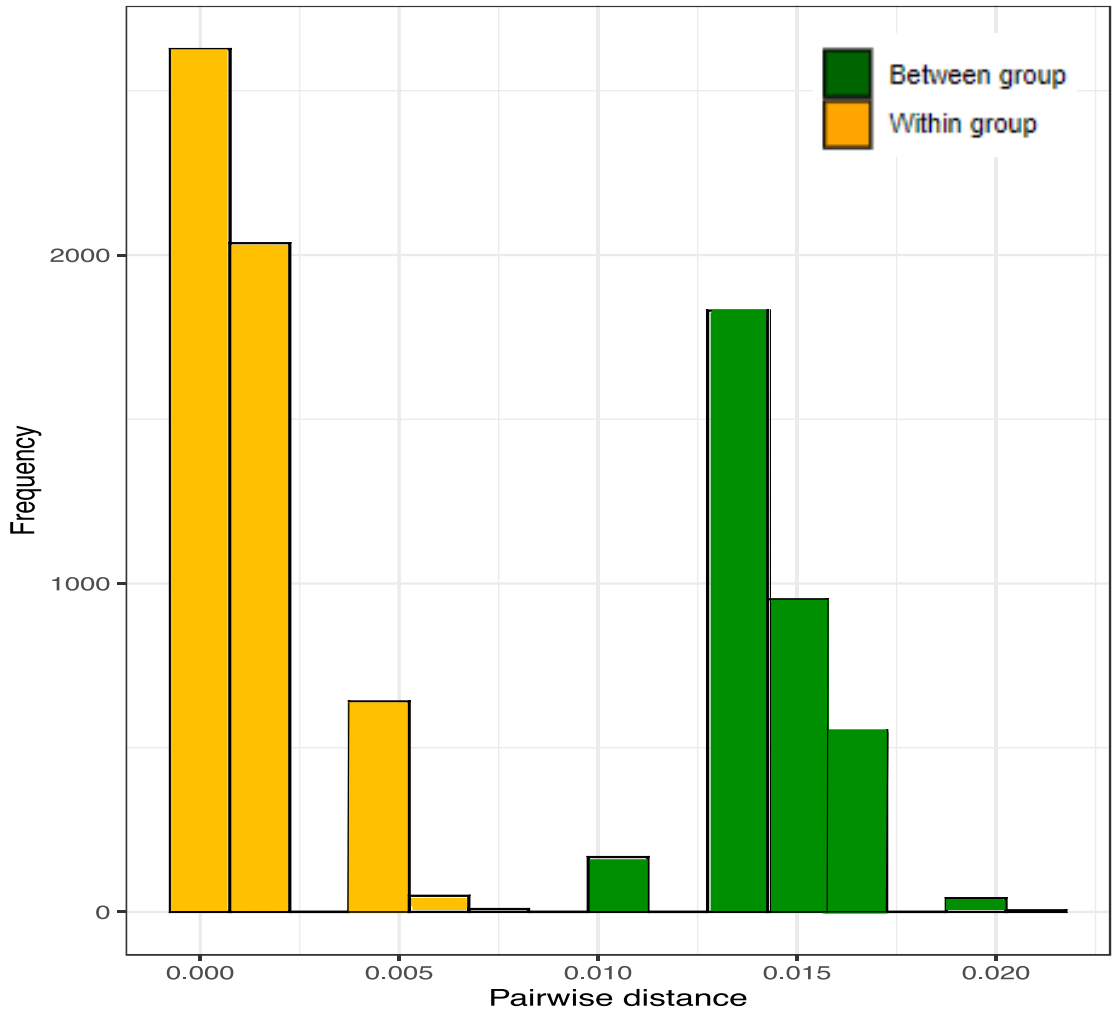

Figure 3.2 Pairwise genetic distances between 16 distinct haplotypes across 135 COI sequences identified from field specimens of *Pegomya*. Between group genetic distances are highlighted in green and within group genetic distances highlighted in yellow.

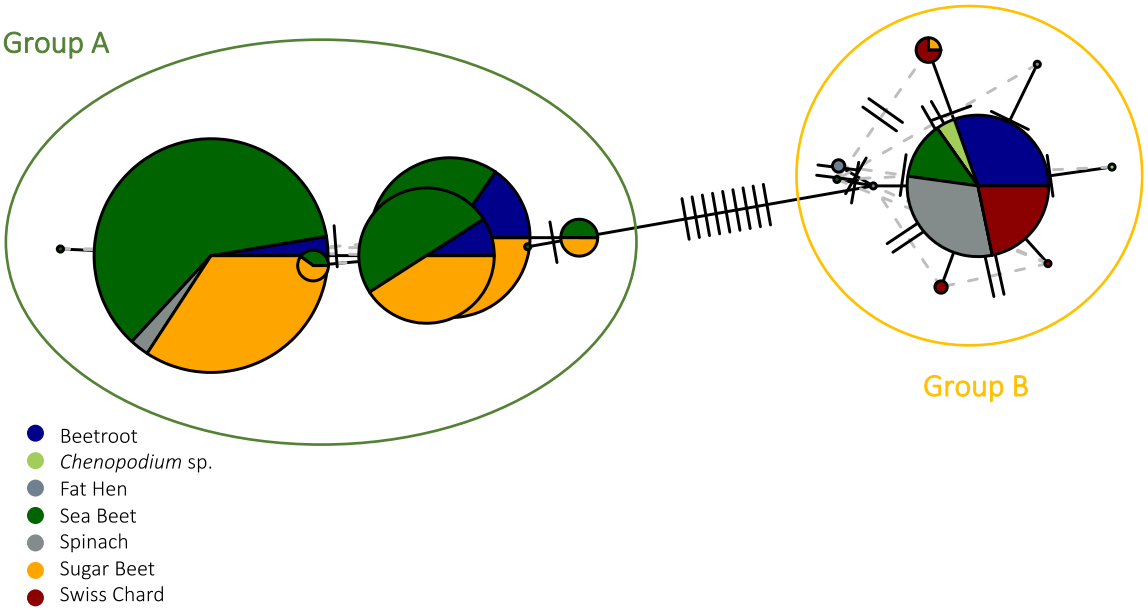

Figure 3.3 Haplotype Network of 135 Cytochrome C Oxidase Subunit I (COI) sequences of *Pegomya* field specimens. There are 16 haplotypes distributed across 2 groups (A and B, as defined in Figure 3.1), with colour coded nodes referring to host plant association. Node size represents number of sequences clustered within a haplotype, with tracks showing the number of base pair differences between each haplotype.

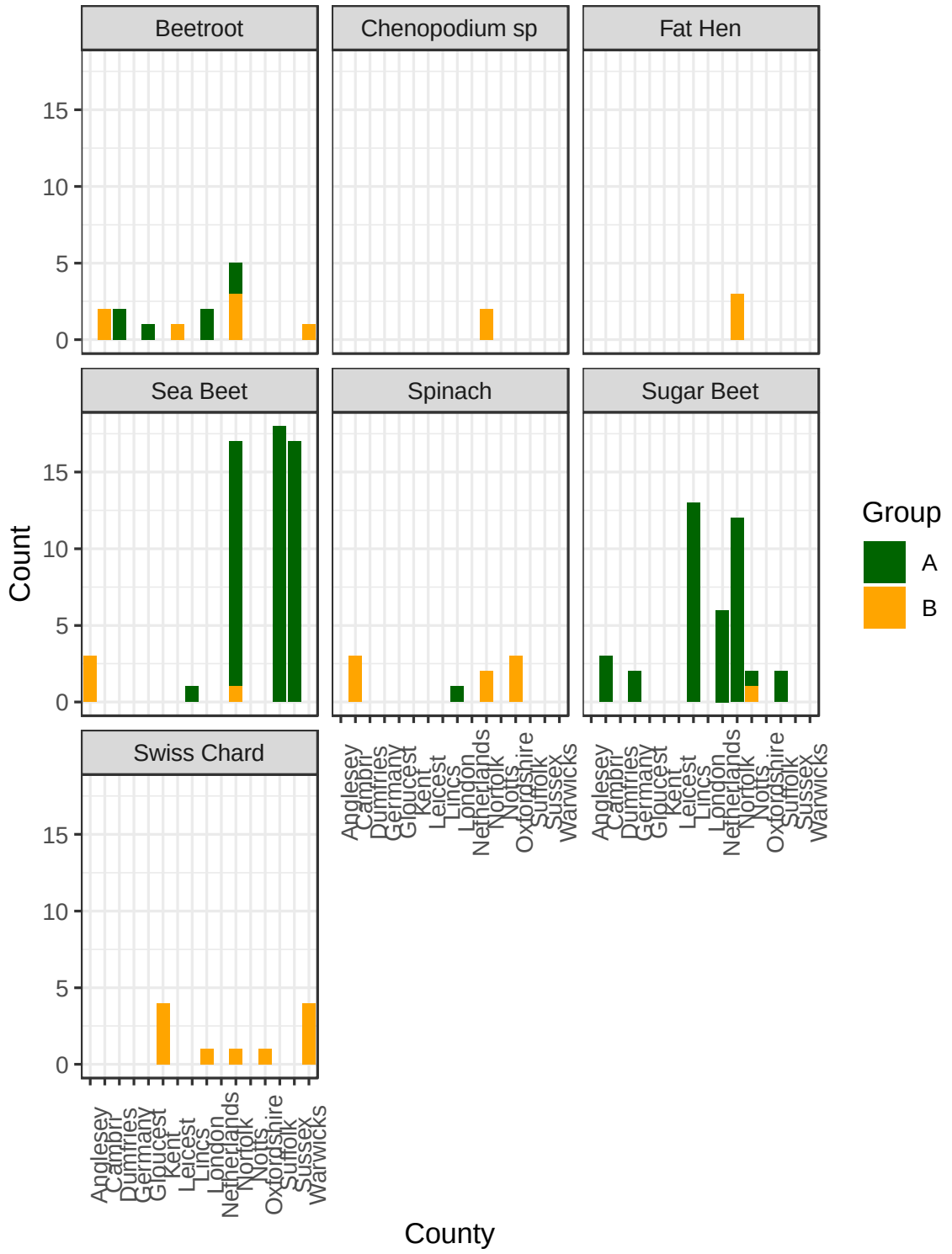

Figure 3.4 Barplots showing counts of sugar beet leaf miner across geographical locations and host plants. The count is coloured based on the groups each sequence was allocated (A or B, as defined in Figure 3.1). UK samples have been scaled to county level, with non-UK samples scaled to country, to protect grower and individual anonymity.

3.6 Discussion

In this study, I provide the first genetic characterisation of leaf mining flies of sugar beet and related plants. My results indicate that there are two, genetically distinct groups, which differ in both host plant range, and (likely to a lesser extent) geographic distribution. One genetic group comprised predominantly of specimens collected from sugar beet and sea beet, while the other group was mainly found in Swiss chard, spinach and wild *Chenopodium* spp. Here, I discuss these findings in terms of the evolution of these flies, and in the context of pest management.

It is possible that the two genetic groups observed represent distinct species. The large mean K2P distance calculated between the two groups (0.014), in addition to the long branch lengths, indicates sufficient divergence between sequences to identify the groups as two individual species (Nolan *et al.*, 2007; Kumar *et al.*, 2007). Moreover, there is clear geographic overlap between the two, with both being found in the same county, and even at the same sampling location. This suggests that there are no geographic barriers to admixture. However, additional analyses of nuclear genes, ideally combined with laboratory cross experiments, are needed to confirm whether these are distinct species. Morphological analysis suggests some level of distinction in identifying the species complex as defined by Ackland *et al.* (2017), but upon assessing the placement of these identifications within the maximum likelihood tree there is some crossover between the two species, *P.betae* and *P.cunicularia*, within the branches of group A and group B. There is at least a single representative of both species identified here as *P.betae* and *P.cunicularia* in both groups A and B. This might suggest the possibility of 'cryptic species',

two or more species previously identified by the same or similar morphology as a single species, but that are distinct from one another (Bickford *et al*. 2007; Pfenninger & Schwenk, 2007). Cryptic species have now been discovered across many taxa, including some important agricultural pests, for example the globally invasive *Liriomyza huidobrensis* leaf miner that affect vegetable and flower crops (Scheffer & Lewis, 2001; Xu *et al*. 2021). It is now understood that morphology does not always evolve in tandem with speciation, though the theories behind why vary somewhat.For example, when morphological evolution is a result of similar selection pressure (morphological convergence hypothesis) (Trontelj & Fišer, 2009; Fišer *et al*. 2018), when niche-related traits within a lineage are conserved across speciation events (phylogenetic niche conservatism (PNC)) (Crisp & Cook, 2012; Fišer *et al*. 2018) and that cryptic species may have recently diverged (recent divergence hypothesis) and therefore morphological distinctions have not yet manifested (Fišer *et al*. 2018) . As understanding how many leaf miner species are associated with sugar beet is fundamental in the management of pest outbreaks, it is important to understand whether the sugar beet leaf miner are actually cryptic species and how this may influence what is seen in the field. However, this is currently just a hypothesis, as there are only five specimens with adult identifications within this tree so I cannot be fully certain. To ascertain the possibility of cryptic species within the sugar beet leaf miner complex, further analyses of the COI gene, in tandem with nuclear data, on a wider range of identified adult samples should be conducted. With regards to the potential presence of four species as described by Michelson (1980) based on morphology (*P.hyoscyami, P.betae, P.cunicularia* and *P.exilis*), I found that, within the two divergent groups, there was limited divergence as indicated by shallow branch lengths, low bootstrap support values and low levels of intragroup distance, which

do not support the hypothesis of four species (Figure 3.1, Figure 3.3). Again, however, more genetic and experimental evidence is required to conclusively say whether each of our two groups represent one or multiple species, and more sampling is required to identify if other species occur in different geographical regions.

It is worth noting that the branching patterns of the haplotype networks within Group A and Group B differ. Group A is linear in shape, whereas Group B is a single haplotype at high frequency, with multiple low frequency haplotypes branching off in a star-like pattern. This could be indicative of differences in the evolutionary history of the two groups. Group A is linear in structure, with one and two step mutations between haplotype groups. This solid genetic structure is indicative of populations with historical gene flow (Nardi *et al*., 2005) which will naturally occur through the movement of individuals between populations (Slatkin, 1987). Star-like haplotype networks tend to arise as a result of recent demographic events such as population expansion or colonisation (Huang *et al*., 2007; Montecinos *et al*., 2012; Chroni *et al*., 2019). It is possible that Group A is an ancestral or founder population to group B, with some individuals undergoing range expansion through accidental means, resulting in the reduced frequency of haplotypes found within group B compared to the higher genetic variation found within group A (Birungi & Munstermann, 2002; Dlugosch & Parker, 2007). More detailed sampling and genetic analysis would provide additional insight into patterns of recent demographic history of these flies.

I observed strong differences in host plant association between the two genetic groups (Table 3.1, Figure 3.3, Figure 3.4). Individuals from Group B were found across a larger

range of host plants than in Group A, despite a smaller overall sample size. Individuals from group B were predominantly found on Swiss chard and spinach, while individuals from group A were found mainly on sugar beet and sea beet. These are not purely taxonomic groupings; for instance, Swiss chard is a cultivar of the same subspecies as beetroot, and group B was found on the former while both groups were found in roughly equal frequencies on the latter. Host plant range expansions are an important driver of diversification in phytophagous insects (Janz *et al*., 2006; Freedman *et al*., 2020), and the patterns of genetic diversity and host plant association in group B are consistent with range expansion. Again, however, more evidence is needed to verify this.

I had some success in amplifying leaf miner DNA from empty leaf mines, albeit with a low success rate. Other studies have shown that it is possible to extract DNA from leaf mines that no longer contain their inhabitants, however the success rate was also fairly low (Derocles *et al*., 2015; Mlynarek *et al*., 2017). It is also important to recognise that the time in which the mine had been left unoccupied was unknown as the samples were collected from the field, in comparison to other studies on DNA extraction and sequencing of empty leaf mines where the time the mine was left unoccupied was a known factor and was only relatively recently abandoned (Mlynarek *et al*., 2017). Even with the lower success rate of sequencing DNA from empty leaf mines, there is high potential in using molecular methods as a tool for species identification from such samples, as mines can be virtually impossible to distinguish to species level (Edmunds, R., personal communications, 11th January 2021).

Sequencing of larvae and pupae also uncovered samples with hymenopteran DNA (Chapter 6). Two sub-families within the Braconid wasps were detected here (sub-families Opiinae and Alysiinae), both of which have great potential as biological control agents with species from both sub-families recorded as parasitoids of major insect pests (Gisloti & Prado, 2012; Hazini *et al*., 2015; Suárez *et al*., 2019; Ridland *et al*., 2020). Future work will aim to identify these species and determine whether they have potential as biological control agents for sugar beet leaf miners. Finally, leaf mine eDNA also has potential in the identification of parasitized larvae from leaf mine material (Derocles *et al*., 2015) which may prove useful in future studies on identifying leaf miner-parasitoid interactions.

The genetic characterisation of sugar beet leaf miners presented here can form the basis of future work to aid pest control. Understanding the wider ecological picture will enable more precise and sustainable methods to be implemented in the field in the control of sugar beet leaf miners. Immediate questions include determining whether there are behavioural differences between the two groups, what influences the cyclical emergence and infestation of leaf miner in sugar beet - does this vary between groups, whether one group is more capable of longer distance dispersal because of generalist feeding behaviours and one more restricted and specialist, and what effects these factors may have on their host plants in terms of crop yields. These elements of leaf miner behaviour may define how they differ in their responses to conventional and novel control methods. Such investigations may pave the way for sustainable approaches to insect control in the future.

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CHAPTER 4

Phylogenetics of the *Pegomya* genus

Pegomya specimens from the collection at Liverpool World Museum (2022): 1 – *P.flavifrons*, 2 – *P.solennis* , 3 & 5 – *P.rufina*, 4 – *P.geniculata*, 6 – *P.provecta*, 7 & 9 – *P.haemorrhoum*, 8 – *P.hyoscyami* (Photos by Siobhan Hillman)

4.1 Contributions

Dr. Phil Brighton, National Museums Scotland, Liverpool World Museum and Manchester Museum provided the *Pegomya* specimens included in this chapter. I conducted the molecular work and analyses and Dr. Tony Irwin verified the identifications of some of the anomalous specimens from the molecular analyses.

4.2 Abstract

The genus *Pegomya* (Anthomyiidae) contains a number of species with extremely diverse larval feeding behaviours and includes species of economic importance in the agricultural industry. *Pegomya* are generally understudied and neglected in many cases due to difficulties arising from their identification, which has resulted in a lack of ecological information. In this chapter I aim to use genetics, in association with morphology, to understand the relationships within the *Pegomya* and how other species may relate to the sugar beet leaf miners. Amplification of a small fragment length of the COI gene (172bp) was successful for a number of specimens, while nuclear loci amplification was unsuccessful in recently collected specimens. Many specimens dated back to the 1900s, with the oldest sequence obtained from a specimen collected in 1910. Maximum likelihood reconstruction confirmed monophyletic clades within the *Pegomya* data, with some grouping at species level. A single clade contained multiple species and was largely unresolved. Morphological analyses confirmed the original identifications allocated to anomalous specimens isolated from the maximum likelihood tree and so misplacement due to possible misidentification of these specimens was ruled out. This study provides an assessment of the phylogenetic relationships of the *Pegomya* genus, suggesting the possibility of cryptic species within unresolved clades, and potential for species groupings defined by larval feeding behaviours. Future work should undertake multi-locus analyses of the group and include larger numbers of representative species.

4.3 Introduction

Historically, the taxonomic standing of many insect groups has been largely disputed. The foundation of the universal Linnaean system of classification has traditionally been based on the morphological descriptions of a select number of representative individuals in order to determine the unique characteristics found between species and to ascertain identification thereafter (Balakrishnan, 2005; Kumar *et al*., 2007). Identification of species from morphology alone however has proven to be problematic in some cases. Cryptic species and homoplastic characteristics can make the morphological identification of species challenging, so the use of molecular techniques can be used to help resolve the phylogeny of such groups (Austin & Melville, 2006). The combination of morphological homoplasy and phylogenetic data can sometimes be conflicting and lead to potential misinterpretation though (Mott & Vieites, 2009), so it is important to analyse the morphological and phylogenetic relationships from multiple angles (e.g., Huang *et al.*, 2019). At its most basic level, phylogenetics can been used to determine the relationships between species, but now phylogenetics is commonly used as a tool in understanding the evolutionary history of species such as speciation events (Barraclough & Nee, 2001). Phylogenetics has also been used in previous studies to determine and to remedy the known taxonomy of complicated and disputed species complexes (Hurtado *et al*., 2019), similar to that of the *Pegomya* sugar beet leaf miner complex.

