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Toll-like receptor variation in the bottlenecked population of the Seychelles warbler: computer simulations see the 'ghost of selection past' and quantify the 'drift debt'

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Abstract

Balancing selection can maintain immunogenetic variation within host populations, but detecting its signal in a post-bottlenecked population is challenging due to the potentially overriding effects of drift. Toll-like receptor genes (TLRs) play a fundamental role in vertebrate immune defence and are predicted to be under balancing selection. We previously characterised variation at TLR loci in the Seychelles warbler (*Acrocephalus*

sechellensis), an endemic passerine that has undergone a historical bottleneck. Five out of seven TLR loci were polymorphic, which is in sharp contrast to the low genome-wide variation observed. However standard population genetic statistical methods failed to detect a contemporary signature of selection at any TLR loci. We examined whether the observed TLR polymorphism could be explained by neutral evolution, simulating the population's demography in the software DIYABC. This showed that the posterior distributions of mutation rates had to be unrealistically high to explain the observed genetic variation. We then conducted simulations with an agent-based model using typical values for the mutation rate, which indicated that weak balancing selection has acted on the three TLR genes. The model was able to detect evidence of past selection elevating TLR polymorphism in the pre-bottleneck populations, but was unable to discern any effects of balancing selection in the contemporary population. Our results show drift is the overriding evolutionary force that has shaped TLR variation in the contemporary Seychelles warbler population, and the observed TLR polymorphisms might be merely the 'ghost of selection past'. Forecast models predict immunogenetic variation in this species will continue to be eroded in the absence of contemporary balancing selection. Such 'drift debt' occurs when a genepool has not yet reached its new equilibrium level of polymorphism, and this loss could be an important threat to many recently bottlenecked populations.

Introduction

Balancing selection can maintain variation in a gene pool in the face of genetic drift, yet the relative impact of both evolutionary forces in bottlenecked populations remains unclear (Acevedo-Whitehouse & Cunningham, 2006). As well as being important from a conceptual evolutionary perspective, a better understanding of this interaction is required in conservation biology, where levels of genetic variation can influence individual fitness and population persistence as a result of inbreeding depression and adaptive potential (Frankham *et al.,* 1999; Reed & Frankham, 2003).

Genetic drift is thought to outweigh the effects of selection in small, bottlenecked populations, resulting in a loss of genetic variation and leading to population isolation and differentiation and isolation (Miller & Lambert, 2004; Grueber *et al.,* 2013). Indeed, genetic variants with selection coefficients (s) smaller than the reciprocal of twice the effective population size ($s < 1/(2N_e)$) are considered to behave neutrally (Kimura, 1979). This implies that in a population that has gone through a single generation bottleneck of just 10 individuals, alleles with selection coefficients $s < 0.05$ have an equal probability of becoming lost or fixed as neutral alleles, assuming they have the same initial frequency. However, selection acting before the bottleneck will have changed the allele frequencies at loci under selection compared to the frequencies at neutral loci. For example, some types of selection create an allele frequency spectrum with a relative excess of intermediate-frequency alleles – hence the term "balancing selection". Such intermediate frequency alleles are less likely to drift to fixation (i.e. being lost from the population) than the (generally low-frequency) neutral alleles. Consequently, a locus that is under balancing selection in the pre-bottlenecked population may continue to show a relatively elevated level of gene diversity compared to a neutral gene after the bottleneck, even in the

absence of contemporary balancing selection. This elevated level of polymorphism could be a "ghost of selection past" that does not reflect the contemporary regime of selection. To put this another way, a recently bottlenecked population is unlikely to be in mutation-drift-selection equilibrium, and the currently observed levels of polymorphism at genes under balancing selection might overestimate future genetic diversity. In this paper, we refer to this phenomenon as the "drift debt".