Museum collections provide a historically diverse and species rich resource which can be utilised in a range of different research areas (Winston, 2007). Their use facilitates access to many species, including those that are rare or even extinct (Payne & Sorenson, 2003;

Tin *et al*., 2014). Collections such as these also present a range of specimens across a potentially much larger time scale than is obtainable from present-day scientific endeavours. The continuous advancement surrounding ethics and collection permits may prevent the collection of species today that were readily accessible decades ago. Similarly, some locations may no longer be easily accessed for biological study, but specimens from such locations may be accessed through museum collections. Historical collections also allow the study of specimens collected over decades, a timeframe which may not be feasible for many scientific studies (Suarez & Tsutsui, 2004; Lalonde & Marcus, 2020). Museum collections not only contain morphological but genetic data (Vaudo *et al*., 2018) from different time periods, which can play a vital role in studying evolutionary changes within species, population dynamics, biodiversity, biological invasions and species decline (Shaffer *et al*., 1998; Suarez & Tsutsui, 2004; Winston, 2007). Although DNA is often fragmented in historical specimens and the effort required to obtain DNA sequences is much greater, it is still possible to extract DNA from museum collections using molecular techniques (Helbig, 2002). A major concern when using any historical specimens for genetic research is that the extraction of DNA often requires some level of destructive sampling that may damage specimens in a permanent manner, removing key morphological features, which will be problematic in the long-term sustainability of museum collections (Thomsen *et al.*, 2009; Hofreiter, 2012). Extracting DNA from such valuable collections can now be achieved using smaller tissue samples and even using non-destructive methods, causing minimal amount of damage in the retrieval of genetic data (Wandeler *et al*., 2007; Gilbert *et al*., 2007; Rohland *et al*., 2018; Sugita *et al*., 2020). However, the error rate when trying to obtain DNA sequences from museum collections is much higher than with freshly collected specimens. The success

rate in obtaining genetic data can moreover be greatly influenced by the age and preservation method in which specimens have been stored (Wandeler *et al*., 2007; Besnard *et al.*, 2016). Nonetheless, studies show that it is possible to obtain DNA from historical specimens and the scope of natural history specimens available in museum collections are an invaluable, and too often overlooked, resource to current biological research.

Many dipterans are ecologically and economically important and revising the taxonomy of some family groups using phylogenetics can provide insights into a deeper understanding of many species. The phylogenetics of some dipteran families which contain medically important vector species, and forensically important flies, have been assessed to help resolve and maximise the accuracy of species identification and determine familial relationships which is vital in such fields (Nelson *et al.*, 2012; Aragão *et al*., 2019; Ren *et al*., 2020). Agriculturally important dipteran groups, such as the genus *Liriomyza* (Agromyziidae), have also undergone phylogenetic assessment due to ambiguous morphological characteristics and overlapping ecological niches, which renders species identification and familial relationships hard to ascertain without the aid of genetic data (Ferreira *et al*., 2017; Carapelli *et al*., 2018). The genus *Pegomya* (Diptera: Anthomyiidae), divided into two sub-genera *Pegomya* and *Phoraea*, contains an assortment of species with remarkable larval feeding behaviours (Table 4.3). Larval feeding behaviours in this family of flies include leaf miners (sub-genera *Pegomya*), stem borers and fungal feeders (sub-genera *Phoraea*), with each species associated with a range of host plants (Ackland *et al*., 2017). Yet, the Anthomyiidae are grossly understudied, with the majority of research focused on pest species within the family.

They are particularly difficult to identify by their morphology, with genitalia often the most reliable characteristic. Combining the known taxonomy of this genus with phylogenetic analyses may provide a deeper understanding of the evolution of *Pegomya* species and how the sugar beet leaf mining complex fits in within its genus. Due to limited knowledge of the Anthomyiidae compared to other more well-known groups of dipterans, phylogenetic reconstruction of one of its more economically important genera will help answer vital questions surrounding such an intriguing group. It will help to clarify the number of species when compared to current knowledge about the genus and gauge the accuracy of species identification based on current descriptions. Moreover, the behaviours and host choices of individual species within the genus, and how these factors are related to one another, may have implications economically.

In this chapter I aimed to analyse the relationships between the sugar beet leaf mining complex and other *Pegomya* species through using molecular tools in association with traditional taxonomic identification and associated ecological information. I investigated and analysed the genus *Pegomya* through the genetic characterisation of field and loaned museum specimens. Amplification of the mitochondrial COI region, as well as the amplification of nuclear loci, allowed for comparisons between phylogenies and a greater understanding of the genus. I optimised the identification of *Pegomya* species through phylogenetic analyses, in association with the accompanying meta data for each specimen. By constructing and analysing a phylogenetic tree of the genus I aimed to clarify key relationships between species within the genus *Pegomya*. I also aimed to examine external morphological characteristics of *Pegomya* specimens in association with their genetic characterisation, and to isolate key external morphological features

that may be used in identification at species level. A comparison of how both methods of identification could help to provide future insights to species biology and ecology and how this may benefit the understanding of relationships between species. Through this work I hope to be able to reveal relationships between *Pegomya* species and therefore associate these relationships with previously known information about morphology, larval biology and host plant associations that are unique to species within this genus.

4.4 Methods

4.4.1 Sample Summary

32 species within the *Pegomya* genus were loaned from museums and from the personal collection of Dr. Phil Brighton, the former organiser of the Anthomyiidae Recording Scheme (Dipterists Forum) for phylogenetic analyses. 74 specimens across 19 species were loaned from Liverpool World Museum, 105 specimens across 25 species from Natural Museums Scotland, 22 specimens across 2 species from Manchester Museum and 18 specimens across 10 species from the private collection of Dr. Phil Brighton. Within the loaned samples there were representative specimens for 3 of the 4 species that make up the sugar beet leaf miner complex. National Museum Scotland, Manchester Museum and specimens from the Phil Brighton collection were loaned as whole, dry pinned specimens. Manchester Museum also provided a number of larval samples which were stored in an unknown liquid. Samples from Liverpool World Museum were taken as legs, preserved in 70% ethanol and later stored at -82°C.

4.4.2 Molecular Methods

DNA was extracted following a modified salt extraction technique used by Richardson *et al.* (2001). All equipment used throughout the molecular process was initially UV sterilised and specimen handling equipment, such as forceps, were sterilised with ethanol and flame between handling each sample. 1.5ml Eppendorf tubes and micropestles were first submerged into liquid nitrogen before the addition of any specimen material. The left prothoracic (foreleg) leg, or in cases where the removal of the prothoracic was impossible or would cause potential damage to the specimen, the mesothoracic (middle leg) was then carefully removed using sterilised forceps and placed into the Eppendorf tube and ground using the micropestle. The tube containing the sample was then submerged into liquid nitrogen again twice, grinding the sample with the micropestle in between submerging the tube each time. 265µl DigiMix (250µl DigSol + 15µl Proteinase K) was then added to each sample and the samples were incubated overnight at 55°C. After incubation, 1.5µl of RNase was added to each tube, followed by 300µl of pre-warmed 4M ammonium acetate. All samples were then centrifuged for 20 mins at 13000rpm. Samples were then transferred into fresh, UV sterilised Eppendorf tubes, adding 1ml of ice cold 100% ethanol to each in order to separate the DNA from the supernatant, and centrifuged again for 20 mins at 13000rpm. The supernatant was then carefully decanted and each tube carefully blotted on tissue paper. 500µl of ice cold 70% ethanol was then added to each tube, now containing a DNA pellet, and centrifuged again for 20 mins at 13000rpm. This process was repeated once more to ensure the remaining DNA was fully cleansed. The samples were then air dried for an hour at 55°C before the addition of 50µl of Low TE, incubating samples again for a further hour at 55°C. DNA

quality and quantity of each sample was then checked using the NANODROP 8000 (Thermo SCIENTIFIC). Due to the degradation of DNA in historical specimens (Lalonde & Marcus, 2020), the previous threshold for DNA quantity (10 – 50 ng/µl) in field specimens was not anticipated from these samples. Therefore, any samples containing a DNA quantity within the region of 1µl were further processed for sequencing.

Amplification of the COI gene

In museum samples the amplification of the COI region using the universal primers LCO1490/HCO2198 (Folmer *et al*., 1994) and the same PCR conditions that were used for field specimens (Chapter 3) was unsuccessful for all initial reactions, most likely due to the general degradation of the DNA available from the tissue sample and the primer amplicon being too large. New primers that amplify smaller fragments of DNA were applied in the reamplification of museum samples based on a study by Lalonde and Marcus (2020) on DNA recovery in historical specimens. The primer pairs miniCOIF2/HC02198 (569 bp), miniCOIF2/miniCOIR3 (501 bp), miniCOIF2/miniCOIR2 (339 bp) and miniCOIF3/miniCOIR3 (258 bp) were tested following PCR protocols used by Gemmell *et al*. (2014). The PCR program was as follows; an initial denaturation stage of 95°C for 2 mins, followed by 5 cycles of 95°C for 1 min, 46°C for 1 min, 72°C for 30 secs and then 35 cycles of 95°C for 1 min, 53°C for 1 min, 72°C for 30 secs, and then a final extension of 72°C for 5 mins. Two museum samples that had previously not been successfully amplified using the universal primer pair LCO1490/HCO2198 were run through this PCR protocol alongside two positive controls from relatively fresh (collected in 2018), dry mounted samples that had already been successfully sequenced. Bands

were obtained for both museum samples when using primer pairs miniCOIF2/HCO2198 and miniCOIF3/miniCOIR3 which were then run on a 1.5% agarose gel and prepared for sequencing. Successful sequences were achieved for both museum samples that had been amplified using the miniCOIF3/miniCOIR3 primer pair and so these primers were selected for future PCR of museum specimens.

Optimisation of mini-barcoding COI primers

Amplification of museum samples following the PCR protocol by Gemmell *et al.* (2014) was initially successful for approximately 1/4 of the samples. To optimise the potential number of sequences I could obtain from our DNA data set, I performed a gradient PCR based on the protocol set out by Gemmell *et al*. (2014), altering the annealing stage to a gradient between 48°C and 58°C (53°C ± 5°C) for 35 cycles. I found that I could obtain bands from some of the samples which had previously been unsuccessfully amplified when using the original PCR protocol. The PCR protocol for the reamplification of unsuccessfully sequenced samples is as follows; an initial denaturation stage of 95°C for 2 mins, followed by 5 cycles of 95°C for 1 min, 46°C for 1 min, 72°C for 30 secs and then 35 cycles of 95°C for 1 min, 48°C for 1 min, 72°C for 30 secs, and then a final extension of 72°C for 5 mins. In addition to these samples, samples which were sent off for sequencing but did not produce a successful sequence, were also reamplified using the new PCR protocol. Samples which produced a clear band during gel electrophoresis were further processed for sequencing.

Previous attempts to amplify DNA using primer pairs that produce larger fragment lengths were mostly unsuccessful. 3 primer pairs were tested in the amplification of the COI gene based on the study by Lalonde & Marcus (2020); miniCOIF2/HC02198 (569 bp), miniCOIF2/miniCOIR3 (501 bp) and miniCOIF2/miniCOIR2 (339 bp). I initially had some success in obtaining sequences from primer pair miniCOIF2/miniCOIR3, but ultimately these primer pairs proved to be problematic. I believe that the problem I initially had with obtaining sequences from the above 3 sets of primers was due to a problem with the forward primer miniCOIF2 itself and acquired a fresh batch to test. Following this, I tested each primer pair using the PCR protocol by Gemmell *et al*. (2014) with a modified annealing temperature. The same conditions were set for each primer pair, with the annealing temperature fixed to a gradient between 48°C and 58°C (53°C ± 5°C) for 35 cycles. The new miniCOIF2 primer stock worked for all primer sets, but I had the most success with primer pairs miniCOIF2/HC02198 (569 bp) and miniCOIF2/miniCOIR3 (501 bp).

Amplification of nuclear loci

Amplification of a nuclear locus was attempted on the most recently collected specimens (Phil Brighton samples/field samples with representatives from groups A and B). I identified four genes to amplify (Table 4.1). The museum collections were excluded from this part of the analyses due to the fragmentation of their DNA and general difficulties in amplifying nuclear loci compared to mitochondrial ones (Lin & Danforth, 2004). I initially tested primer sets for 3 different genes (numbers 1 – 4, Table 4.2), a mixture of primers amplifying shorter to longer fragment lengths of DNA. I used a gradient PCR (±5°C either

side of the original annealing temperature) to find the annealing temperature that worked best for each primer set. Number 5 was never tested in the end due to failures in obtaining sequences using the other primers.

Table 4.1 Summary of nuclear loci and PCR conditions selected for testing in the amplification of nuclear DNA from a select set of samples from the *Pegomya* collection.

	Gene Amplifying	Primers & PCR protocol	Source
1.	BZF	BBZF_F. 5′-CTCGCACGTCAAGGTGAGT-3′ BBZF_R. 5′-GATCCGAATGTGGATTTGCT-3′ PCR protocol: 94°C for 3 mins, 35 cycles of 94°C for 30 secs, 55°C for 30 secs, 72°C for 45 secs and finally 72°C for 10 mins	Takaoka et al. (2018)
2.	*White*	5'-TGYGCNTATGTNCARCARGAYGA-3'a 11404-11426 (S) 5'-ACYTGNACRTAAAARTCNGCNGG-3"a 11975-11997 (A) PCR protocol: 35 cycles of 94°C for 1 min, 47°C for 1 min and 72°C for 1.5 mins	Baker et al. (2001)
3.	EF-1α	EF-40. 5′-GTCGTGATCGGACACGTCGATTCCGG-3′ EF-53. 5′-GCGAACTTGCAAGCAATGTGAGC-3′ Internal primers EF-46. 5′-TGAGGAAATCAAGAAGGAAG-3′ EF-50. 5′-ACTTCCTTCTTGATTTCCTC-5′ PCR Protocol: 92°C for 1 min, 35 cycles of 92°C for 30 secs, 45°C for 30 secs, 72°C for 1 min and finally 72°C for 7 mins	Scheffer & Lewis (2001)
4.	EF-1α	EF1. 5′-ACAGCGACGGTTTGTCTCATGTC-3′ EF2. 5′-CACATTAACATTGTCGTGATTGG-3′ EF3. 5′-CCGATACCACCGATTTTGTA-3′ EF4. 5′-CCTGGTTCAAGGGATGGAA-3′ (J.K. Moulton, Tennessee, pers. Comm) – unmodified versions of the primers used	McDonagh, L. M. 2009. (Thesis)

		Modified PCR protocol based off Hoelzel, (1992): 95°C for 5 min, 35 cycles of 55°C for 1 min, 72°C for 1 min , 94°C for 30s and finally 55°C for 1 min, 72°C for 10 min	
5.	28S rRNA	D1±D2 fragment Amplification primers D1.F: 5'-CCCCCTGAATTTAAGCATAT-3' (20-mer)a D2.R: 5'-GTTAGACTCCTTGGTCCGTG-3'(20-mer)b Internal sequencing primers D1.R: 5'-CTCTCTATTCAGAGTTCTTTTC-3'(22-mer)a D2.F: 5'-GAGGGAAAGTTGAAAAGAAC-3' (20-mer)c D3±D7 fragment Amplification primers D3-5.F: 5'-GACCCGTCTTGAAACACGG-3' (19-mer)d D7.R: 5'-CGACTTCCCTTACCTACAT-3' (19-mer)a Internal sequencing primers D3-5.R: 5'-TTACACACTCCTTAGCGGA-3' (19-mer)d D3-5.486.R: 5'-TCGGAAGGAACCAGCTACTA-3' (20-mer)e D3-5.742.F: 5'-TCTCAAACTTTAAATGG-3' (17-mer)d D7.F: 5'-GACTGAAGTGGAGAAGGGT-3' (19-mer)a PCR Protocol: 94°C for 3 mins, 30 cycles of 94°C for 30 secs, 55°C for 30 secs, 68°C for 1 min (D1±D2 fragment)/1 min 30 secs (D3±D7 fragment) and finally 68°C for 15 mins	Stevens & Wall (2001)

4.4.3 Analyses

Sequences were aligned by eye using AliView v 1.26 (Larsson, 2014) and all segregating sites were reviewed together with the accompanying chromatograms. BLAST (Altschul *et al.*, 1990) was then used to check the taxonomic identification of each sequence against the sequence database available in GenBank (Benson *et al.*, 2013). Additionally, representatives of the two groups found from the genetic characterisation of *Pegomya* field specimens were also included within the phylogenetic analyses. Any failed sequences were removed prior to further analyses. This process was repeated once more

to check alignments and to filter out and remove any remaining low quality sequences in the alignment. Sequences were then trimmed to 172bp.

A Maximum Likelihood (ML) tree was constructed using the IQ-Tree Web Server (Trifinopoulos *et al*., 2016) for all 72 successful *Pegomya* sequences, in addition to those procured from both Groups A and B of our field specimens. To find the model best fitted to our data (K3Pu+F+I) based on Bayesian Information Criterion (BIC) (Schwarz, 1978) I used ModelFinder (Kalyaanamoorthy *et al*., 2017). The ML tree was then constructed using 1000 ultrafast bootstrap (Hoang *et al.*, 2018) replicates to assess node support. Bootstrap values of less than 50 were not included in the final tree which was edited using figtree (Rambaut, 2010).

4.4.4 Morphological Analysis

I identified key specimens that required morphological analyses based on inconsistencies observed within the original (prior to the removal of 11 sequences) COI (162bp) phylogenetic tree. The morphological examination of each of the isolated specimens was supported by the work of Ackland *et al.* (2017) on the identification of British Anthomyiidae Part 1 (Text). Examination was initially conducted by myself, and then latterly by Dr. Tony Irwin. Genitalia dissections of museum specimens were not possible due to the destructive manner that is required for such examination as these were loaned specimens, however, some of the samples isolated for examination had previously undergone genitalia dissection and so could be examined this way. Detailed photographs of the external morphology were taken of each specimen (excluding Liverpool World

Museum Specimens) using GX Capture 8.5 (https://www.gtvision.co.uk/Cameras/GXCAM-Camera-Drivers). Of the collections, only the Liverpool World Museum specimens were not available for in person morphological analyses as the specimens remained in the collection within the museum. These however, had previously been examined by Dr. Phil Brighton or verified by Michael Ackland and detailed photographs were taken at the museum for later reference. Stacked photographs were taken and finalised using the Helicon Focus 7.5.8 (registered pro unlimited) and Helicon remote ver. 3.9.7.W (Method c – pyramid, smoothing ¼, varying shots, 20 intervals). Results of the morphological analyses were then compared to the original COI phylogenetic tree to confirm if the anomalies were then resolved. The morphology of specimens of any sequences remaining unresolved after this comparison were then compared to the morphology of conflicting species that each was grouped with.

4.5 Results

Sequencing of the mitochondrial COI gene was successful for 54 museum specimens and all 18 specimens from Dr. Phil Brighton's collection, with a total of 17 species out of a possible 32 from the genus included in the final analyses. Within our final sequence alignment there was at least one sequence to represent each of the 17 species, with a number of species with multiple representative sequences (see summary Table 4.2). The age of specimens that were successfully sequenced also varied somewhat. The oldest specimen I sequenced was collected in 1910, with a number of other specimen collection dates ranging in the 1900s (Figure 4.2, Table 4.2). Though I obtained bands for the field

specimens during the tests for suitable PCR conditions for loci and primer pairs 1 – 4 (Table 4.1), sequencing of the nuclear loci was unsuccessful.

Maximum Likelihood phylogenetic reconstruction revealed that there was monophyletic clustering within the data set. For ease in discussing the results the tree has been divided into four groups based on branching and sequence clusters (Figure 4.1). In general, there are strong bootstrap support values on many of the species' groupings within the tree. Going back to the basal branches of the phylogeny indicated lower bootstrap support. Group number 1 is the largest of the groups and had strong bootstrap support with sequences of multiple different species grouped together within a single branch. The relationships between individual species amongst clades in groups no. 2 – 4 had strong bootstrap support and were mostly resolved relative to association between species groupings. I also observed that in groups no. 2 – 4 there was a degree of grouping of species with similar larval feeding behaviours, but with poor bootstrap supports for placement of these groupings within the tree (Figure 4.1, Table 4.3).

The phylogenetic tree was assessed for species outliers that would be the focus of the morphological analysis or reamplification of the COI gene. These were identified based on contradicting tree topology and morphological descriptions of the species, e.g. sequences of species that were not grouped with other sequences of the same species were isolated. 21 specimens were identified based on this criterion. Four reamplifications were successful and compared to the original sequences. Reamplification of these four specimens confirmed the original placement of the sequences within the tree. Within group 1 (Figure 4.1) the common species was *P.bicolor*, therefore I worked under the

assumption that species different to *P.bicolor* grouped within this clade were due to possible misidentifications or poor sequences resolution. From this, I isolated specimens of *P.cunicularia, P.geniculata, P.haemorrhoum* and *P.hyoscyami* also based on the grouping of sequences of the same species elsewhere in the tree. A larger number of sequences of *P.geniculata* and *P.haemorrhoum* can also be seen grouped with higher bootstrap support values (Figure 4.1), resulting in the rationale behind isolating the sequences of these species within group 1 for morphological analysis. In addition to this, sequences of *P.rugulosa, P.rufina, P.provecta* and *P.setaria* were isolated from group 1 for morphological analysis. The justification for this was due to the differences in larval feeding behaviour which has traditionally been used to divide both sub-genera within the genus *Pegomya* (Ackland *et al*., 2017). I was also limited in my ability to cross-reference these sequences against others of the same species as there were few representatives, or in some cases they were the only representative, of each within the tree.

A total of six National Museums Scotland specimens and the four specimens collected by Dr. Phil Brighton were isolated due to anomalous placement in the original tree. Dr. Tony Irwin confirmed the identifications of all specimens, with no re-allocation of species names. For all but two specimens (*P.hyoscyami* (3) and *P.pulchripes* (1)) the original identification was verified. *P.hyoscyami* (3) (NMS55) was confirmed to have the same identification as it was originally labelled with. The grounds for this was that no genitalia examination had taken place, that this was the species description it best fitted, and that it had matching breeding information with its neighbouring specimen of the same identification. The only tentative confirmation of morphological identification lay with *P.pulchripes* (1) (NMS84) as the genitalia did not quite match what is described in the

Anthomyiidae key (Ackland *et al.*, 2017). Liverpool World Museum samples were previously examined by Dr. Phil Brighton or some had been verified by Michael Ackland (or other entomologists) and access to the specimens came at a late stage so examination of these specimens in detail was not possible. Detailed stacked photographs (see title page) of the 9 specimens I identified for re-examination (two were removed from analysis) have been kept for reference at a later date if required.

Table 4.2 54 loaned specimens from the genus *Pegomya* included in the final phylogenetic analyses with associated tree ID name, species and specimen source. Highlighted in grey are the species which make up the sugar beet leaf mining complex.

Tree ID	Species	Specimen Source	Date Collected
P.bicolor__1_	*P.bicolor*	Phil Brighton	2017
P.bicolor__2_	*P.bicolor*	Phil Brighton	2018
P.deprimata__1_	*P.deprimata*	Phil Brighton	2016
P.geniculata__1_	*P.geniculata*	Phil Brighton	2016
P.geniculata__2_	*P.geniculata*	Phil Brighton	2016
P.haemorrhoum__1_	*P.haemorrhoum*	Phil Brighton	2017
P.haemorrhoum__2_	*P.haemorrhoum*	Phil Brighton	2017
P.haemorrhoum__3_	*P.haemorrhoum*	Phil Brighton	2017
P.haemorrhoum__4_	*P.haemorrhoum*	Phil Brighton	2018
P.rubivora__1_	*P.rubivora*	Phil Brighton	2018
P.rubivora__2_	*P.rubivora*	Phil Brighton	2018
P.rugulosa__1_	*P.rugulosa*	Phil Brighton	2018
P.setaria__1_	*P.setaria*	Phil Brighton	2018
P.solennis__1_	*P.solennis*	Phil Brighton	2018
P.solennis__2_	*P.solennis*	Phil Brighton	2017
P.vanduzeei__1_	*P.vanduzeei*	Phil Brighton	2018
P.vanduzeei__2_	*P.vanduzeei*	Phil Brighton	2017
P.winthemi__1_	*P.winthemi*	Phil Brighton	2017
P.bicolor__3_	*P.bicolor*	Liv. World Mus.	1942
P.bicolor__4_	*P.bicolor*	Liv. World Mus.	1935
P.bicolor__5_	*P.bicolor*	Liv. World Mus.	1943

P.bicolor__6_	*P.bicolor*	Liv. World Mus.	1943
P.bicolor__7_	*P.bicolor*	Liv. World Mus.	1957
P.bicolor__8_	*P.bicolor*	Liv. World Mus.	1996
P.bicolor__9_	*P.bicolor*	Liv. World Mus.	1996
P.bicolor__10_	*P.bicolor*	Liv. World Mus.	1997
P.flavifrons__1_	*P.flavifrons*	Liv. World Mus.	1936
P.flavifrons__2_	*P.flavifrons*	Liv. World Mus.	1943
P.flavifrons__3_	*P.flavifrons*	Liv. World Mus.	1999
P.geniculata__3_	*P.geniculata*	Liv. World Mus.	2008
P.haemorrhoum__5_	*P.haemorrhoum*	Liv. World Mus.	1941
P.haemorrhoum__6_	*P.haemorrhoum*	Liv. World Mus.	1957
P.haemorrhoum__7_	*P.haemorrhoum*	Liv. World Mus.	2002
P.hyoscyami__1_	*P.hyoscyami*	Liv. World Mus.	1936
P.provecta__1_	*P.provecta*	Liv. World Mus.	2004
P.rubivora__3_	*P.rubivora*	Liv. World Mus.	1951
P.rufina__1_	*P.rufina*	Liv. World Mus.	1959
P.rufina__2_	*P.rufina*	Liv. World Mus.	1996
P.geniculata__4_	*P.geniculata*	Liv. World Mus.	1996
P.solennis__3_	*P.solennis*	Liv. World Mus.	1943
P.vittigera__1_	*P.vittigera*	Nat. Mus. Scot..	2010
P.hyoscyami__2_	*P.hyoscyami*	Nat. Mus. Scot.	1993
P.hyoscyami__3_	*P.hyoscyami*	Nat. Mus. Scot.	1989
P.laticornis__1_	*P.geniculata*	Nat. Mus. Scot.	1910
P.geniculata__5_	*P.geniculata*	Nat. Mus. Scot.	2010
P.geniculata__6_	*P.geniculata*	Nat. Mus. Scot.	2010
P.geniculata__7_	*P.geniculata*	Nat. Mus. Scot.	2010
P.geniculata__8_	*P.geniculata*	Nat. Mus. Scot.	2010
P.solennis__4_	*P.solennis*	Nat. Mus. Scot.	1981
P.solennis__5_	*P.solennis*	Nat. Mus. Scot.	1990
P.pulchripes__1_	*P.pulchripes*	Nat. Mus. Scot.	1954
P.bicolor__11_	*P.bicolor*	Nat. Mus. Scot.	1981
P.bicolor__12_	*P.bicolor*	Nat. Mus. Scot.	1970
P.geniculata__9_	*P.geniculata*	Nat. Mus. Scot.	2010

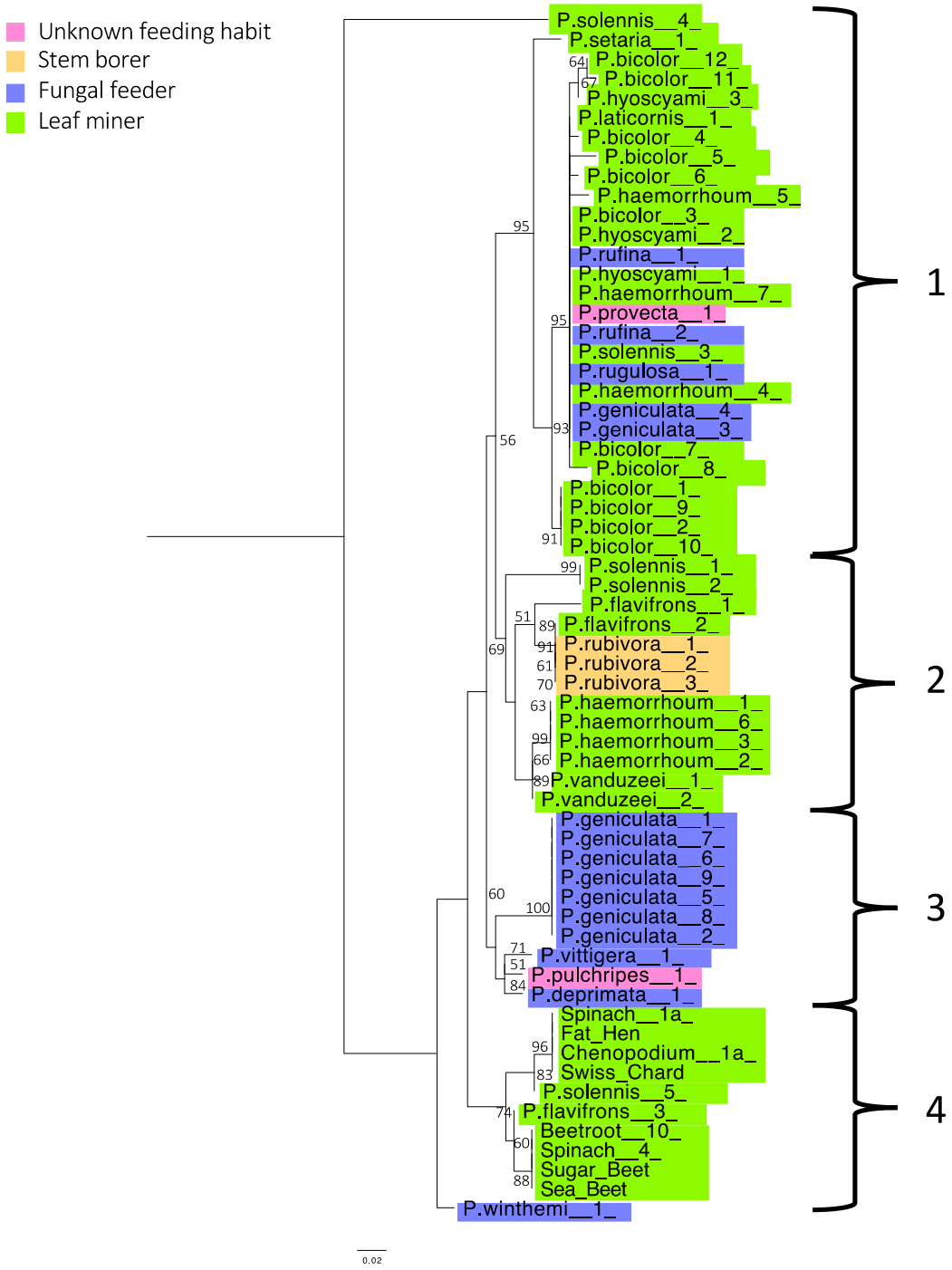

Figure 4.1 Maximum Likelihood tree of 62 Cytochrome C Oxidase Subunit I (COI) *Pegomya* sequences spanning 17 species, constructed using the model of best fit K3Pu+F+I with 1000 ultrafast bootstrap values. Larval feeding behaviours for each species within the genus *Pegomya* are coloured based on the key indicated. Species names and numbers are reflected from those defined in Table 4.2, with the addition of 8 sequences identified from field specimens in chapter 3 under host plant names. Groupings 1 – 4 were defined based on branching and sequence clustering within the tree.

Table 4.3 *Pegomya* larval feeding behaviours of species in this analysis with associated host data, including the information source.

Species	Larval Feeding Behaviour	Host	Source
P.betae	Leaf miner	*Beta* spp.	Ackland et al. 2017
P.bicolor	Leaf miner	Polygonaceae	Ackland et al. 2017
P.cunicularia	Leaf miner	*Beta* spp. Often on coasts.	Ackland et al. 2017
P.deprimata	Fungal feeder	Fungus	Ackland et al. 2017
P.flavifrons	Leaf miner	Caryophyllaceae	Ackland et al. 2017
P.geniculata	Fungal feeder	Fungus	Ackland et al. 2017
P.haemorrhoum	Leaf miner	*Rumex* spp.	Ackland et al. 2017
P.hyoscyami	Leaf miner	*Beta* spp.	Ackland et al. 2017
P.laticornis	Leaf miner	*Arctium* sp.	Ellis, 2020
P.provecta	Unknown	n/a	Ackland et al. 2017
P.pulchripes	Unknown	n/a	Ackland et al. 2017
P.rubivora	Stem borer	*Rubus* spp.	Ackland et al. 2017
P.rufina	Fungal feeder	Fungus	Ackland et al. 2017
P.rugulosa	Fungal feeder	Fungus	Ackland et al. 2017
P.setaria	Leaf miner	*Polygonum*	Ackland et al. 2017
P.solennis	Leaf miner	*Rumex* spp.	Ackland et al. 2017
P.vanduzeei	Leaf miner	*Rumex* spp. Formerly *P.versicolor* *Polygonum* & *Rumex* spp. (Polygonaceae) (*P.versicolor*)	Ackland et al. 2017 Griffiths, 1997
P.vittigera	Fungal feeder	Fungus *Leccinum* (Boletaceae)	Ackland et al. 2017 Griffiths, 1997
P.winthemi	Fungal feeder	Fungus Wide range of fungi. In particular *Leccinum* & *Boletus* (Boletaceae)	Ackland et al. 2017 Griffiths, 1997

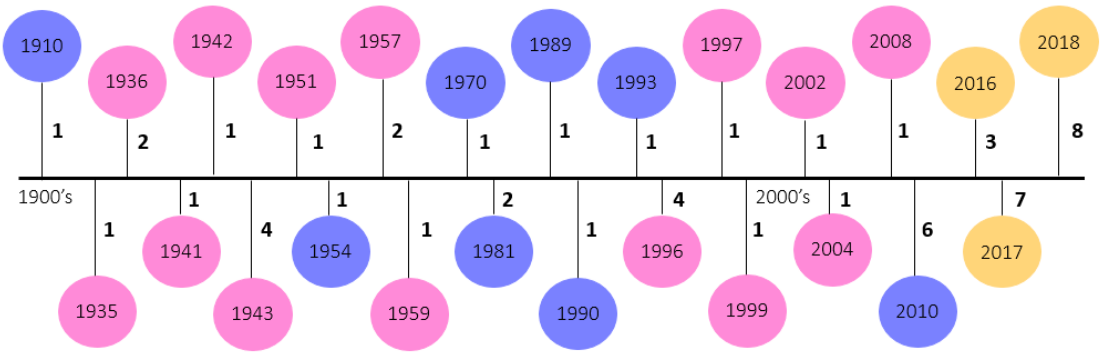

Figure 4.2 Visual timeline of collection dates for the *Pegomya* specimens sequenced in this chapter. The number of specimens collected on each year is to the right of each branch (colour coded based on source: purple – National Museums Scotland, pink – Liverpool World Museum, orange – specimens collected by Dr. Phil Brighton).

4.6 Discussion

Genetic analyses of museum and field specimens showed that the constructed phylogeny grouped the majority of samples to species level. Amplification of a smaller fragment of the mitochondrial COI gene was successful for a number of historical specimens, some of which dated back to the early 1900s. Some level of resolution was attained for several species groups within this analyses, however, one of the monophyletic clusters had multiple species grouped within a single branch. Amplification of two nuclear loci of recently caught specimens was unsuccessful. Examination of the morphology of anomalous specimens identified from the tree, most situated within the mixed species branch in group 1, confirmed original species identification. Whether the 172bp fragment length sequences obtained contained sufficient variation to distinguish species and fully resolve the *Pegomya* phylogeny remains somewhat unclear.

Of the *Pegomya* species that were sequenced, 8 species have leaf mining larvae (excluding specimens from our own collection), 6 species with fungal feeding larvae, 1 with stem boring larvae and the larval feeding behaviours of 2 species being currently unknown. Relatively little is known about many of the species included in this analysis, with much of the information available in the descriptions of species checklists. The genus *Pegomya* has formerly been divided into two subgenera based on feeding habits and ovipositor length, with the sub-genera *Pegomya* largely comprising of species with leaf mining larvae, and the genus *Phoraea* comprising of species with fungal feeding and stem boring larvae (Ackland *et al*., 2017). Within the data set there is a level of grouping between same species, and to a degree, those with similar larval feeding behaviours. However, as the bootstrap values of the ancestral branches are low I cannot say for certain whether this is actually the case. In addition to this, I had significantly more sequences from specimens with leaf mining larvae compared to those that have fungal feeding or stem boring larvae, creating potential bias within the data set. It is also possible that due to the shortness of sequence length that I did not fully capture the variation required between sequences to fully delineate the relationships between species with differing larval feeding behaviours.

Future comparisons of mitochondrial and nuclear phylogenies of specimens collected in the field, accompanied by museum data, may confirm or reject the possibility of grouping species with similar larval feeding behaviours. Multi-locus analysis has proven to be more effective when constructing phylogenetic relationships. Studies have shown that multi-locus phylogenies are more stable, having higher boot-strap support values for individual tree branches, therefore enabling a superior level of discrimination between individuals

(Guo *et al.*, 2008). Both mitochondrial and nuclear loci have their strengths and weaknesses (see summary Table 4.4) and so uniting the two helps to counteract their failings. Although sequencing of nuclear loci was unsuccessful in this chapter, this was most likely in part due to time restrictions not allowing for optimisation of the molecular protocol. Testing the amplification of different loci, using different primers, altering PCR conditions are just a few of the factors that I could have adjusted to achieve higher resolution. In addition to this, examination of a wider range of species, both native to the UK and non-native, and from a broader range of British and international collections, could have provided additional knowledge of this genus.

Table 4.4 Benefits and limitations to the amplification of mitochondrial vs. nuclear DNA (adapted from Lin & Danforth, 2004).

MITOCHONDRIAL PRO'S	MITOCHONDRIAL CON'S	NUCLEAR PRO'S	NUCLEAR CON'S
Easier to amplify. Conserved primers widely available. Lack non-coding regions. Clonally inherited (through the maternal lineage). Non-recombining: makes recombination, paralogy and heterozygosity (heteroplasmy in mit. genes) less of a problem for phylogenetic analysis. Mitochondrial genes evolve at higher rates – good for analyses of closely related taxa with recent divergence.	Nuclear copies of mitochondrial genes may exist. Mitochondrial genes evolve at higher rates - good for analyses of closely related taxa with recent divergence. All mitochondrial genes linked on the same chromosome – don't provide an independent estimate of phylogeny. Have attributes that can lead to high levels of homoplasy when analysed by standard phylogenetic methods – E.g. extreme A/T bias in the 3rd positions.	Less biased base composition. Generally evolve more slowly than mitochondrial genes. Include both slowly evolving regions (exons) and more rapidly evolving regions (introns). Generally observed to provide greater phylogenetic resolution when used in combination with mitochondrial genes (especially at deeper taxonomic levels). Show lower levels of homoplasy (measured by consistency index (CI)). Greater bootstrap and Bremer support.	Non-coding regions (E.g. introns) common in single copy nuclear genes. Occur in lower copy number and can be more difficult to amplify. Often involve two or more paralogous loci that can cause problems for phylogenetic analyses. à occurs in at least 5 copies in insects so when analysing *Wingless* sequences some caution is necessary.

The phylogenetic tree seen in Figure 4.1 showed that there was some resolution across the data set, but with the majority of group 1 with mixed species grouping along one branch in particular. The majority of sequences grouped here were that of *P.bicolor*, with some of the smaller branches within this clade having good bootstrap support and same species grouping. Within the long branch there was the addition of sequences from 8 other species; leaf miners *P.hyoscyami, P.laticornis, P.haemorrhoum* and *P.solennis*, fungal feeding *P.geniculata, P.rufina* and *P.rugulosa*, and *P.provecta*, whose larval feeding habits are largely unknown. Other sequences from some of the species here (for example *P.geniculata*) have been grouped elsewhere in the tree with other specimens of the same species and with strong bootstrap support. These were the specimens that made up the majority of those I isolated for morphological re-examination as there was potential for misidentification. The specimens shown here were confirmed to be true to their original identifications however, ruling out this possibility. It is important to note that the sequences of these specimens with differing species identifications have been grouped together based on similarities in the mitochondrial DNA sequences I obtained. Whether there is a degree of morphological ambiguity amongst the specimens that have been grouped together here remains unclear, but perhaps should be investigated in the future. As seen in chapter 3, there is a possibility that cryptic species are hidden within the Anthomyiidae due to morphological similarities between species. Cryptic species are two or more species previously identified by similar morphology as a single species, but that are distinct from one another (Bickford *et al.*, 2007; Pfenninger & Schwenk, 2007). Cryptic species are more common than previously thought and should not be ignored when assessing the phylogenies of morphologically similar species (de León & Nadler, 2010).