Immune genes are ideal candidates with which to investigate the link between genetic variation and fitness because of their direct effects on survival (Sorci & Moller, 1997; Merino *et al.,* 2000; Moller & Saino, 2004; la Puente *et al.,* 2010) and reproductive success (Pedersen & Greives, 2008; Kalbe *et al.,* 2009; la Puente *et al.,* 2010; Radwan *et al.,* 2012). Variation at immune genes can have an important impact on the demographic structure of populations (Hudson, 1986; Redpath *et al.,* 2006; Deter *et al.,* 2007; Pedersen & Greives, 2008). They are also thought to evolve faster than the rest of the genome as a result of host-pathogen co-evolution (Trowsdale & Parham, 2004). Much work has been done investigating how balancing selection can maintain genetic variation at genes of the Major Histocompatibility Complex (MHC) (for reviews, see Piertney & Oliver, 2006; Spurgin & Richardson, 2010). However, the MHC is a large multigene family with a complex evolutionary history and with many evolutionary forces acting simultaneously on the various gene members (see, van Oosterhout, 2009). For example, gene conversion (e.g. Spurgin *et al.,* 2011; Eimes et al., 2011) and the generally unknown locus-affiliation of alleles can complicate population genetic analysis of the MHC in wild populations. In contrast, many other immune genes remain relatively understudied, yet these genes are increasingly recognised as important candidates for investigating functional variation and selection (Acevedo-Whitehouse & Cunningham, 2006; Turner *et al.,* 2012; Grueber *et al.,* 2013; Chapman *et al.,* 2016).

Toll-like receptors (TLRs) are membrane-bound sensors of the vertebrate immune system that recognise pathogen-associated molecular patterns (PAMPs) and help trigger an immune response (Akira *et al.,* 2001; Werling & Jungi, 2003). Vertebrate TLRs fall into six different families depending on the specific PAMPs they recognise (Takeda & Akira, 2005; Kawai & Akira, 2010). Different TLRs bind to different elements, ranging from bacterial lipoproteins (Takeuchi *et al.,* 2002; Jin *et al.,* 2007), lipopolysaccharides (Bihl *et al.,* 2003; Kim *et al.,* 2007), DNA motifs (Keestra *et al.,* 2010; Brownlie & Allan, 2011) and viral RNA (Yoneyama & Fujita, 2010). Studies have shown evidence of positive selection acting within TLR loci across a range of vertebrate taxa (Ferrer-admetlla *et al.,* 2008; Nakajima *et al.,* 2008; Areal *et al.,* 2011; Palti, 2011; Grueber *et al.,* 2014) and it appears that this selection largely targets the TLR extracellular domain responsible for binding PAMPs (for reviews, see Takeda & Akira, 2005; Kawai & Akira, 2010). If TLRs are involved in a co-evolutionary arms race with pathogens, it is likely that balancing selection operates at these genes. This idea is also supported by the direct links that have been made between *in vitro* nucleotide variation at these genes with differential disease outcome (Basu *et al.,* 2012; Netea *et al.,* 2012).

Avian models are widely used for looking into patterns of functional variation both within and between populations (for examples, see Hellgren *et al.,* 2010; Bonneaud *et al.,* 2011; Kyle *et al.,* 2014; Gonzalez-Quevedo *et al.,* 2016), including studies that have looked into the relationship of this variation with

anthropogenic factors (e.g. Wright *et al.,* 2014; Gonzalez-Quevedo *et al.,* 2016). A study on the entire TLR multigene family in seven phylogenetically-diverse avian species has inferred polymorphic TLRs to be under strong balancing selection (Alcaide & Edwards, 2011) but only recently have studies been able to link this variation to drivers of selection in avian populations, particularly those that have undergone a genetic bottleneck (e.g. Bonneaud *et al.,* 2011; Grueber *et al.,* 2014; Gonzalez-Quevedo *et al.,* 2015).