In groups 2 and 3 (Figure 4.1) sequences of the same species mostly group closely together. In group 2 there are 5 species present; *P.solennis*, *P.flavifrons, P.haemorrhoum* and *P.vanduzeei,* with leaf mining larvae, and *P.rubivora* with stem boring larvae. Both *P.haemorrhoum* and *P.vanduzeei* are known to be associated with hosts in the genus *Rumex* (Polygonaceae) (Ackland *et al*., 2017) and were found to have a recent common ancestor which is supported by strong bootstrap values. Sequences of *P.solennis*, which is also known to mine *Rumex* spp. in its larval stage (Ackland *et al*., 2017) likewise shares an ancestor with *P.haemorrhoum* and *P.vanduzeei*. The evolutionary race between phytophagous insects and host plant is well documented as playing a role in speciation events (Janz & Nylin, 2008; Forbes *et al*., 2017) and the possibility should be explored further with a wider range of samples of these species and amplification of multiple loci. Contrasting this is the relationship within this clade between *P.rubivora* and *P.flavifrons*. *P.rubivora* has stem boring larvae which have been recorded as associates of *Rubus* spp. (Rosaseae) (Ackland *et al*., 2017), whilst *P.flavifrons* are recorded as mining members of the Caryophyllaceae (pink) family (Ackland *et al*., 2017). Interestingly, the stem boring larvae of *P.rubivora* theoretically should have been more closely related to those species with fungal feeding larvae based on previous approaches to dividing *Pegomya* species between the two sub-genera. But as the only representative species with stem boring larvae, conclusions cannot be fully made as to the evolutionary implication of the placement of this species within this tree.

There are several placements of individual species sequences within, or near clades grouping the same species. In group 3 (Figure 4.1) three sequences of individual species; *P.deprimata, P.pulchripes* and *P.vittigera*. *P.vittigera* sequences from GenBank appear to

be related to the single *P.vittigera* (1) from our own data set and this was supported by a strong bootstrap value when adding these GenBank sequences into the analyses. The placement of the remaining species is less certain. *P.pulchripes* (1) was isolated alongside other sequences for morphological verification. It was the only sample out of the 9 Dr. Tony Irwin verified that left some uncertainty as to its original identification. The original species identity was tentatively confirmed based on the specimen not fitting other species descriptions as well as it did the description of *P.pulchripes* (Ackland *et al*., 2017). This specimen was female, which in the Anthomyiidae are generally understood to be the harder sex to identify. It is recommended that in future analyses of this specimen that its identification is compared with that of a female of the same species raised from a mating pair (Dr. Tony Irwin, personal communications, 2021). *P.flavifrons* (3) and *P.solennis* (5) (group 4, Figure 4.1), were grouped within the same clades as my sugar beet leaf miner sequences, with strong bootstrap support values for both branches. Neither of these two sequences underwent additional morphological analyses so it is possible that either their original identification is incorrect, or there is morphological ambiguity and similar sequence variation to the sugar beet leaf miner sequences they were grouped with. There was low bootstrap support for the placement of the single *P.winthemi* (1) specimen. *P.winthemi* (1) underwent morphological verification and has been categorised as a certainty in relation to its original identification. Therefore, sequencing of a wider sources (historical and recent collections) of *P.winthemi*, as well as larger fragment length sequences, may resolve the position of this species within the tree.

The addition of four randomly chosen representative samples from groups A and B that were defined in Chapter 3 were used to assess the relationships of the sugar beet leaf

miners and other *Pegomya* species. The resulting positions of these sequences corresponds to the two groups I identified in Chapter 3, though due to low bootstrap support on ancestral branches I can't be certain of their relationships with the other *Pegomya* species in this phylogeny. The three *Pegomya* specimens obtained from museum collections are grouped within the unresolved branch of group 1, when theoretically they should be grouped near the sequences from leaf miner I collected in the field. When looking at the dates these specimens were collected, two were collected after the publication of Michelson's paper on the *P.hyoscyami* complex (1980), while one was collected in the 1930s. I can be fairly certain that the two collected post-1980 were subject to morphological identification based on Michelson's (1980) description of the complex. The specimen collected from the 1930s was possibly grouped within this part of the tree due to misidentification, but as the sample originated from the Liverpool World Museum collection it is likely to have been verified since then. Whether the placement of these three sequences in the tree is again due to some level of genetic distinction and morphological ambiguity remains to be seen. More data on historical specimens and those collected in the field should help to clarify this.

Of the samples I sequenced I had more success in obtaining sequences from the Phil Brighton and Liverpool World Museum collections relative to the number of specimens I sampled. I obtained sequences from all 18 of the Phil Brighton specimens. As these were fresher specimens than those of the museum collections, with the oldest specimens being from 2016, this reflects that the age of specimens can be key in the attainment of DNA (Wandeler *et al*., 2007; Besnard *et al*., 2016). Out of all the collections, the Liverpool World Museum collection was kept in wet storage after the removal of the tissue sample

from dry, pinned specimens. I obtained 22 sequences out of a possible 74 from the Liverpool World Museum collection, and 14 out of a possible 105 from National Museums Scotland. Although this is relatively few compared to the total number I could have achieved, the specimens in this collection are older than some of the more recently collected samples I obtained sequences from. Again, reiterating the importance of age as a factor in the success rate of sequencing specimens. Notably, I recovered sequences from samples with DNA quantity less than 1 ng/µl when quantified with the NANODROP 8000 (Thermo SCIENTIFIC). After obtaining sequences from several where the nanodrop readings were very low I tested all samples regardless. In addition to this I ran my samples on a 1.5% agarose gel. This method has been suggested as not sensitive enough to fully capturing smaller fragments from degraded samples, and that a capillary electrophoresis might be better suited (Sutrisno, 2012). It may be worthwhile altering the methods of testing DNA samples obtained from historical specimens in order to better capture the data they contain in future. Overall, though the number of sequences I included in the final analyses from historic specimens is a significant achievement and reflects the importance of using historic specimens for phylogenetic analyses.

This study provides an initial insight into the relationships of several *Pegomya* species. Though there are many gaps in this chapter it may form a basis for future phylogenetic studies. In particular, the use of museum data is of particular interest as I have successfully obtained the DNA sequences of several older specimens. Museum collections contain a fountain of untapped knowledge and should be considered in any future studies. Of the gaps in this chapter, the lack of nuclear data is particularly noticeable, as the addition of this data to what I represent here may rectify some of the

unresolved clades or clarify species groupings elsewhere. It is imperative that the combination of both mitochondrial and nuclear DNA in the phylogenetic analyses, as well as the morphological data, of such a morphologically challenging family as the Anthomyiidae is utilised to the fullest extent as this may uncover any cryptic speciation, evolutionary patterns and relationships in species with different larval feeding behaviours.

4.7 References

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CHAPTER 5

Rearing method for *Pegomya* species mining leaves of *Beta vulgaris* and associated host plants

Leaf miner collected from Sea Beet at Burnham Ovary Staithe, Norfolk, 2020 (Photo by Siobhan Hillman)

5.1 Contributions

The work in this chapter was done by myself, with help in maintaining the host plants from the JIC insectary team; Anna Jordan, Jake Stone, Susannah Gill and Darrell Bean.

5.2 Abstract

Cultivating pest species within a laboratory environment can allow more detailed study of the target species. Many studies have focused on understanding the life history traits of pest species. This is a fundamental aspect of any management strategy for any pest because if the identification is wrong then costs can be catastrophic, both in terms of crop and monetary loss. The sugar beet leaf miners have historically been regarded as a number of different species, most commonly either *P.hyoscyami* and *P.betae*. Though generally known to be a minor pest of sugar beet, it is surprisingly difficult to obtain information regarding the life histories of either species, especially with regards to how it may influence modern day sugar beet management. In this chapter I report findings on how to culture sugar beet leaf miners, including the problems faced with setting up a laboratory culture. I also discuss what plans were made for the use of the culture, with particular attention to experiments relating to sugar beet yield and host plant choice.

5.3 Introduction

The sugar beet leaf miners (commonly referred to as mangold fly in the sugar beet industry) have generally been considered minor and sporadic pests of sugar beet in the UK (White, 2016a). Recent outbreaks however stimulated the need to understand more about these species. Problematic leaf miner activity late in the sugar beet growing season was reported in 2011 (Stevens *et al*., 2012), and 2014 (Stevens, 2014; Stevens; 2015), though this was not widespread at the time. It was only in 2015 and 2016 leaf miner became a real issue within sugar beet crops, with estimates of up to 50-70% canopy loss as a result of feeding damage in afflicted fields (White, 2016b). In British Agriculture and Horticulture the sugar beet leaf miners are usually referred to as a single species (Stevens 2012, 2014, 2015; White, 2016a, 2016b). This differs from an entomological view on the *P.hyoscyami* species complex which refers to four species; *P.hyoscyami, P.betae, P.cunicularia* and *P.exilis,* as published by Michelson (1980). In earlier chapters I discuss the importance of understanding what species truly mine sugar beet and how different species may interact with one another. This can be shortly followed by the basic, but fundamental need to understand life histories of pest species, and how these may differ between species.

Sugar beet leaf miners are recorded as having up to three generations per year. The first generation occurs usually around April/May, the second in June/July and the third in August/September (Figure 5.1). Larvae of the third generation drop down into the soil and overwinter as pupae until the adults emerge again at the start of the year around March time. Adult *Pegomya* will then lay their eggs on the underside of the leaf in rows

of between 2-10 (average). These eggs are small (<1mm), white and oval in shape. Shortly after laying the eggs the first instar larvae will hatch and burrow into the leaf at the point where the egg adheres to the leaf surface, this usually takes up to 5 days. From there the larvae will undergo rapid growth through three larval instars, reaching the final instar within approximately two weeks. Upon reaching the final instar larvae will then emerge from the leaf, drop into the soil and pupate, where they stay until emerging again as fully formed adults (Cameron, 1914; White, 2016a).

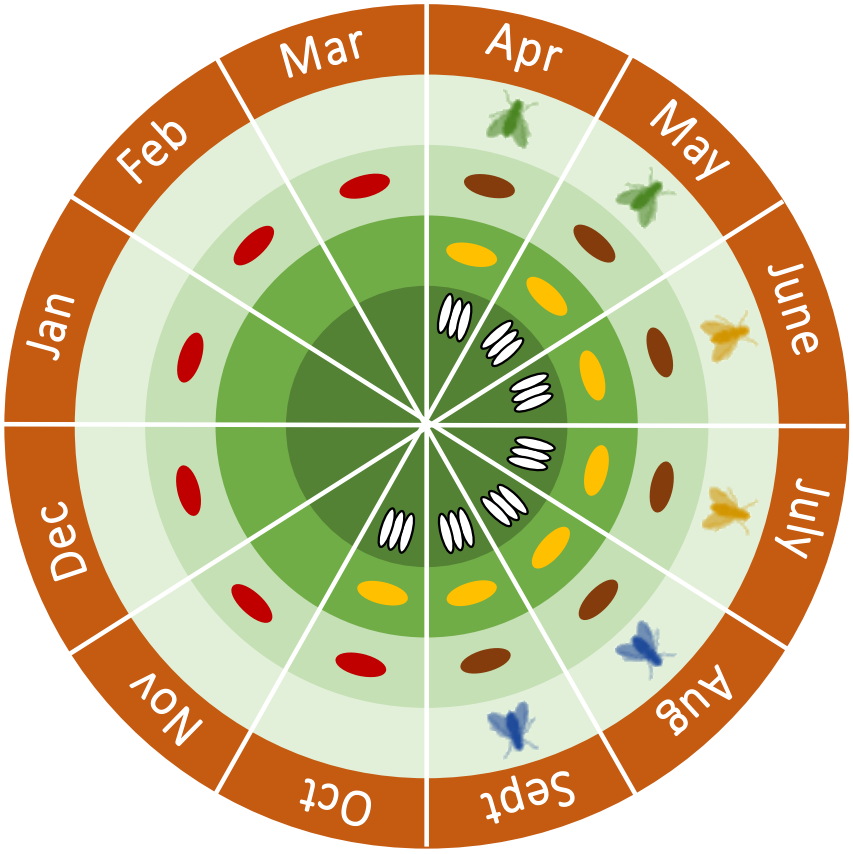

Figure 5.1 Leaf miner life cycle as seen in UK sugar beet. 1ST generation adults: April/May, 2nd generation adults: June/July and 3rd generation adults: August/September. The flies overwinter as pupae during the winter months (red pupae). Eggs (white) are laid by newly emerged adults and hatch as larvae (yellow), feeding on the leaves of the host plant and pupate in the soil between generations (brown pupae) before emerging again as adults.

Cameron (1914) is amongst the first available records of rearing a culture of one of the sugar beet leaf miners, *P.hyoscyami*. Here *P.hyoscyami* is referred to as the *Belladonna* leaf miner due to the authors experiments in rearing the species on a *Belladonna* host. It is a comprehensive study on not only the life history of *P.hyoscyami*, but also the previous taxonomic history of the species, as discussed in Chapter 2. This study differs from others of its kind in that specimens were reared past adulthood onto the next generation and conditions the flies were kept in were recorded. In his experiments on *P.hyoscyami*, adults were fed on a sugar solution and kept in a laboratory at about 70 °F, where he managed to witness adults mating, laying eggs and subsequently larval and pupal development. Observations were made on these behaviours, and he recorded that an interval was necessary between mating and the laying of eggs, and that sometimes more than one batch of eggs may be laid on a single leaf (three or four in some cases). He also observed that whilst laying eggs adult flies seemingly prefer the top shoots of the host plant, with egg laying on radical leaves occurring more frequently towards the latter end of the season. Cameron published another paper on *P.hyoscyami* in 1916, where larger scale trials (similar to that of a glasshouse trial) in rearing the species were performed. The aim in this paper appeared to be to clarify the misinterpretation that *P.hyoscyami* mined dock plants as published in a different article by different authors. In these experiments *P.hyoscyami* and *P.bicolor* (a species known to mine docks) reared on a specific host plant were then introduced to a different host and the behaviours of the flies were recorded. The results of this experiment showed, to a degree, some preferences in both species and their host plant associations. *P.hyoscyami* reared on *Belladonna* would oviposit and complete its life cycle on mangold hosts, but *P.hyoscyami* reared on mangold would not oviposit on that of sugar beet.

In this chapter I provide an overview of culturing methods for leaf miner collected from sugar beet and related hosts. The purpose of starting a leaf miner culture was to gain a deeper understanding of leaf miner life history, as well as to determine the effects of leaf miner infestations on different host plant species, specifically on sugar beet. Previous attempts at culturing species that comprise of the sugar beet leaf miners have been made (Cameron, 1914; Cameron, 1916). I wanted to put this into the context of the present-day taxonomic standing of the sugar beet leaf miners by investigating the consequences of leaf miner colonisation on host health and end yield, and the resulting implications for the sugar beet industry in the UK. I outline future experimental plans for sugar beet leaf miner cultures. These experiments, if conducted, would provide a greater depth of knowledge into the sugar beet leaf miners' behaviours and relationships with their hosts, as well as how different species of sugar beet leaf miner potentially interact with one another.

5.4 Methodology

5.4.1 Collection of leaf miner

Larvae were obtained for the first line of the culture from beetroot and Swiss chard (*Beta vulgaris* spp. *vulgaris*) leaf samples from a garden in Norfolk in June 2019, and from leaves of sugar beet (*Beta vulgaris* spp. *vulgaris*) at a site in Suffolk in June 2019. After this line failed, a second line was set up from samples obtained by the BBRO at a site in Norfolk in September 2019. Like in Chapter 3, the locations have been scaled up to county level to protect individual anonymity.

5.4.2 Host plants

Two non-treated sugar beet seeds var. Cayman, obtained from the British Beet Research Organisation, were sown per pot in John Innes No.11 compost, labelled and then placed within watering trays inside a mesh cage within the JIC glasshouses. Pots in which two plants had successfully germinated were separated and planted individually inside new pots. Plants were watered weekly until reaching the 8 – 10 leaf stage, suitable for addition to maintain the insect culture. In instances where there were insufficient sugar beet hosts present in the JIC facility, host plants of a similar stage were available from the BBRO stock.

5.4.3 Pilot Culture

A pilot culture was commenced in 2018 with the few individuals (±10) that were collected that year. Larvae were reared through from samples collected in 2018 from Swiss chard and beetroot in Norfolk as described above. The culture was kept in similar incubation conditions to the ones outlined below with the exception of a different diet. Adults were fed a diet of sugar-water (50:50). However, this did not support them, and all adults subsequently died before mating and thus the next generation was not produced and the culture became defunct. Following this pilot culture, a revised protocol was created.

5.4.4 Rearing of Leaf Miner

Larvae were initially reared through in quarantine to prevent any introduction of plant disease and other potentially damaging invertebrates into the controlled temperature rooms from leaf material obtained from outside the institute. This was made as an additional revision to the protocol after spider mites infested the host plants in the first colony and ultimately caused the collapse of that line. Leaves containing larvae were set up in glass conical flasks containing water to sustain the leaves. The top of the conical flask was sandwiched with sponge to prevent larvae from falling into the water (Figure 5.2). These flasks were then placed and contained within a Perspex rearing cage with mesh doors on a bench in the laboratory at natural room temperature and conditions. Larvae were reared through to pupae (average developmental time ~ 2 weeks) in these conditions before being removed from quarantine using soft forceps and a paintbrush. Pupae were then placed in a petri dish in preparation to be added to the culture cages. Any parasitoids then emerging were easily removed before the addition of the adult flies into the culture.

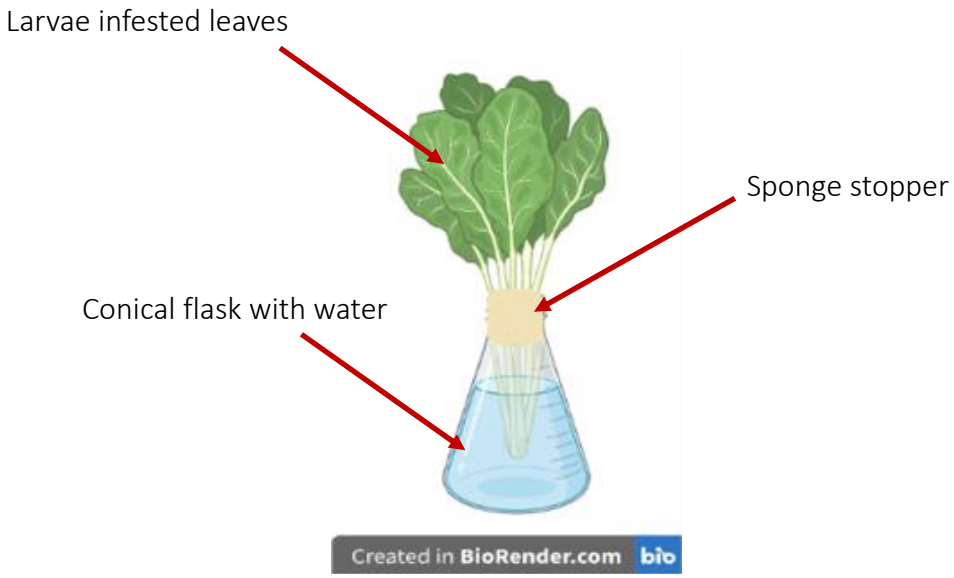

Figure 5.2 Image of conical flask containing infested leaves. The opening to the conical flask is stoppered with sponge to prevent larvae mining the leaves from falling into the water. Created with BioRender.com and edited in PowerPoint.

5.4.5 Culturing of sugar beet leaf miners (Line 1)

A fresh cage of the same dimensions was set up within a controlled temperate room (CE2 of the JIC facility) maintained at 22° day/20° night on a 14-hour day cycle (04:00 – 18:00). A plastic drip tray was placed inside the fresh cage and blue tissue paper laid in layers inside to prevent any subsequent damage to the pupae during removal. A single sugar beet plant at approximately the 8-leaf stage was placed within a watering dish containing perlite near the rear of the drip tray. A small conical flask containing cotton wool and water and a small petri dish of pollen (Agralan) were placed near the front of the cage to act as a source of food for the adult flies. Initially, pupae were kept in a large petri within the cage and allowed to emerge (Figure 5.3). I later changed this part of the protocol as

the emergence of the adults was very staggered and resulted in larvae mining the host plant before the majority of the adults had emerged. Pupae were later kept in separate containers and upon emergence adults were then added to the cage and allowed to mate. Once the sugar beet was at maximum capacity with a number of eggs and mines, adults were removed. Larvae were allowed to complete their full larval development inside the rearing cage, with the necessary addition of another host plant if required, until their removal at the pupal stage and placed into a petri dish. The steps above were then repeated for the next generation (See protocol 1, appendix).

Figure 5.3 Top-down view of initial culture set up. Pupae were kept within the culture cage and allowed to emerge and mate. Blotch mines on sugar beet leaves are of larvae already present within the culture. In the top right is a water dish with cotton wool to prevent drowning, and the top left (not seen in this photo) is a dish of pollen. Enlarged pupal photo is of a pupae that pupated on a leaf.

5.4.6 Culturing of sugar beet leaf miners (Line 2)

The first and second generations of line 2 were maintained as in the above section, with generation 3 of line 2 maintained with the revised protocol below. Two cages, labelled A and B, of the same dimensions as the quarantine cage were set up in a controlled temperature room (CE5 of the JIC facility) maintained at 24° day/20° night on a 14 hour day cycle (05:02 – 19:01). Blue tissue paper was added to line the bottom of the cage to prevent any escaping pupae and old plant matter sticking the pupae to the Perspex or bottom of the tray. A single sugar beet plant grown to the 8 – 10 leaf stage was added into the cage inside a watering dish containing perlite. The addition of a small conical flask containing cotton wool and water as well as a small petri dish of pollen was also added to each cage. 130 pupae collected from the previous generation were split into two petri dishes of equal number for one petri dish per rearing cage. Adults were allowed to emerge within the petri dishes and once a set quota of adults (29) was reached within each, these were then added to the rearing cages. Adult flies were initially allowed to mate for up to seven days before removal from the cage but this was extended to 10 – 14 days due to a lack of eggs on the hosts. Once the adults had been removed any remaining eggs and larvae were allowed to complete their larval development within the rearing cages. Pupae from each cage were collected and placed inside clean petri dishes.

As pupae from the third generation did not reach high enough numbers from each cage the fourth generation was the last of this line. Should there have been enough pupae to continue the line the following part of the protocol would have been followed. Each batch of pupae must be split again into two halves and labelled. One half of the pupae from

Cage A was added to the pupae obtained from Cage B, and vice versa. The rearing cages were removed and cleaned during this process. Fresh water and pollen was added to the cages along with each batch of pupae. Adult flies were allowed to emerge in the rearing cage without a host plant. At a set number of adults (X males, X females), the host plant was then added to each rearing cage and adults were allowed seven days to mate before their removal. The process above is then repeated for the next generation (Figure 5.4, see protocol 2, appendix).

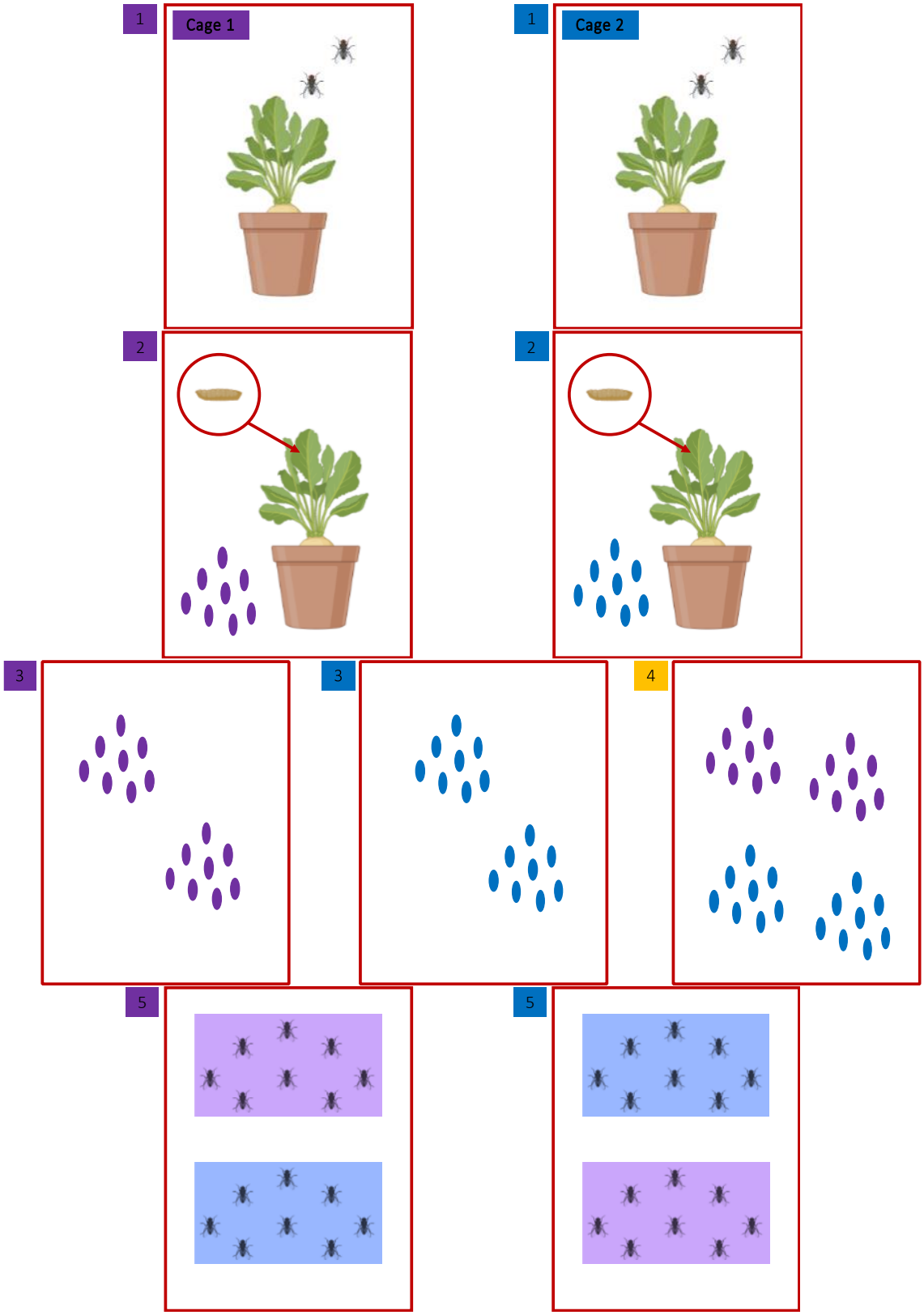

Figure 5.4 Modified culture protocol for generation 3 (Line 2). See appendix for step-by-step guide. Created with BioRender.com and edited in PowerPoint.

5.5 Results

The change of diet from sugared water to pollen was a success and adults bred and produced the next generation successfully. Adult flies could happily survive in the conditions provided for > 2 weeks. The first line concluded after 3 generations due to two issues; the accidental release of several individuals from the main culture, and the infestation of spider mites (Family: Tetranychidae) on the sugar beet host. Initially in the 3rd generation of this line there were approximately 30 adults, but due to the gap in the cage lining the final number of adults was a third of what it had started as (Table 5.1). In addition to this, a sugar beet host from the BBRO glasshouses was used to supplement feed the larvae. This plant had previously been treated for spider mite but an infestation broke out again regardless and this led to the reassessment of quarantining material from outside the JIC facility before addition to the cultures. Due to the infestation all material within the culture, including the adults and infested host plant, had to be disposed of to prevent spread to other parts of the facility as spider mites are highly prolific pests.

The second line of leaf miner resulted from a presumed late emerging third generation from a single sugar beet field in Norfolk. Quarantine of the samples resulted in the emergence of several parasitoids which were separated from the remaining larvae to prevent decimation of any future generations. Like the previous line, the second line died out after 4 generations (Table 5.1). As part of this second line the aim was to reach a set number of adults to the culture, with the goal of regulating certain parts of the culture methodology. It was during this aspect of the initial set up that there were some issues as the emergence of the adults was very staggered. The resulting wait time to reach

sufficient numbers of adults prior to addition into the controlled temperature cage was problematic and the numbers of adults were not ideal. This was further exasperated when it was decided that the culture should be divided into two separate cages. The reasoning behind this modification to the protocol was to prevent inbreeding in later generations. Furthermore, the adults from the third generation did not breed as prolifically as in previous generations. It took longer than anticipated for the adults to begin mating and consequently longer before any first instar larvae were detected.

Table 5.1 Approximate number of leaf miner adults per line and generation *generation with escaped adults. **Adults split into two cages during this generation following protocol 2 (see appendix), total number of adults is therefore double.

Line	Generation	Start date for each gen.	Average No. Adults
F1	1	June 2019	50
F1	2	July 2019	40
F1	3	Aug 2019	10*
F2	1	Sept 2019/Oct 2019	60
F2	2	Oct 2019/Nov 2019	30
F2	3	Nov 2019/Dec 2019	30**
F2	4	Dec 2019/Jan 2020	10**

Table 5.2 Problems that arose while attempting to culture sugar beet leaf miners, how they were solved during this process and suggestions for how to solve unresolved issues in future. Each point is discussed in more detail within the discussion.

No.	Problem	Proposed solution
1	Diet of sugar-water	Diet of pollen.
2	Cryptic species	Separate samples based on host plants larvae were collected from. See chapter 3 for reference as to genetically distinct groupings.
3	Adult Emergence	Alteration of day light hours/storage temperature.
4	Host turnover	Trial of alternate host to sugar beet.
5	Loss of pupae	Modification of cage layout.
6	Parasitoids	Quarantine of fly specimens before adding to the main culture.
7	Unwelcome pests	Quarantine of any plant material from outside the facility so contamination of any other pests is limited.

5.6 Discussion

Adults showed the capacity to mate once the diet was changed to pollen and appeared to have no issues laying eggs on a sugar beet host. Most eggs also hatched without any problem and throughout each generation the larvae prospered on feeding on sugar beet. During the last generation of line 1 the turnover of sugar beet in the JIC glasshouses was insufficient and additional host plants of a similar age were sourced from the BBRO sugar beet stocks. The BBRO stock had previously undergone management for spider mite infestation and was deemed to be clear of infestation. Upon addition of the new host plant to our culture at JIC there were also no obvious signs of spider mite. However, after some time spider mites emerged on the host plants and spread to other hosts within the JIC incubation chambers. Due to the incubation chambers being used communally for

different experiments, and the voracity of the spread of spider mites potentially across other cultures, this line was quarantined. The infestation on the sugar beet host plant in a communal incubation chamber meant that the culture had to be neutralised and any material associated with this quarantined and removed from the vicinity. The lack of hosts at a suitable age for addition to the culture also led to the idea of trialling the propagation of another, faster growing host (spinach or beetroot etc.). Sugar beet is a slow growing host, and the colony may not have been fully supported at each life history stage because of the turnover of host plants during culturing attempts. There was limited space for growing host plants, which restricted the number of plants grown at any one time. Hence the propagation of a faster growing host would have been an advantage, though this was not tested in the end. There was similar success in producing a second line of leaf miner from samples collected from sugar beet, though staggered emergence of adults resulted in adult numbers of lower quantities than anticipated which may have ultimately led to the extinction of this line. I discuss attempts at culturing sugar beet leaf miners in more detail below.

During the pilot culture (2018), adult flies were fed a mixture of sugar-water which they did not thrive on and adult flies died without producing the next generation. Once the diet was changed to pollen, kept in the same state used to culture bumblebees, and water with no added components, adult flies mated and produced the next generation successfully (see Table 5.2). Initially the diet of sugar solution was used in a study by Cameron (1914) which showed that rearing *P.hyoscyami* on this diet seemed to prolong the life of the adults as they lived for 3 weeks, rather than 2. After failure to get adults to feed or mate I tried the adults on a diet of pollen as they are also regarded as a group of

underrated pollinators. Yasumatsu & Sasagawa (1953) report that during their experiments on *P.hyoscyami* (here referred to as the spinach leaf miner) adult flies were fed on honey, though they do not report the success of this as a diet. This change from sugar-water to pollen in their diet worked and mating between adults of the culture ensued. It is highly probable that *Pegomya* adults will feed on other substances that pollen though. If diet is optimised, it may in turn result in fitter adults and therefore higher reproductivity within the culture.

It is possible that the issues faced during the maintenance of line 1 may have resulted, at least in part, from an inability to reproduce due to the presence of more than one species. The results found in Chapter 3 indicated that there were two genetically distinct groups, seemingly distinguished by host plant range. One of the groups was found primarily on sugar beet, the other on Swiss chard, with a mix between the two groups of leaf miner found on beetroot. As this line originated from a mix of samples from all three of these hosts it is possible that there was more than one species present which in turn reduced the potential number of individuals within the population that could interbreed (see Table 5.2). In both lines, the original individuals used to start the culture were from a limited range of areas and number. Line 1 was more diverse than the second, in that larvae from different hosts were present and there were samples from two different locations. However, as the samples of Swiss chard and beetroot were collected from a single garden the actual geographic range was fairly limited. Likewise, the sugar beet samples used in line 1 were collected from different county but also from a single field. In the Line 2, we had a larger number of individuals present, but they were also collected from a single field in Norfolk. It is therefore possible that many of the individuals used in

both lines came from a single, or relatively few parents, potentially resulting in a degree of inbreeding between subsequent generations.

The set-up implemented during the last generations of the Line 2 may have been more successful if a greater number of individuals were available for addition to the main culture prior to mating. Staggered emergence was observed across each generation and in both lines and was one of the main problems faced during maintenance of the leaf miners (Table 5.2). Although some generations started off with larger numbers of pupae, emergence of adults became problematic as the proportion of adults that would emerge at any one point was significantly lower. Adults would often emerge after the first batch of adults were already integrated into the culture cage. This was particularly pronounced when adults were divided into two separate cages as this stage as dividing the available adults resulted in fewer adult flies per cage. More adults did emerge over time but the addition of late emerging adults at a later stage within the set-up was not ideal. It should be noted that at any point during the culturing attempts no more than 100 adults were present from any generation, and this number was reached only once within a single generation, within a staggered timeframe. In order to conduct any future experiments a larger number of adults would need to be present at the same stage of sexual maturity (>150). Finding ways to speed up adult emergence, and possible reasons for any pupal death, should be a priority in order to achieve this.

Based on the findings in chapter 3, trialling the growth of beetroot plants as the main host appears to be the best plan of action due to both species groups being found relatively evenly distributed across this host plant. There were occasions where the

turnover of sugar beet hosts was not routinely high enough (see Table 5.2), this was in part due to limited space and the fact that sugar beet is a fairly slow growing crop. Due to the infestation of spider mites from an external source it also became apparent that quarantining material from outside sources was necessary. As this step was taken to prevent the spread of any undesirable pest species across the JIC facility it would be reasonable in future to grow a faster maturing crop within the limited allocated space assigned to us.

Some additional alterations should also be made to the final protocol as the temporary solutions made to prevent larvae pupating in difficult areas did not fully resolve the problem (Table 5.2). Several larvae pupated in parts of the Perspex cage which made it hard to remove them without damage. Blocking off possible escape routes for the larvae with different material may reduce the number of larvae pupating in undesirable areas of the cage, e.g. under the watering trays. This was partially solved by putting down layers of tissue paper under the trays as a temporary solution but could have been done better as regardless of this larvae still managed to move into small crevices within the cage. I propose that the host plant be maintained within a watering dish as before, but also placed on a plant stand with grates, so that larvae cannot pupate underneath the watering tray. The main tray lining the base of the cage should also be filled with a soft sand, kept dry, for larvae to drop down from the host and pupate in. The dry material should also help with the stickiness of pupae due to old plant matter and reduce the possibility that pupae will stick to surfaces. I suggest sand instead of soil as soil can become moist and humid which would encourage the growth and infestation of fungi within pupae. Long strips of foam, or a similar product, should also be used to seal off the

sides of the base tray in order to prevent larvae from pupating underneath on the surface of the Perspex cage. See Figure 5.5 for an amended cage set up.

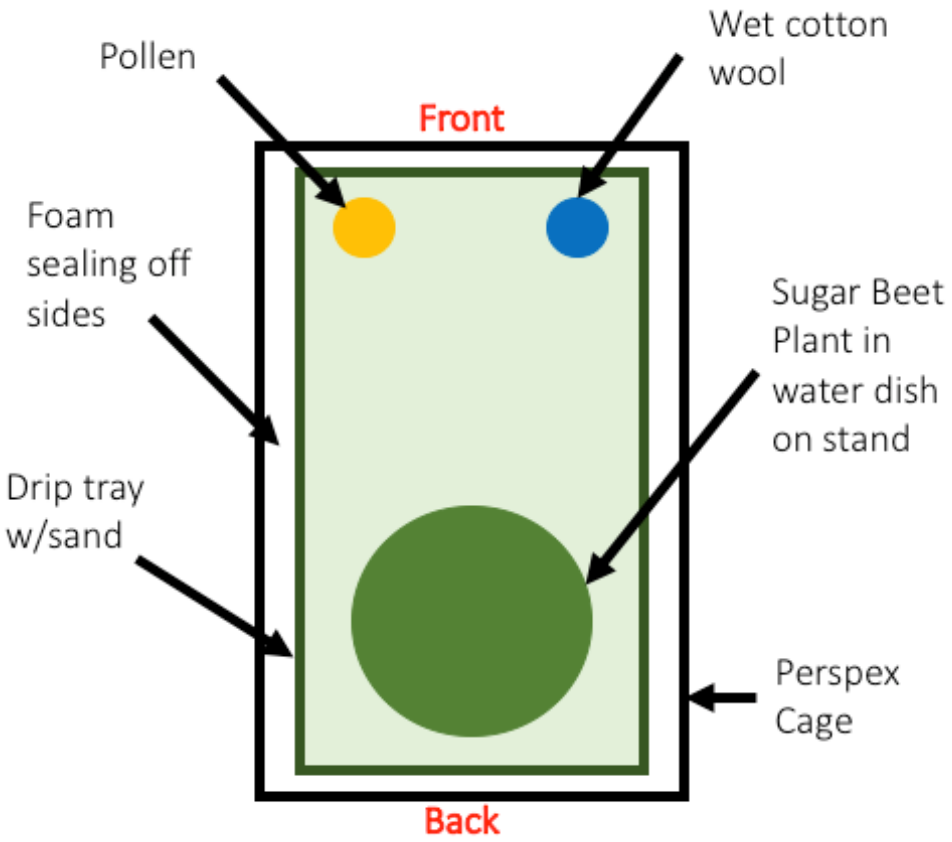

Figure 5.5 Suggestions for the future layout of the leaf miner culture with annotated modifications.

5.6.1 Future experiments with leaf miner cultures

One of the goals for successfully maintaining a culture of leaf miner was to provide a continuous population of leaf miner to use in laboratory experiments. Through these experiments more detailed analysis of the life histories of the leaf miner complex would have facilitated research into the full extent of the effects of leaf miner on sugar beet and related hosts. The rationale and aims of some of these planned potential experiments are discussed in more detail below.

Effect of timing of leaf miner infestation on host pant health

In regard to host health, the effect of leaf miner colonisation at varying stages of plant growth is critically important as the relationships between plant defences in response to herbivore attacks may vary as the plant develops (Boege & Marquis, 2005). Initially, leaf miner could be introduced to isolated sugar beet crops at stages mirroring those seen in the field at times of colonisation. Three generations of leaf miner are potentially observed within the sugar beet crop annually; generation 1 occurs during April/May when the crop is still young and therefore more vulnerable, generation 2 occurs around June/July when the crop is more established, and generation 3 occurs around August/September when the crop is fully established within the field. A predetermined number of leaf miner could be introduced at a single stage of the host's development in order to observe the effects of plant health and survival, as well as end yield, when the host has only had to endure a single generations infestation throughout its annual lifecycle. Individuals could then be additionally subjected to multiple infestations of leaf miner throughout all three stages

when generations may be seen within the field, recording any observations on plant health, canopy loss and end yield. Canopy loss is particularly problematic as it results in the crop diverting energy to restore lost leaves instead of into sugar storage (Stevens, 2014). It would also be interesting to see how various levels of infestation effect the host plant at different times of the year, e.g., low infestation earlier in the year and higher infestation later in the year, as previous attack on the plant may make the host more susceptible later in the season. Previous attacks by one pest on the host plant may also more quickly trigger later attacks by a different pest. Furthermore, different types of herbivorous feeding behaviours may initiate different plant responses (Bruce & Pickett, 2007), and so the response of the plant to an aphid compared to that of a leaf miner may be different. Understanding how plant defences respond to leaf miner attack, as well as other pests, and how the defence mechanisms interact may be crucial in pinpointing what mechanisms can potentially be artificially triggered to aid with defence against pests. I predict that host plants subjected to leaf miner infestation during the first stage of colonisation, when the host is more susceptible and vulnerable to attack, as well as multiple infestations, have lower fitness than hosts subjected to single infestations during the later stages of colonisation, when hosts are producing aspects of natural resistance.