The Seychelles warbler, *Acrocephalus sechellensis*, is an island endemic passerine species that went through a population bottleneck of less than 30 individuals on a single island during the last century (Collar & Stuart, 1985). In a previous study, we characterised variation at TLR genes in this population (Gilroy *et al.,* 2016). We found that despite the considerable losses of genome-wide variation due to the bottleneck (Spurgin *et al.,* 2014), considerable polymorphism remained at five different TLR loci (*TLR1LA, TLR1LB, TLR3, TLR5* and *TLR15*), while two loci were monomorphic (*TLR4* and *TLR21*). Remarkably, four functional variants (alleles) were found at a single locus (*TLR15*), which calls into question the inferred neutrality of this locus.

Due to the overwhelming effect of stochastic processes, detecting the signature of selection in bottlenecked populations using standard population genetic statistical methods is difficult. This includes population genetic summary statistics such as Fst outlier analysis, dN/dS ratio and allele frequency spectrum tests, which may not always be able to identify the signatures of selection. Furthermore, these methods make unrealistic demographic assumptions, such as assuming constant population size and no population structure (Nielsen, 2001), and they fail to distinguish historic selection from current selection (Nielsen, 2005). Several reviews have attempted to disentangle selection effects from those of demography, but they all come to the same conclusion that no single statistical method can convincingly separate the forces (for examples, see Stajich & Hahn, 2005; Wegner, 2008; Alcaide, 2010; Sutton *et al.,* 2011). Additionally, a rejection of neutral evolution only indicates that a population is not in mutation-drift equilibrium. Such deviation from equilibrium is consistent with both the effects of selection as well as a post-bottleneck population expansion, making the interpretation of such tests complicated (Ramírez-Soriano *et al.,* 2008). This problem is particularly acute in relation to conservation genetics given that, by definition, endangered populations are not in equilibrium. In an attempt to resolve this problem, we re-analysed the Seychelles warbler data with an agent-based model that uses forward-in-time simulations to account for the stochasticity during the population bottleneck, as well as the effects of balancing selection and other evolutionary forces before the bottleneck in the ancestral population. As explained above, this is important because balancing selection does not only affect the population genetic dynamics of alleles during the bottleneck, but also affects the initial allele frequency and polymorphism at a locus in the ancestral population. By simulating the exact bottleneck scenario, as previously inferred through neutral markers and historic data (Spurgin *et al.,* 2014), we estimate the strength of balancing selection acting on these genes before, during, and after the bottleneck. In addition, we estimate the predicted future loss of genetic variation at the TLRs, i.e. the 'drift debt', which is likely to occur until the Seychelles warbler has reached its new mutation-drift-selection equilibrium state.

Materials and Methods

Study species

The Seychelles warbler (*Acrocephalus sechellensis*) is a small (ca 12-15 g) passerine bird endemic to the Seychelles archipelago (Safford & Hawkins, 2013). This species underwent a recent bottleneck when the world-wide population was reduced to < 30 birds on the island of Cousin by the 1960s (Crook, 1960). This bottleneck reduced the effective population size (*Ne*) of Seychelles warblers from ca 6900 in the early 1800s to < 45 in the contemporary population (Spurgin *et al.,* 2014). As a result of subsequent conservation actions, by 1982 the population on Cousin recovered to carrying capacity of ca 330 adult birds (Komdeur, 1992; Brouwer *et al.,* 2006). This population has since provided an excellent study system for evolutionary, ecological and conservation study (Komdeur, 1992; Richardson *et al.,* 2003; Barrett *et al.,* 2013; Spurgin *et al.,* 2014). Since 1997, > 96% of the Cousin population has been caught and given unique colour ring combinations and a metal British Trust of Ornithology ring (Richardson *et al.,* 2002). Blood samples (ca 25 µl) are taken at each catch via brachial venipuncture, placed in absolute ethanol in a 2 ml screw-top Eppendorf tube and kept in the fridge at 4^{o}C.