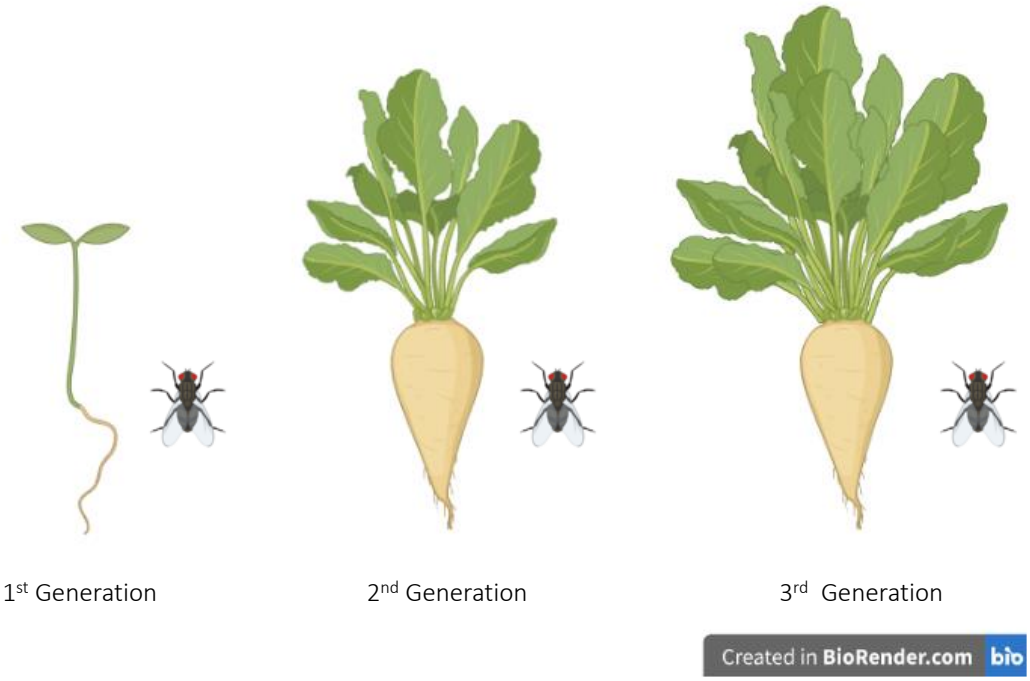

Figure 5.6 Timed releases of a controlled number of adult *Pegomya* on sugar beet hosts to mimic generations seen in the field and investigate effects on host plant health and yield. Created in BioRender and edited in PowerPoint.

Effect of leaf miner infestation on different sugar beet varieties

Much research has gone into the cultivation of different varieties of sugar beet. Varieties (both those bred, and from genetic work on varietal traits) have been tested for tolerance against beet cyst nematode, aphids and even leaf miners, which were reported to find the smoothness of the leaves in some varieties unattractive which discouraged oviposition by females (Zhang *et al*., 2008). *Beta trigyna,* one of the 'wild beets', also has reported tolerance towards leaf miner (Van Geyt *et al*., 1990; Bartsch, 2010. But little research has gone into how different varieties may cope with minor pests such as leaf miner. This experiment idea was aimed at studying the effects of leaf miner on the growth of different varieties, which would have used selected varieties commonly used in the field. Similar to studying the effects of leaf miner at different stages of host growth,

different varieties would have been subjected to leaf miner infestation throughout their development. This would have again reflected the development of sugar beet within the field and followed the patterns of leaf miner generations in their natural environment. The end yield would have also been calculated to record any significant reductions. Unique physiological traits, as well as any other specific adaptations of each variety, would have also been taken into consideration based on any findings from these experiments. Earlier in this chapter I mention Cameron's (1914) observation on the adults initially preferring to lay eggs on the top shoots. It is possible that with certain canopy architecture the top shoots may be more difficult to attack, for example if they are surrounded and therefore protected by other leaves. Or like in the above study where the smooth surface of the leave appeared to deter female oviposition, a rougher leaf surface may also prevent the landing of any adult flies and prevent oviposition in a similar manner.

Effect of leaf miner infestation on different host plant species

Testing host plant preference at various stages of leaf miner life histories, and subsequent generations, would have indicated how choice in the field may vary and would be comparable to our findings on host plant relations to possible species groups in chapter 3. Larvae from the culture had been initially reared on sugar beet as this was the primary host of concern. However, the sugar beet leaf miners are known to attack other host plants as is documented in chapter 3. Potential host plants isolated for this experiment would include; sugar beet (the main host of interest), sea beet (a wild relative of main host), Swiss chard (another cultivar of *B.vulgaris*) and spinach (another species from

within the same family). Individual plants would be separated and a controlled number of larvae allowed to feed on them until reaching the final larval instar. Larvae would then be removed from their first host and then a set number would then be placed on a new host, with a proportion of the larvae retained and placed on a fresh host plant of the same species. This would result in larvae that were allowed to feed from the same host throughout their larval development, and three sets of larvae that were removed from their initial host after completing the second larval instar and placed on an alternate host to feed (Figure 5.6). The following factors would then be recorded after the move to the new host plant or species; mortality rate of larvae, pupation success rate and pupal emergence rate. This generation of larvae would be named as the first generation (e.g. G1). For the generations that successfully emerged as adults, I would then proceed with the following steps. Next, I would have liked to test how being reared on an alternate host effected the success of mating in adults and possible effects in later generations. Should any adults emerge from generation 1, they would be allowed to mate in a space containing the same host plant from when they completed their larval developments. The following information would be recorded; number of eggs laid by adults from generation 1, success rate of generation 2 (G2) eggs hatching, number of G2 larvae that completed larvae development/larvae mortality rate, G2 pupation success rate, G2 pupal emergence rate, and so on for any resulting generations. In addition to this, adults may have been presented with the option of 2 or more host plants and allowed to choose which host they preferred. The host plant range in this series of choice tests could have been further extended to other hosts such as *Chenopodium* species, or even other sugar beet varieties, to examine a wider range of potential hosts.

Figure 5.7 Host plant preference experiment. Colour coded to indicate the origin of each *Pegomya* population in association with host species and introductions into alternative host plants.

Environmental effects on pupal survival and adult emergence

Another key component in the life history of the species, as well as in potential management of leaf miner in the field, is any potential effects of soil types and environmental conditions on the survival rate of pupae. Survival rate could have initially been tested using various soil types only, such as sandy soil vs. clay. Other factors could have then been added in, such as the pH of the soil, dampness of the soil, density of the soil and temperature of the soil across all soil types. Soil temperature could have been measured across the pupation period to see which temperature pupae started to respond to and what temperature triggers adult emergence.

On a similar note, knowledge of what effects the overwintering pupal stage could also play an important role in the future management of leaf miner in sugar beet. This could then lead to more detailed examination into pupal diapause within sugar beet leaf

miners. A study on summer and winter diapause (and parasitism) in *P.mixta* by El-Serwy (2008) found variable levels of diapause, between 2% and 37%, at different locations during different years. The larvae were collected from 6 untreated sugar beet fields across the Governates of Kafr El-Sheikh and reared through in plastic containers while being monitored for emergence of adults and parasitoids. Similar experiments could be conducted in the UK by assessing the conditions for pupal survival in a laboratory environment and determining what environmental factors trigger pupal diapause. A study by Hanski & Stâhls (1990) on prolonged diapause in fungivorous species of *Pegomya* suggests that resource availability is what triggers a higher percentage of prolonged diapause within populations, and so species which may be subjected to higher scarcity or variable levels of food resources available are those more likely to enter into extended diapause. Identifying similar elements within UK sugar beet may explain the cyclical behaviours of the sugar beet leaf miners and provide a greater understanding into the unseen element of their life cycles. This in turn may allow the future manipulation of the diapause phase in leaf miner and avert the necessity of chemical control. For example, the artificial manipulation of environmental factors such as light, chemistry and hormones to trigger used in the prevention of diapause (Denlinger, 2008).

Predicting emergence patterns of adults from these pupae would also help in the forecasting of possible future outbreaks of leaf miner. Altering the day light hours and temperature of pupae currently hibernating will help identify the thresholds in which *Pegomya* development occurs. Once this has been established the use of forecasting tools, such as degree days may be used to predict emergence of *Pegomya* in the field. Degree days are the accumulated temperature that occurs above the base temperature

(usually the lower end of the threshold at which insect development can happen) that occurs within a 24-hour period. By monitoring the minimum and maximum temperature occurring each day from a set start date to the date in which the pest appears, the number of degree days can be calculated and then used in the future forecasts of the target species (Wilson & Barnett, 1983; Herms, 2004; Murray, 2020). Furthermore, once the triggers that initiate emergence are identified, steps can be taken to manipulate adult emergence in a laboratory setting which may help with limiting staggered emergence.

Overall, the methods described in this chapter were successfully implemented in the rearing of leaf miners within a culture for a number of generations. The first line failed as a result of human error and may have possibly produced further generations if these errors hadn't occurred. Issues that arose with regards to the maintenance of the culture could be easily resolved by optimising host plant production and cage set up to reduce pupal mortality from accidental damage. Although the methods described here could be successfully used in the culture of leaf miner in the future, some of the suggested alterations may be required in order to achieve a higher number of adults within the general populace of each generation. A key feature of this would be to examine in more detail how staggered emergences can be utilised without becoming detrimental to a controlled culture setting. Further investigation into synthetically initiating adult emergence should be considered for any future culturing methods as this was possibly the most problematic factor faced during these trials. This would be a requirement should any future cultures of leaf miner be used in laboratory experiments.

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CHAPTER 6

Molecular characterisation of hymenopteran parasitoids associated with the sugar beet leaf miner complex

A rather dusty looking *Pegomya* parasitoid – Norfolk, 2018 (Photo by Siobhan Hillman)

6.1 Contributions

The work in this chapter was conducted by myself, with samples collected from field work that I conducted collecting leaf miner, through the BBRO and from Levine de Zinger at the Institute for Research in Sugar Beet (IRS) in the Netherlands. Identification of adult parasitoids was conducted by Professor Charles Godfray, with identification of adult *Pegomya* specimen again conducted by Dr. Tony Irwin.

6.2 Abstract

Parasitoids are extremely diverse and range across a number of different insect orders, with such varying behaviours and host ranges that they are coined as guilds. They are differentiated from parasites in that they almost always kill their hosts, which makes them suitable candidates for biological control. Parasitoids have been used effectively as biological control agents for many decades, though they are yet to have been incorporated within the control strategies used by the sugar beet industry as potential alternatives to conventional chemical controls. This is partly due to a lack of understanding of the parasitoids associated with sugar beet leaf miners, and partly due a perceived lack of need for alternative control measures, at least until recently. Providing effective control through the use of biological agents such as parasitoids in the field can be more complicated than in a controlled environment such as a glasshouse. In order to understand the potential of parasitoids as biological control agents in sugar beet, it is important to know what species are associated with them and what species naturally occur in this environment. As parasitoids are difficult to identify from morphology, molecular tools can aid with the genetic characterisation of species. In this chapter I used genetic tools to help characterise morphologically identified adult parasitoids that were reared from leaf miner samples included in this thesis. Four species were identified based on morphological analyses, two from the UK and two from the Netherlands. Of the four species identified from the morphological analyses I successfully sequenced samples from the two species collected in the Netherlands. These sequences will provide a sequence database for future work on parasitoids associated with sugar beet leaf miners,

and will lay a foundation for further study into the potential use of these parasitoids as alternative control measures to conventional chemicals.

6.3 Introduction

Parasitoids are extremely diverse. They currently represent approximately 10% of insect species, though this figure it expected to rise to as much as 25% as relatively little is known about many species (Mills, 2009). Among the various hosts, leaf miners support amongst the highest number of parasitoid guilds amongst all other insects. Leaf mining species are thought to be particularly susceptible to parasitism due to their sedentary nature, high visibility and limited protection provided by the barrier of leaf epidermis (Gauld, 1988; Salvo & Valladares, 2007). Parasitoids are found most notably within the Hymenoptera (approx. 78%) and Diptera (approx. 20%), as well as the Coleoptera, Lepidoptera, Neuroptera, Strepsiptera and Trichoptera (Mills, 1994; Feener & Brown, 1997). Parasitoids show complex life cycles, with varying behaviours between species. Female parasitoids may oviposit in, on or near a host. The hatched parasitoid larvae will then feed on the host tissues, typically ending in death for the host species (Eggleton & Belshaw, 1992; Mills, 2009). Such primary parasitoids may feed alone or gregariously either directly on a host (ectoparasitoids) or within a host (endoparasitoids) (Waage & Hassell, 1982; Godfray & Shimada, 1999). Some parasitoids are generalists, feeding on a number of different hosts, and some are specialists, focusing on one or two host species (Snyder & Ives, 2001). Parasitoids can likewise be grouped depending on the host stage they attack. Parasitoids which kill their hosts or halt the hosts development are known as idiobionts, and those that allow the hosts to continue developing are known as koinobionts (Hawking *et al*., 1990; Brodeur & Boivin, 2004). Obligate, or direct, secondary parasitoids (hyperparasitoids) are species that can only feed or develop on or in a primary parasitoid. Indirect secondary parasitoids feed on the same host as a primary parasitoid

and so are indirectly attacking the primary parasitoid itself (Sullivan, 1987). Parasitoid species with similar behaviours (e.g., mode of parasitism, form of parasitism or attacking the same host stage) and those that attack the same hosts are grouped together within parasitoid guilds (Smith, 1993; Hawkins & Mills, 1996).

Broad-spectrum pesticide use is widely acknowledged to have played a role in reducing the abundance of beneficial insects in agricultural landscapes (Wilkinson *et al*., 1975; Desneux *et al*., 2007; Bueno *et al*., 2017). The peculiarity of leaf miner behaviour means pest leaf mining species have the advantage of protection from the leaf itself, which defends against non-contact insecticides and many are also resistant to chemicals that harm natural enemies present within the crop environment (Salvo & Valladares, 2007). Dunning (1953) suggests that the cyclical nature of sugar beet leaf miners is probably the result of parasitism, and so understanding the parasitoids of sugar beet leaf miners is vital in any future management of the species. IPM, or integrated pest management, aims to employ the use of biological and chemical controls, not in the eradication, but in the maintenance of pest populations below levels where crop damage is potentially significant (Barzman *et al.*, 2015; Stenberg, 2017). Using a multifaceted approach to pest control reduces the possibility of dependence on a single method in the regulation of pest populations, and any deleterious consequences, such as pesticide resistance (Thomas, 1999; Barzman *et al*., 2015). The most important factor in utilising biological control agents in the field is the accurate identification of the natural enemies themselves, as failure to accurately identify biological control agents can result in the breakdown of management strategies (Gariepy *et al*., 2007). This can be achieved more effectively with the aid of DNA-based tools.

A previous study by Dunning (1953) on the parasitoids associated with *P.betae* found both parasitic coleopteran and hymenopteran species. Some of these parasitoids occurred only occasionally or regularly in small numbers at low frequency and were egg, larval and pupal parasitoids. The list includes species from the Braconidae and Ichneumonidae such as; *Biosteres rusticus, Adelura balteata*, *Opius fulvicollis* (more common outside the UK), *Phygadeuon destator, Pseudoeucoila* sp., *Asaphes vulgaris*, *Pachyneuron* sp., *Trichogramma* sp., and the Staphylinidae species *Aleochara bipustulata*. Other species were more commonly found and include the Staphylinidae species *Aleochara bilineata,* the Braconidae species *Biosteres carbonarious* and *wesmaeli*, *Opius nitidulator and ruficeps,* and the Ichneumonidae species *Phygadeuon pegomyiae.* While it is noted that some of these species are likely to be hyperparasitoids, presence of hyperparasitoids would generally be expected within such a large number of samples.

DNA-based methods have been successfully employed in many biological control and integrated pest management studies in order to identify potential biological control agents, resolve phylogenies amongst related taxa, assess cryptic diversity and measure impact on pest populations (Agustí *et al*., 2005; Heraty *et al*., 2007; Zhou *et al*., 2013; van Nouhuys, 2016; Franck *et al.*, 2017). Traditional identification may entail rearing hosts through and dissecting adult parasitoids that emerge, which can be time consuming and requires a high level of expertise in the identification of the related taxa (Zhou *et al*., 2013). Moreover, DNA-based methods can detect small amounts of DNA in situations where the physical examination of a specimen may not reveal the obvious presence of any parasitoids, for example in the detection of parasitoid eggs within the host (Agustí *et al*., 2005; Gariepy *et al*., 2007).

In order to develop a greater understanding of the relationships between parasitoid and *Pegomya* leaf miner species associated with sugar beet and related hosts, we first need to identify which parasitoids are associated with the sugar beet leaf miner complex. In this chapter I use both traditional morphological means, as well as molecular methods, to identify adult parasitoids reared from *Pegomya* leaf miner in sugar beet and sea beet samples collected as part of chapter 3. Morphological and molecular analyses of adult *Pegomya* hosts taken from the same host plants from which the parasitoids emerged was also done in order to later compare parasitoid species assemblages to associated sugar beet leaf miner samples. This allowed me to investigate differences between parasitoids associated with the two *Pegomya* groups found in my investigation into the molecular characterisation of sugar beet leaf miners (Chapter 3).

6.4 Methods

6.4.1 Sample Summary

Pegomya leaf miner were collected from sugar beet and sea beet during 2018 to 2019 (Table 6.1). Larval samples from the leaves collected were reared through in batches, with batches consisting of larvae collected from the same host plant (where only one plant was present), or cluster of host plants (where infestations occurred close to one another), and several parasitoids emerged from pupae. The British parasitoids were collected from sea beet (4 adult parasitoids reared from leaf miner) and sugar beet (13 adult parasitoids reared from leaf miner). The samples from the Netherlands were collected as *Pegomya* larvae from sugar beet leaves by Levine De Zinger (IRS) and reared

through in batches (as stated above). The parasitoids that emerged from these samples totalled 363 adults in identifiable condition (Table 6.1). Adult parasitoids were previously kept in dry storage, initially at room temperature and then in a -80°C freezer. Upon removal legs were placed temporarily in 95% ethanol and the intact specimens were sent off for morphological identification. The morphological identification of the adult parasitoids was completed by Professor Charles Godfray (Oxford University).

During rearing, both adult parasitoids and adult *Pegomya* specimens emerged from the same larval samples. (Table 6.2). Adult *Pegomya* in the UK were primarily found as larvae on sea beet (5 adult *Pegomya* in total). Adult *Pegomya* from the Netherlands were found as larvae on sugar beet on 4 out of 7 sites (109 adult *Pegomya* in total). Morphological identification of adult *Pegomya* specimens was completed by Dr. Tony Irwin (Norfolk County Diptera Recorder) using the key produced by Ackland *et al.* (2017) parts 1 and 2 on the identification of British Anthomyiidae prior to DNA extraction. Adult *Pegomya* specimens were kept initially in dry storage and temporarily frozen at -82°C prior to the removal of a single leg during DNA extraction.

Table 6.1 Sample summary of the 380 parasitoids collected in the UK and Netherlands from *Pegomya* leaf miner on Sugar beet and sea beet. Samples are scaled up to county in the UK, and to country from non-UK samples to protect grower anonymity.

Host Plant	Area	Month Collected	Year Collected	Number of Samples
Sea Beet	Suffolk	July	2019	3
		September	2019	1
Sugar Beet	Norfolk	September	2019	13
	Netherlands (site 1)	July	2018	17
	Netherlands (site 2)	July	2018	8
	Netherlands (site 3)	July	2018	11
	Netherlands (site 4)	July	2018	5
	Netherlands (site 5)	July	2018	30
	Netherlands (site 6)	July	2018	5
	Netherlands (site 7)	July	2018	287

Table 6.2 Sample summary of the 114 adult *Pegomya* collected from sugar beet and sea beet at locations where parasitoids where also present. *Pegomya* from sea beet samples were predominately *P.cunicularia* (with the exception of one *P.betae* specimen), and those from sugar beet were all identified as *P.betae*. Samples are scaled up to county in the UK, and to country from non-UK samples to protect grower anonymity.

Host Plant	Area	Month Collected	Year Collected	Number of Samples
Sea Beet	Suffolk	July	2019	4
		September	2019	1
Sugar Beet	Netherlands (site 1)	July	2018	2
	Netherlands (site 2)	July	2018	2
	Netherlands (site 5)	July	2018	50
	Netherlands (site 7)	July	2018	55

6.4.2 Molecular Methods

DNA Extraction

DNA was extracted using a modified salt extraction technique (Richardson *et al*., 2001) as used previously in Chapters 3 and 4 with some slight modifications. Where possible the middle leg was removed from each adult parasitoid, if the middle leg was not present then either a front or hind leg was removed. In very few cases, 2 legs were removed and used in the DNA extraction. All equipment was initially UV sterilised prior to use, using 70% ethanol and flame on tools in between the handling of each induvial sample. As in the work described in chapters 3 and 4, 1.5µl Eppendorfs were first submerged into liquid nitrogen before the addition of any material, and then submerged again with the addition of the sample until the material was sufficiently crushed. Due to the size of the material when working with parasitoid legs the Eppendorf containing the leg was submerged into liquid nitrogen between two to four times. Adult *Pegomya* specimens had already been identified before the removal of any leg material, with the removal of a single leg to be used during DNA extraction. Samples from one location were identified as two different species and therefore in that instance two legs were removed from each of the three specimens in order to ensure at least one successful DNA extraction and amplification from each. In one instance a thorax was used for the DNA extraction due to the damage on a specimen resulting in no legs present for the molecular analyses. Leg and thorax material was added immediately into UV sterilised 1.5µl Eppendorfs prior to submersion into liquid nitrogen. If the leg material was not sufficiently crushed after the first submersion, then an additional submersion into the liquid nitrogen was completed.