Molecular methods

Blood samples used in the present study were from adult birds (> 1-year-old) chosen at random from the contemporary 2000-2008 population. Genomic DNA was extracted using a salt extraction method (Richardson *et al.,* 2001) and sex was confirmed using a molecular sexing protocol (Griffiths *et al.* 1998). The following TLR loci were amplified: *TLR1LA, TLR1LB, TLR3, TLR4, TLR5, TLR15* and *TLR21,* in 22-30 individuals, as detailed in Gilroy *et al.* (2016).

Demographic scenario

The demographic scenario of the Seychelles warbler has been reconstructed by Spurgin *et al.,* (2014), and this has been used to evaluate whether the observed polymorphism can be explained by neutral evolution (i.e. no selection). First, to establish the genetic variation in the ancestral population, each locus could accumulate polymorphisms in an ancestral population with an effective population size *Ne* = 6900 (Spurgin *et al.,* 2014). The burn-in consisted of 200 000 generations during which the population reached a mutation-drift-selection equilibrium value of polymorphism (Fig. 1). We also explored the minimum and maximum estimates of the ancestral effective population size (*Ne* = 2600 and 9700; see Spurgin *et al.* 2014), again to account for variance in this estimate. After the burn-in, we applied a bottleneck of 22 generations at *Ne* = 50, followed by three generations of population expansion with *Ne* = 100, 150 and 200, and finishing with nine generations at *Ne* = 250 (Spurgin *et al.,* 2014). We assume there is no recombination and that variation is solely introduced by mutation. This was based on previous tests using Genetic Algorithm Recombination Detection (GARD) analyses (Pond *et al.,* 2006) on sequences for all TLR loci in the Seychelles warbler, which failed to detect any evidence of recombination using site-by-site analysis (Gilroy *et al.,* 2016).

Testing neutral evolution with DIYABC

To assess whether an elevated mutation rate could explain present-day Seychelles warbler TLR diversity in the absence of selection, we implemented the demographic model as described above in the program DIYABC v2.1 (Cornuet *et al.,* 2014) and then examined the posterior distributions of mutation rates. This was done on the premise that if posterior mutation rates came out unrealistically high, balancing selection may be responsible for maintaining the variation, partially countering the effects of drift. The programme bases its posterior distributions on the simulated scenarios that most closely match the observed data in terms of a set of user-defined summary statistics. We tested each locus separately, using a Jukes Kantor mutation model with a uniform prior of 10^{-12}-10^{-6}, leaving all other mutation parameters as default. For summary statistics, we enabled all except number of private segregating sites (redundant for single-sample analyses). After a preliminary run of 1 M simulation to evaluate the validity of prior settings and to check the potential for matching the summary statistics to observed value, we ran 5 M simulations for each locus and used the closest 20 000 in the local linear regression for posterior estimation (Cornuet *et al.,* 2014).

Forward-in-Time Computer Simulations

An agent-based model was built to simulate the loss of genetic variation at an autosomal locus under balancing selection (symmetric overdominance) in a diploid panmictic population that experienced a bottleneck of known size and duration. Briefly, TLR loci with known number of base-pairs were simulated. Individuals were diploid, and the fitness (w) of an individual with a homozygous locus was given by $w = 1 - S$, with S being the selection coefficient. Based on preliminary findings (Gilroy *et al.,* 2016), we explored the parameter space across selection coefficients $0.0 \leq S \leq 0.1$. The fitness of heterozygote individuals was equal to unity. Generations were discrete, and selection acted on individuals once per generation. Individuals survived if their fitness was larger than a randomly drawn value from a uniform distribution between zero and one (U [0,1]). Genetic drift was simulated by randomly drawing individuals so that the population size was equal to the user-defined effective population size of that generation. Surviving individuals could reproduce and contributed gametes to the next generation. Gametes unified randomly to make the next generation of individuals. In a population of constant size, this procedure results in a Poisson distribution of the number of offspring with a mean and variance of two. Given the small amplicon size ($\bar{x}$ = 637 bp) of TLR loci, recombination was set to zero. The mutation rate was $\mu = 10^{-9}$ per base pair per generation, a well-justified estimation for this taxonomic group (Kumar & Subramanian, 2002), but we also examined a mutation rate ten times higher ($\mu = 10^{-8}$) to account for uncertainty in this parameter. The bottleneck scenario was started at random points in time after the burn-in, and these sampling points were separated by >100 generations to avoid pseudo-replication. Finally, a subsample equal to the number of genotyped birds was randomly drawn from the simulated contemporary population, and heterozygosity (H_{sim}) in this sub-sample was assessed and compared to the observed value (H_{obs}).

Simulations were first run with a selection coefficient $S = 0.0$ to test whether the null model of neutral evolution could be rejected. If neutral evolution was rejected, different selection coefficients were explored to

examine the parameter space and determine the strength of selection that was most consistent with the observed heterozygosity in the post-bottleneck Seychelles warbler population. We therefore compared the simulated value (H_{sim}) to the observed heterozygosity (H_{obs}) in the contemporary Seychelles warbler population using a total of 1000 independent sampling points to calculate the distribution of H_{sim} for each selection coefficient S of each locus. To determine the minimum selection coefficient required to explain the observed heterozygosity, the percentage of simulations with $H_{sim} < H_{obs}$ was calculated, and if this was less than 5%, the selection coefficient was rejected as being too weak.

To examine whether we could also detect the effect of selection since the bottleneck, we ran a second set of simulations to examine the effect of reducing parasite mediated balancing selection on TLR variation. This is relevant for conservation genetics given that isolated bottlenecked populations may lose a component of their parasite fauna (Bergstrom *et al.,* 1999; Fairfield *et al.,* 2016) and so the strength of parasite-mediated selection will be reduced and have genetic consequences. We focused on *TLR15,* the most polymorphic locus in the Seychelles warbler, and compared the loss of heterozygosity under modest balancing selection (S = 0.03) with that under neutral evolution (S = 0.00) during and after the bottleneck. Observing a continued decline in gene diversity under the neutral scenario, we then performed forecast modelling to predict the future loss of genetic variation at TLR loci in the original Cousin island population of the Seychelles warbler. This part of the study was performed to quantify the 'drift debt' by analysing the loss of genetic polymorphism still required to reach the mutation-drift-selection equilibrium value appropriate for the given effective populations size. In these simulations, we assume a 'no-change' scenario regarding the future demography and population size of the Seychelles warbler. The model predicted the amount of genetic variation at *TLR15* in 2050 and 2100. We assumed an average four-year generation time and an effective population size *Ne* = 250 (Wright, 2014). All simulations were run using Minitab 17 statistical software (2010); macros are available on GitHub (https://github.com/DGilroy89/TLRsims).

Results

DIYABC's posterior distributions of mutation rates for each of the five polymorphic TLR loci are given in Table 1. The programme was unable to estimate posteriors for the two monomorphic loci, even when we reduced the mutation rate prior range to 10^{-15}-10^{-9} and only sought to match one summary statistic (number of haplotypes). To account for the observed level of polymorphism, the posterior distribution of the mutation rates exceeds the expected values for birds, reported in Nobholz *et al.* (2009). This suggests that neutral evolution can be rejected in two out of seven loci.

Simulations with the agent based model show that populations reached mutation-drift equilibrium after 100 000 generations with $\theta = 2.76 \times 10^5$ (which is equivalent to *Ne* = 6900 and $\mu = 10^{-9}$), and that H_{sim} reaches a plateau (Fig. 1). Values of H_{sim} increase with an increased coefficient of balancing selection (S). When simulating neutral evolution, the mutation-drift equilibrium value of heterozygosity is as close to, if not, zero ($H_{sim} \approx 0.0$). In contrast, even a small selection coefficient of S = 0.01 results in high gene diversity ($H_{sim} \approx 0.7$).