265µl of DigiMix solution (250µl + 15µl Proteinase K) was then added to each individual specimen after completion of submersion into liquid nitrogen. Samples were then digested overnight at 55°C. After removal from incubation 1.5µl RNase was added to each sample, followed by 300µl of pre-warmed 4M ammonium acetate. Samples were then centrifuged for 20 minutes at 13000rpm. The resultant supernatant was then poured into newly UV sterilised 1.5µl Eppendorf tubes. To achieve separation between the DNA and supernatant 1ml of ice cold 100% ethanol was added to each sample, then all samples centrifuged for 20 minutes at 13000rpm. The separated supernatant was then carefully discarded and each Eppendorf blotted on tissue paper before adding 500µl of ice cold 70% ethanol to each sample. All samples were then centrifuged again for 20 minutes at 13000rpm. The addition of ice cold 70% ethanol to each sample was repeated once more with all samples centrifuged again for 20 minutes at 13000rpm. Once complete, samples were air dried in an incubator for one hour at 55°C. 50µl of Low TE was then added to the dry Eppendorf tubes and further incubated at 55°C for one hour.

Amplification of the COI gene in parasitoid and Pegomya leaf miners

Due to previous success in obtaining sequences from insect leg material I amplified the ~658bp fragment of the mitochondrial cytochrome c oxidase I (COI) gene using the universal primer pair LCO1490 (5' GGT CAA ATC ATA AAG ATA TGG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') (Folmer *et al*., 1994) in both parasitoid and *Pegomya* specimens. The PCR reaction was as outlined in Chapter 3; 1µl of DNA template, 0.5µl of each primer (Sigma-Aldrich), 3µl autoclaved H_20 and 5µl PCRBIO Taq Mix Red (PCRBIOSYSTEMS). The PCR protocol was as follows; an initial denaturation stage

of 95°C for 1 min, followed by 40 cycles of 95°C for 15 secs (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (amplification) and then a final extension at 72°C for 5 mins. All PCR reactions were conducted using the DNAEngine TETRAD 2 (MJ Research). The resulting PCR products were then run on a 1.5% agarose gel to confirm if DNA amplification was successful. PCR products which had been successfully amplified were prepared for sequencing with the addition of a 10µl clean up reaction mix; 0.1µl Exo I, 0.2µl FastAP (PCRBIOSYSTEMS) and 9.7µl autoclaved H_20. This was followed by a heat treatment of 37°C for 15 mins and then 80°C for 15 mins, and then the addition of 2µl of forward primer to each of the end reactions. Samples were sent for sequencing at Eurofin Genomics (Eurofins Scientific, 2018).

6.4.3 Analyses

All sequences were aligned by eye using AliView v 1.24 (Larsson, 2014), with segregating sites examined alongside associated chromatograms. Sequences were then checked against the GenBank (Benson *et al.*, 2013) database using BLAST (Altschul *et al.*, 1990) to check broad scale taxonomic identification. Failed sequences (approximately ½ to ¾ of the total number of specimens) were removed before any further analyses. All parasitoid sequences were trimmed to 568bp.

Pegomya sequences were added into the phylogenetic and haplotype analyses from Chapter 3 (see Figures 3.1 and 3.3) and allocated a group based on sequence clustering (allocation to either Group A or B). A haplotype network of the 145 parasitoid sequences was constructed using the pegas package (Paradis, 2010) in RStudio v.1.3.959 (R Core

Team, 2020). Networks were visualised based on site location. Genetic distance was measured by the number of base pair differences in the nucleotide composition of sequences.

6.5 Results

Four species of parasitoids, all within the Braconid family Opiinae, were identified from the morphological analyses of field samples (Figure 6.1). The UK samples predominately consisted of the species *Phaedrotoma nitidulator* (15 specimens), with 2 specimens identified as *Biosteres carbonarius*. The Netherlands samples were identified as *Utetes fulvicollis* (the majority) and *Biosteres wesmaeli/rusticus*. 84 parasitoids from the Netherlands were successfully sequenced. The haplotype network corroborated the morphological identification of two species found in the Netherlands and identified 3 haplotypes. Haplotype 1 was identified from 79 sequences found across all sites, haplotype 2 was identified from 4 sequences and was found across sites 1, 3, 4 and 7 and haplotype 3 was identified from a single sequence found within samples collected from site 7 (Figure 6.2). Although the adult parasitoids came from both sugar beet (majority) and sea beet, only sequences obtained from sugar beet fields in the Netherlands were successfully amplified and so analyses of host plant range in association with parasitoid species was not possible. All adult *Pegomya* specimens from each of the Netherlands sites were identified as *P.betae*, while all but one of the *Pegomya* specimens from the UK were identified as *P. cunicularia*, with one female identified as *P. betae* (see Chapter 3). Of the adult *Pegomya* samples I sent for sequencing 2 were successful and included in the phylogenetic tree from Chapter 3 (see Figure 3.1). Inclusion of the new *Pegomya*

sequences into the Maximum Likelihood Tree from Chapter 3 placed both specimens within 'Group A'. Likewise, due to all parasitoid sequences successfully obtained originating from the Netherlands, and due to low success rate in sequencing adult *Pegomya*, analyses of host fly species and parasitoid assemblages was not possible as all Netherlands host *Pegomya* were identified as *P.betae*.

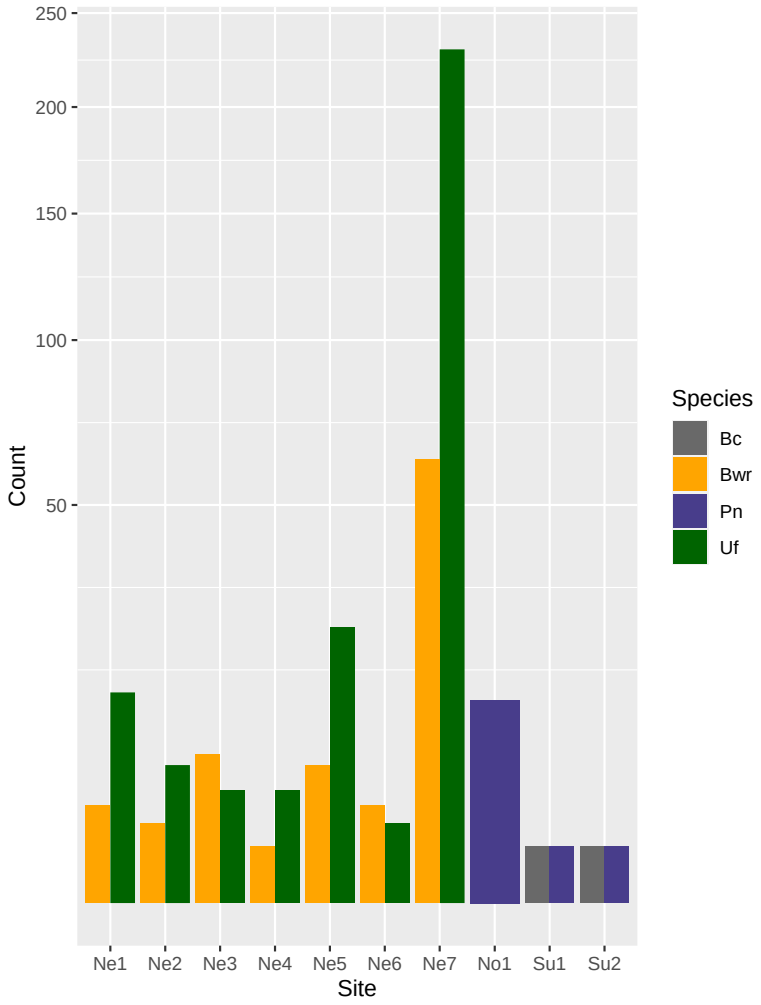

Figure 6.1 Bar chart showing the number of parasitoid adults (count), colour coded by species, identified by morphology from each site in the Netherlands and the UK. Location codes are as follows; Ne: Netherlands, No: Norfolk and Su: Suffolk. Species codes are as follows; Bc: *Biosteres carbonarius,* Bwr: *Biosteres wesmaeli/rusticus,* Pn: *Phaedrotoma nitidulator* and Uf: *Utetes fulvicollis.*

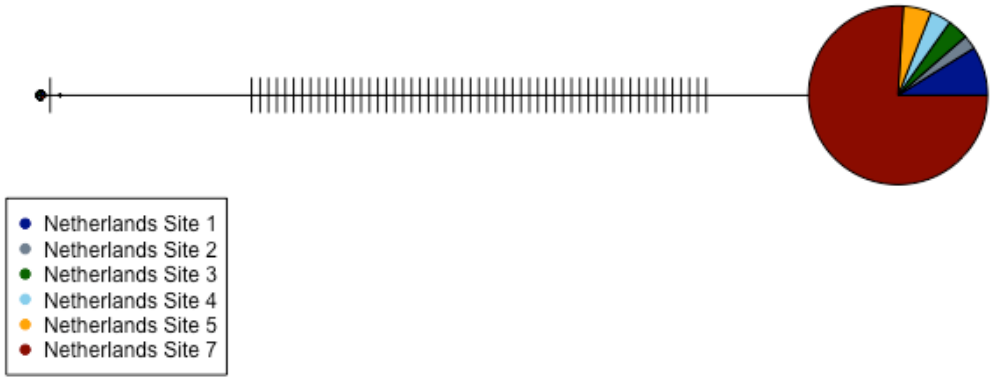

Figure 6.2 Haplotype Network of 84 Cytochrome C Oxidase Subunit I (COI) sequences of *Pegomya* parasitoids. There are 3 haplotypes distributed across 2 species groups, with colour coded nodes referring to site location. Node size represents number of sequences clustered within a haplotype, with tracks showing the number of base pair differences between each haplotype.

6.6 Discussion

Four species were identified from the morphological analyses of parasitoid specimens collected in the UK and the Netherlands, with success in sequencing the two species that originated from the Netherlands. The two *Pegomya* specimens were both grouped within Group A (Figure 3.1) and were both representatives of a single site. Other samples from the Netherlands were also included within the analyses of Chapter 3 but had not been subject to morphological analyses. These were also grouped with Group A and included samples from different sites. I obtained 84 parasitoid sequences that were included in the final alignment. Samples were identified as predominately *Utetes fulvicollis*, with a small number of *Biosteres* sp. sequences obtained also. All parasitoid sequences originated from the Netherlands sugar beet samples.

Construction of a haplotype network of the sequence data set confirmed the presence of two species of parasitoids. One node was that of a single haplotype at high frequency. Though all sequences were identified, only a proportion from Netherlands site 7 were individually matched to their identifications. The first 50 samples from Netherlands site 7 were identified individually and so morphological identifications could therefore be matched with the associated sequence. However, samples from number 51 (Ne7) onwards were identified and then a total number of each species from the 237 remaining parasitoids from this site was given. Matching sequences to identifications associated with each individual specimen was therefore not possible. Sequences from parasitoids without an individual identification were therefore matched to sequences from specimens which had been identified. There was unevenness in the distribution of the sequence data as there were only 5 sequences representing the *Biosteres* sp., and many representing that of *U.fulvicollis.* In addition, the majority of the sequences came from a single site so it was not possible to analyse the geographical boundaries of parasitoid populations and differences in species assemblages. Identification of the *Biosteres* sp. specimens was narrowed down to a possible two species, *B.wesmaeli* and *B.rusticus,* based on morphology. Cross references to sequences of confirmed specimens of these two species was not possible as they do not currently exist in any online sequence databases. Should sequences of either species be produced in future, the comparison of sequences included in this chapter may confirm species identity of the two possible identifications provided.

All four species of parasitoids that were identified fall within the Braconidae (Ichneumonoidea), of which there are >1300 species in the United Kingdom alone (Broad

et al., 2016). The Opiinae is one of the largest braconid families (>20000 species). They are primarily koinobiont endoparasitoids of cyclorrhaphous Diptera, including the Anthomyiidae (Khajeh *et al*., 2014; Dolati *et al*., 2018). Although the sequenced specimens were reared from leaf miner from the Netherlands, both *B.wesmaeli* and *B.rusticus* are recorded as being present in England, Scotland (both species) and Ireland (*B.rusticus* only) (Broad *et al*., 2016). Indeed, Dunning (1953) found *Biosteres carbonarious, B*.*wesmaeli* and *Opius nitidulator* during his survey of parasitoids of *P.betae* in the UK. A survey of the Opiinae of Finland states that *B.rusticus* and *B.wesmaeli* both parasitize members of the Anthomyiidae, specifically *Delia* and *Pegomya* sp. (in the case of *B.wesmaeli* both *P.betae* and *P.hyoscyami* are specified) (Fischer & Koponen, 1999). *U.fulvicollis* is also present in England (Broad *et al*., 2016). Both *P.betae* and *P.hyoscyami* have been recorded as host species for *U.fulvicollis* from a study in Iran (Ameri *et al*., 2020), though specific host information is fairly scarce. Monitoring the species identified in this chapter over time will be important in identifying the full scope of natural enemies associated with the sugar beet leaf miners, their distribution, how they interact with one another and other species and how they may be affected by various environmental or anthropogenic factors.

In relation to the use of such parasitoids in any future biological control, these species would most likely fall within the biological control process of 'augmentation' as they naturally occur in the UK and attack the leaf miners upon which I focus in this thesis. Augmentation is defined by the release of native species in order to bolster the number of individuals in populations that are already present. The 'introduction' method, also known as classical biological control, of introducing an exotic species in order to suppress

a pest (Waage & Greathead, 1988) could also be considered in the future management of these pests. This chapter focuses on parasitoids reared from the leaf miner samples under study, but there are undoubtedly species of parasitoids that under atypical conditions (e.g., differing environments) will survive and thrive on the sugar beet leaf miners. Identifying these species may prove important in the future management of sugar beet leaf miner. For example, in a situation where the natural enemies of these pests are not providing sufficient regulation of population numbers. In addition to this, studies on the timing of parasitoid releases within sugar beet crops should be investigated as the prime time for leaf miner mortality caused by parasitoids has been identified as the later stages of crop development (Salvo & Valladares, 2007). This may prove useful in future as an alternative to chemical controls for the later generations of leaf miner which can prove particularly damaging. Further work should also be conducted on providing habitats for parasitoids within crop fields, for example field margins. Monocultured crops are reported as having negative influences on parasitoid density in agricultural settings, as parasitoids often have preferences in habitats and host plants (Salvo & Valladares, 2007). Identifying what these plant preferences are and how to adapt sugar beet fields to accommodate them will naturally encourage higher density parasitoid populations and therefore higher rates of leaf miner mortality.

Many of the limits of the findings in this chapter were due to time restrictions on this part of the project and there are several ways in which the research could be improved and extended. One drawback was the limited collection of parasitoid samples during fieldwork, with samples being obtained from collections focussed on the leaf miners themselves. Because the samples were obtained 'passively,' the exact, broader

representation of parasitoid species remains to be confirmed. The majority of parasitoids included in this chapter were collected from the Netherlands on sugar beet, with relatively few from the UK and from sea beet or other host plants. Comparisons between species assemblages across different hosts and countries was consequently not possible, but future research into this may shed light into how parasitoid guilds affecting sugar beet leaf miners may vary between geographical region and host plant range. It is also important to note that the species I identified were of hymenopteran parasitoids. Although these make up the majority of parasitoid species, parasitoids from other insect orders are also possible. As parasitoid samples were not actively collected, the study of these species over time was also not possible. Long term study of parasitoids associated with sugar beet and related hosts may show how the diversity of parasitoids in an agricultural setting can change over time and may indicate the varying ecological factors that may affect this. Finally, optimisation of primers and PCR conditions was limited by time constraints. With additional alterations to the molecular process, the number of successful sequences could be higher. Obtaining a larger sequence data set of both morphologically identified adult, as well as immature parasitoids (e.g. within the host larvae/pupae), would provide additional information and further insights with regards to the parasitoid assemblages observed.

6.7 References

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CHAPTER 7

General Discussion

Leaf miner on beetroot – Wiltshire, 2020. (Photo by Siobhan Hillman)

7.1 General Findings

In this thesis I identified two genetically distinct groups from *Pegomya* field samples with divergent host plant ranges. Genetic and morphological analyses of adult specimens also showed potential for cryptic speciation within the complex. I also successfully amplified sequences of historic *Pegomya* specimens from various museum collections and the mitochondrial phylogenetic tree that resulted from that work provides a foundation for future phylogenetic studies on the *Pegomya* genus, and the wider Anthomyiidae. Moreover, I genetically characterised two species of parasitoids reared from sugar beet leaf miners which will provide a sequence library for future analyses and genetic comparisons of *Pegomya* parasitoids. The findings in this thesis will be discussed below along with potential future directions for work on the sugar beet leaf miners.

7.2 Thesis Overview

The research programme described in this thesis came into existence from the need to understand more about the leaf miners that were responsible for the 2015/16 outbreaks witnessed by growers within the British sugar beet growing community. When first exploring the 'sugar beet leaf miners', it became clear from previous literature, that it isn't necessarily clear what species, or what pest, it actually is. My systematic search of the literature showed that sugar beet leaf miners appear to comprise two main species: *Pegomya betae* and *P. hyoscyami*. Both of these species are collectively referred to by different common names, such as mangold fly, spinach leaf miner, *Belladonna* leaf miner and so on. Entomological and taxonomic data emphasise the importance of the

P.hyoscyami complex. The naming of this complex gives the most current taxonomic description, from an entomological point of view, of some of the species to which I refer as sugar beet leaf miners in this thesis. A seminal paper describing the *P.hyoscyami* complex was published in 1980 by Michelson and is well known amongst entomologists with a particular focus on the *Pegomya*: Anthomyiidae. However, this understanding contrasts with the general view held among agriculturalists, with many agricultural handbooks referring to only one species as the sugar beet leaf miner, namely *P.betae*. In fact, *P.hyoscyami* appears to be referred to more commonly as the spinach leaf miner in the agricultural literature. On top of this, much agricultural research on sugar beet leaf miners is buried away in arcane and inaccessible literature. This motivated the major aim of my thesis, to provide a detailed review of the literature as well as an updated description of the taxonomy of this group. Although certain aspects of the older literature available are certainly valuable, it was important to bring this information up to date for a number of reasons;

1) The gap between the entomologist's knowledge of these species and what is known in agricultural contexts appears to be very wide and therefore needs resolving and closing.
2) Previously unavailable molecular tools can help confirm and resolve species identification of leaf miners in sugar beet and potentially even uncover new cryptic strains and significantly augment knowledge of these species.
3) Without understanding what pest species are affecting sugar beet it is much harder to effectively control them.

Identifying, through the use of morphology and molecular tools, the species associated with sugar beet was the first priority. Efficient and sustainable pest management is becoming increasingly important within agriculture, as policies, the environment and pests themselves change (Forgash, 1984; Weddle *et al*., 2009; Dewar, 2017; Midingoyi *et al*., 2018; Jactel *et al*., 2019; Mc Namara *et al*., 2020). Key to this is an understanding of the basic biology and ecology of the target species. Accurate identification is the first step in pest control, in order to avoid eradicating or harming non-target species and the environment (Gariepy *et al*., 2007). As my target species was leaf miner of sugar beet, host plants and parasitoids were important, due to the close relationship between the three, and therefore formed an additional major investigation of this thesis.

7.3 Molecular characterisation of an insect pest complex and its parasitoids

Genetic characterisation of *Pegomya* field samples from sugar beet and related hosts identified two distinct genetic groups, these were also distinguished on the basis of their host plant preferences, with additional evidence for differences in geographic location between the two groups, though an influence of bias in the data sampling across different regions also cannot be ruled out. The host plant ranges of both groups of *Pegomya* largely reflects what is seen in the literature with reports that *Pegomya* spp mine sugar beet or spinach (Cameron, 1914; Yasumatsu & Sasagawa, 1953; Michelson, 1980). Again, sampling bias could have some influence, but the overall finding of genetically distinct groupings associated with different plant hosts is robust. Further investigation into the host plant range of both groups can now be conducted. From the perspective of the sugar beet industry, these findings may indicate that during years of higher infestation of leaf

miner in the field, it is possible that more than one species is present as there is a slight overlap in the host plant ranges of both groups. The few adult *Pegomya* that were also sequenced corresponded to two morphologically defined species, *P.betae* and *P.cunicularia*, which were spread across both groups. These two findings combined suggest the possibility of cryptic species, as there is evidence (i) that both groups defined in chapter 3 are genetically distinct, and (ii) of 2 morphologically defined species within each of those groups. Identified adult specimens were few and so the possibility of cryptic speciation should be further investigated, with the addition of nuclear analyses, to confirm this hypothesis.

It is also interesting to see where Michelson's (1980) description of the complex fits within these findings. I have often referred to this paper throughout this thesis, and though Michelson defines the complex as the 'beet leaf miner complex', it contains the species often referred to as sugar beet leaf miners (El-Serwy, 2008a; 2008b; El-Rawy & Shalaby, 2011; Sabbour & Soleiman, 2019; Sabbour *et al*., 2020) or as *Pegomya* species mining *Beta* hosts (Ackland *et al*., 2017; Edmunds, 2022) throughout the literature. This paper is widely acknowledged as the most sound description of the species. The findings in chapter 3 do not support the idea of four distinct species however, but this may in part be due to limited sampling of the known hosts of the complex. Additional sampling of potential hosts across broader geographical regions, including other countries, is needed to confirm these patterns. What is more interesting is the placement of specimens identified as *P.betae* and *P.cunicularia*, within the genetic groups defined in chapter 3. It raises the question of how accurate morphological descriptions may be in identifying specimens that are genetically distinct. Morphologically identifying many anthomyiids is

generally quite complex, especially with regards to female specimens. Whether these descriptions truly define the species described in this thesis remains to be confirmed.

From the genetic analyses of the four parasitoids identified from their morphology by Charles Godfray, I successfully sequenced two, though there were significantly more sequences of *Utetes fulvicollis* than there were of *Biosteres wesmaeli/rusticus*. I sequenced parasitoid samples from seven sites in the Netherlands, with two sites having particularly higher numbers of parasitoids that emerged from the leaf miner pupae that were collected. The majority of the sequences originated from site 7, though these were again the two species above in much higher numbers. Though infestation of *Pegomya* in the UK has been relatively low since starting this PhD, following significant outbreaks occurring in the years prior, it is surprising how few parasitoids emerged from the number of samples that were collected, both from sugar beet and from other hosts, during the fieldwork conducted over the past 4 years. Dunning (1953) found significantly higher numbers of species over the 3 year period in which he studied parasitoids of *P.betae*, and these included both hymenopteran and coleopteran species. Though the work in this thesis didn't actively sample parasitoids of sugar beet leaf miners, like that of Dunning (1953), with the larger samples from the Netherlands (one site having just under 300 parasitoids), it was expected that the species diversity of the parasitoids would be greater than it actually was. As I know of no in depth studies that have been conducted since the work of Dunning (1953), investigating the natural enemies of sugar beet pests should be prioritised in future, as well as monitoring these species over time in order to gauge what population fluctuations may occur and what triggers them.

7.4 Phylogenetic analyses of an understudied genus

The *Pegomya* phylogeny presented in chapter 4 provides a future foundation for phylogenetic studies on the *Pegomya* genus and wider Anthomyiidae. Species level grouping was obtained for the majority of the samples, with one large branch containing multiple species. Reamplification of anomalous sequences and reverification of the morphological identities of the matching specimens strongly supports my proposed current placement of these sequences. The placement of the anomalous specimens could suffer from some lack of resolution, as the amplified sequences were rather short in length. Further multi-locus analyses will help to confirm the tree topology presented in chapter 4. It is also possible that there is cryptic speciation within the genus, as I discussed in chapter 3, though further work is required to confirm this. The possibility that species are grouped together based on similar feeding behaviours, as seen in the division of sub-genera of the *Pegomya* described by Ackland *et al*. (2017), was not observed in this analysis as there was poor bootstrap support on the older branches. The most rewarding part of this work was the success in sequencing several historic specimens from museum collections, including several from the early to mid-1900s. As both age of the specimen and storage methods (Wandeler *et al*., 2007; Besnard *et al*., 2016) can drastically affect the success rate of sequencing specimens this achievement is noteworthy. With alterations to DNA extraction methods and PCR protocols it is very likely that an even higher success rate in obtaining quality DNA sequences from museum specimens of *Pegomya* can be achieved in the future.

7.5 Lessons learned from culturing leaf miner

Though my culturing work was cut short due to COVID restrictions, my findings show that it is possible to culture leaf miner within a laboratory setting. The first attempt at culturing leaf miner failed due to the adults not feeding on the diet provided. Cameron (1914) previously reared *P.hyoscyami* on a sugar solution which I found that the flies ate, but that was insufficient to support their survival. Once this was changed to a pollen diet I successfully managed to get adults to mate and produce subsequent generations. The first line of 2019 was destroyed due to spider mite infestation. Prior to this, if fewer adults had escaped, it is likely that there would have been subsequent generations. Both lines from 2019 died out after 4 generations, due to different reasons. The first line was destroyed to prevent contamination, as stated above, but the second line possibly died out as a result of limited breeding pairs. Further investigation into why this line died out would have identified problems which could then be avoided in future culturing experiments. As there were adults present in the first line from a mix of host plants, presented in chapter 3 as a factor that distinguished both genetically distinct groups, it is possible there were two species/genetically distinct groups present in this line. No examination of adults took place to confirm this but in future it would be advised based on the findings in chapter 3 to rear larvae from different hosts in isolation. Future experiments could also be conducted to see if these two groups will interbreed. With some modifications to the protocol, particularly with regards to number of adults provided for mating, investigating faster grower host plants and looking at alternative diets for the adult flies, it would be possible to produce a continuous line of multiple generations of leaf miner in a controlled environment.

7.6 Conclusions and Future Directions

There is a lack of historical records of leaf miner within British sugar beet and related hosts from which to compare recent outbreaks. Therefore, gathering such records could be a useful task for the future. Leaf miner has generally been considered a minor pest that affects sugar beet. However, major outbreaks in recent years have highlighted the importance of understanding the leaf miners in much greater depth both now and in the past. However, due to the nature of agriculture and agricultural research, historical records of minor pest outbreaks are not always readily kept or easily accessible. Accurate entomological records are also scarce due to the complicated morphology of this family. Investigating and collating data on the sugar beet leaf miners from historical records was not prioritised in my thesis research. Collating data from non-digitalised copies of past BBRO or entomological bulletins and news articles, as well as investigating possible remnants of research from the time when the BBRO was based at Brooms Barn, could be useful to reveal records and information of relevance to the findings in this thesis.

Similarly, original discussions centred on the assembly of meta data regarding the sugar beet leaf miner complex obtained from local biological record centres and county recorders. However, due to restrictions imposed by the COVID pandemic, this exercise was not initiated. Nevertheless, the aim would have been to produce, to the greatest possible extent, a UK distribution map of *Pegomya* and any non-*Pegomya* species associated with sugar beet. Due to the differing recording systems across the UK, it is highly likely that some data on *Pegomya* distributions has not yet been examined and thus that records regarding these species are not in a single system. By requesting records

from local records centres, county recorders, the Anthomyiidae recording scheme and recording systems such as iRECORD, and gathering data, it would be possible to create a UK distribution map and cross examine the associated meta data in relation to the findings reported in this thesis. In particular, data on the host plants associated with each of the 4 potential species in the complex could have been evaluated against our own findings and an assessment made on whether there were any geographical boundaries separating species. Associated museum data could have also been included in this analysis. The absence of physical specimens in this type of analyses is problematic and limits the accuracy of species identifications, even if some records are accompanied by photos. However, this uncertainty could be accommodated through an additional tag for each record on perceived accuracy, for example as represented by a scale of 1 – 5, with 5 being highly likely and 1 being highly unlikely. This scale could have been included as a factor in the final analyses. Furthermore, records of other species in the genus *Pegomya* could have also been incorporated, and host plant association examined against the meta data of the sugar beet leaf miner species complex. Examination of the meta data of all *Pegomya* species could have identified any outliers and potentially discovered patterns within the genus which could be linked to my findings in chapter 4 on *Pegomya* phylogenetics.

Another research strand, which was originally discussed but ultimately not included, was the collection of field data from growers. Such data would include soil type, soil pH, variety of sugar beet crop, crop rotation and management strategies implemented – all of which could influence the success and spread of the crop-damaging larval stage of the leaf miner. The initial plan was to request that any growers sending in leaf miner samples

filled out a short survey form requesting the above data. This proved not to be fully possible due to (i) time constraints, (ii) growers not always knowing the full details of the varieties of sugar beet crop they were sowing, and (iii) doubt about data sharing permissions being granted outside the scope already given to the CASE partner. Some relevant information could instead be gleaned in the future from within laboratory experiments, which were partially initiated in chapter 5. While insights into the impacts of crop rotation and management cannot easily be obtained from within a laboratory environment, the effects of soil type, soil pH and sugar beet variety could have been investigated, as discussed in chapter 5. Though necessarily small in scale, some insights into the potential effects of crop rotations could be gained in a laboratory or glass house context by examining the effects on leaf miner cultures of host plants that had been grown in an area where a different crop had grown previously. Similarly, some management strategies, particularly the effects of pesticides and fertilisers, could also have been replicated and tested on a smaller scale within a laboratory environment, to provide data to feed into future field trials.

In regard to the collection of leaf miner and *Pegomya* specimens, it would be possible in the future to expand the number of samples collected. I received leaf miner samples from allotments and gardens across the UK during the global pandemic. This enabled me to process samples from a wider range of locations and host plants than would otherwise have been possible. Initially the focus for collecting samples was dedicated to leaf miner in sugar beet crops, but during the first two years of this research there was very little leaf miner reported in the field. In retrospect, public engagement could have been deployed earlier to enable me to collect more leaf miner samples from the selected host

plants across a much broader area in the UK. Similarly, in the future spinach growers could also be contacted, to acquire samples of leaf miner from large scale spinach crops across the UK. Sea beet samples could also have been obtained in the same way I obtained samples from Swiss chard, beetroot and spinach, by contacting nature reserves or privately-owned land near the British coast. Enhanced sampling of various *Chenopodium* species would also be useful to provide greater resolution to the phylogenetic placement of results found in chapter 3, and further investigations of the host plant range of the sugar beet leaf miners. Enhanced sampling of various *Pegomya* species from a wider range of British and international museums would also allow a deeper expansion of the phylogenetics of the genus reported in chapter 4. Collection of leaf miner from sugar beet samples worldwide should be a priority in the future, in order to compare species assemblages in different countries and climates to the findings in chapter 3. This could be important for both the British sugar beet growing community, and sugar beet growers worldwide.

Morphological analysis of adult *Pegomya* specimens was performed, as described in chapters 3, 4 and 6. I outsourced identifications to nationally recognised experts to identify the *Pegomya* and associated parasitoid samples. There were some limits to these analyses. For the field specimens, I was not able to rear through many British larvae to adulthood. This was due to time restrictions during the global pandemic and very few samples in the first two years of my study. This led to a decision to dedicate samples that were collected during 2020, when I obtained the majority of the field samples, to processing for molecular identifications, rather than rearing experiments. More detailed morphological analysis, in association with the molecular findings in chapter 3, could be

beneficial to understanding the genetically distinct groups I have identified. Future detailed comparisons of adults found across both groups may yet uncover shared or distinct morphological features. Likewise, the morphological analysis of loaned specimens used in chapter 4 was also originally planned. Due to the molecular work for this chapter being carried out during the pandemic I was not able to analyse the morphology of all the museum specimens and decided instead to restrict those morphological analyses to specimens that corresponded to any outlier sequences in the phylogenetic tree. During the collection of leg samples from specimens housed in Liverpool World Museum, stacked photographs were taken of the first few *Pegomya* specimens in their collection, and at a later date the outlier specimens I had identified from the phylogenetic tree, resulting in highly detailed photographs. Additional photographic sampling of these specimens would have allowed critical examination of the external morphology at a later date without the need for physical specimens. More detailed photographs could have also been taken of other loaned specimens and used in the morphological analysis of the genus.

Refining the molecular techniques used throughout chapters 3/6 and 4 should be considered in any future work. Although I had success with amplifying DNA from all life stages except for *Pegomya* eggs, the success of amplification at each stage varied greatly. In particular, I succeeded in the sequencing of DNA from abandoned leaf mines and this technique has great potential to aid in future analyses. However, the success of amplification was fairly low and increasing the proportion of sequences obtained from leaf mines, regardless of their age, to a sufficient level would advance knowledge of leaf mining in general, without the need for the specimen itself. The amplification of DNA

from leaf mines cannot only identify the species of leaf miner that created the mine, but may indicate the presence of associated parasitoids or predators. This could be applied to other plant-invertebrate relationships where the presence of the individual species involved is not always easy to ascertain. I achieved greater success when amplifying the DNA from larval or pupal specimens. However, this could still be improved through optimisation of the PCR protocol or primers. The killing and preservation methods of samples can significantly affect the success rate of DNA amplification in larval and pupal specimens. Hence a systematic investigation of these factors may also provide further insight into the success rate of DNA amplifications. DNA amplification in adult specimens was also fairly successful, although less so than with larval and pupal samples. Less material was used in the process of amplification of adults (a single leg) compared to larval and pupal samples (whole specimen). Optimising the amplification of a single dipteran leg should be prioritised as the removal of a single legs allows the specimen to retain morphological features which is desirable over the destruction of a whole specimen. This may also be achievable through adapting the PCR protocol and primers used but may also require some alteration to the method of extracting the DNA itself (e.g., alteration of overnight incubation times etc.).

In addition to refining the success of sequencing leaf miner samples, further investigation into population structure would also provide new information on the species complex to help with the sustainable management of leaf mining species in sugar beet. This could include detailed analysis into specific populations where the outbreaks have occurred in the past. Population analyses may also show possible links with other populations on sea beet, as a wild host, Swiss chard and beetroot, as other cultivars of the same species, and

spinach, a species within the same family. Links to other host plant families, specifically those within the Chenopodiaceae, may also uncover new information regarding the complex.

During the sequencing of field samples using the universal primers LCO1490/HCO2198, I uncovered parasitoid DNA from the braconid families Opiinae and Alysiinae when comparing the sequences to the GenBank database. This DNA was recovered from the immature stages of leaf mining larvae collected in the field in the UK. The aims of that chapter were to characterise on a molecular level associated parasitoids of the leaf miner complex and relate this to the findings in chapter 3. During this work I prioritised sequencing of adult parasitoids due to time restrictions and because of this I had fewer British samples. I had planned to dissect several larvae and pupae retained in ethanol at -82°C to test for evidence of parasitoids within the British samples. As I had several larval and pupal samples remaining from British samples across sugar beet, sea beet, beetroot, spinach and Swiss chard, differences in parasitoid assemblage between host plants could have been identified, had this work been possible. Later comparison of sequences from larval and pupal samples to morphologically identify and sequence adults would have then been possible. The discovery of parasitoid DNA within leaf miner field samples, and the emergence of adults from several, would be valuable information for the sugar beet industry. This should be further investigated along with studies of other natural enemies, with the aim to sustainably manage any future outbreaks of sugar beet leaf miners.

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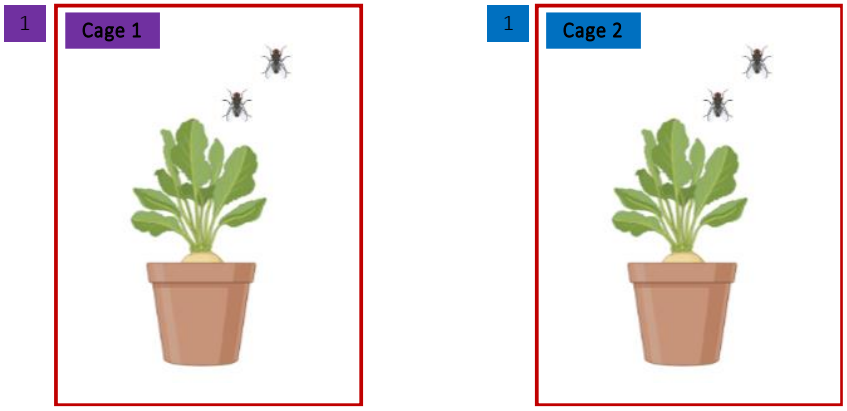
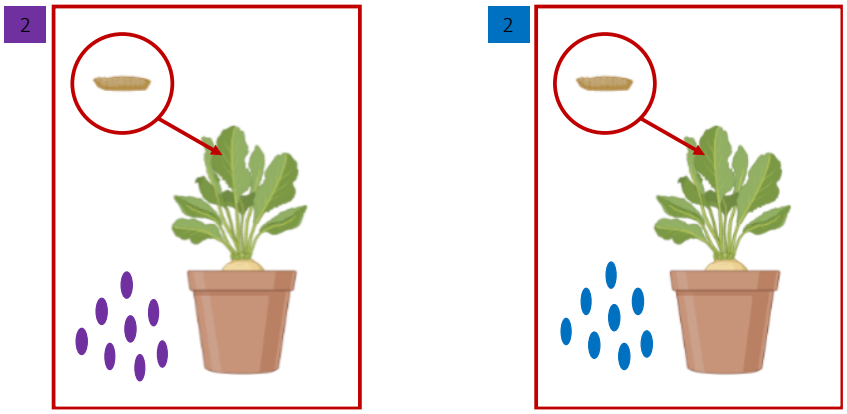
Appendix

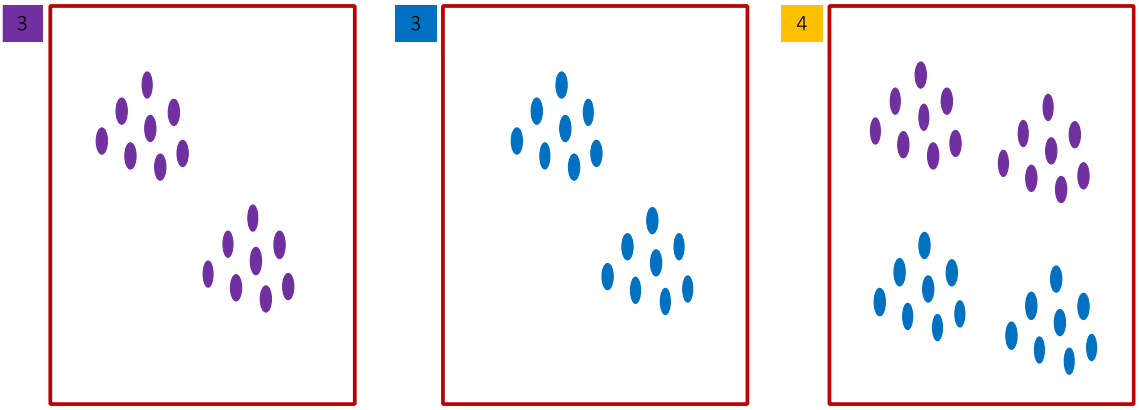
Figure 4. Modified protocol for generation 3 (Line 2). Created with BioRender.com and edited in PowerPoint.



1) A set number of adult flies are allowed to mate for a given number of days and lay eggs on a host plant of suitable age. Once the allocated period for mating and laying eggs is complete, the adult flies are removed.

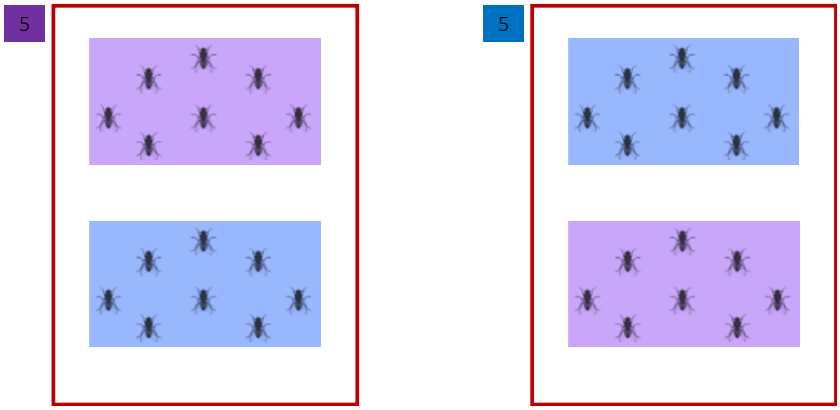


2) Eggs are allowed to emerge as larvae. The larvae are then supplied with sufficient host plant material to feed upon until they pupate.



3) The pupae are removed from the cage. Any wet leaf matter is dried from the pupae to prevent fungal growth.

4) The pupae are divided into two lots. One lot from cage A is added to one lot from cage B and vice versa. The end product in an equal mix of pupae from both cages.



5) Pupal batches are contained in a quarantine cage and allowed to emerge. Once a set number of adults is reached, adults are then added back into the culture cages and the protocol starts at step 1 again.