A previous study showed that five out of seven TLR loci remained polymorphic in the Seychelles warbler population (Gilroy *et al.,* 2016). According to our simulations, we can reject neutral evolution for the three most polymorphic loci (*TLR1LB, TLR3* and *TLR15; P* < 0.001 for all), but not for the two less diverse loci (*TLR1LA* and *TLR5; P* = 0.991, 0.964, respectively; Tables 2 & 3). Both monomorphic loci, *TLR4* and *TLR21*, also appear to be evolving neutrally (*P* = 0.939, 0.996, respectively; Fig. 2, Fig. S1).

We then explored the effects of the ancestral effective population size on the level of contemporary variation, simulating both the minimum and maximum estimates (*Ne* = 2600 and 9700). The *Ne* of the ancestral population does not appear to have a significant effect on the overall conclusions (Fig. S2). However, when we used a 10× higher mutation rate ($\mu = 10^{-8}$), only the two most diverse TLR loci (*TLR1LB* and *TLR15*) have a level of genetic polymorphism that is inconsistent with neutral evolution (Fig. S3).

Next we tested whether we could discern the effects of contemporary balancing selection, i.e. selection during the 34 generations of the bottleneck (for details see Spurgin *et al.,* 2014). We simulated *TLR15*, the most polymorphic TLR locus in our study, and found that there is no discernible difference in the initial decline of heterozygosity between the scenario with and without balancing selection (S = 0.03 and S = 0.00 respectively) (Fig. 3). However, without balancing selection, the gene diversity continues to decline into the future.

To test this further and quantify the 'drift debt', we conducted forecast modelling to predict the amount of genetic variation at *TLR15* that will remain in the Seychelles warbler population over the next decades without balancing selection. Simulations show that in this scenario, genetic variation continues to decline by a further 2.2% and 4.5% by 2050 and 2100 respectively, even with the future Seychelles warbler population size remaining constant at present day levels. In contrast, if balancing selection continues to act at a constant intensity (S = 0.03), the TLR variation is expected to neither decline further nor increase.

Discussion

We first used the program DIYABC v2.1 (Cornuet *et al.,* 2014) to assess whether an elevated mutation rate could explain present-day Seychelles warbler TLR diversity in the absence of selection. We implemented the demographic model inferred for the Seychelles warbler by Spurgin *et al.* (2014), and found that the posterior distributions of mutation rates had to be unrealistically high if mutation rate was to explain the observed variation within the Seychelles warbler population. For example, the most polymorphic locus *TLR15* had its lowest value estimate for the 5% quantile at 4.85×10^{-8}, and a median μ value of 1.63×10^{-7}. Based on what we know of mutation rate estimates in birds, the fastest substitution rate is in the order Passeriformes is equal to 3.8×10^{-8} (and the median is 4.0×10^{-9}), which are both smaller than out 5% maximum estimate. In other words, our posterior estimates significantly exceed the established μ values in Aves (Nobholz *et al.,* 2009), and hence, we argue that balancing selection may be responsible for maintaining the TLR variation.

Our simulations suggest that balancing selection has been acting on five out of seven TLR genes in the past, assuming an expected typical mutation rate equal to $\mu = 10^{-9}$ per base pair per generation (Kumar & Subramanian, 2002). With a higher mutation rate ($\mu = 10^{-8}$), neutral evolution was rejected in only two of the seven loci. The strength of selection inferred in the Seychelles warbler differs between TLR loci but is generally relatively weak ($0.005 \leq S \leq 0.03$) compared to what has been found for TLRs in other avian species (Alcaide & Edwards, 2011; Grueber *et al.,* 2012). However, the model does not discern a signal of contemporary balancing selection during 34 generations of bottlenecking; the change in allele frequencies during the bottleneck is governed only by drift and not by selection. In other words, the rate of loss of variation is nearly identical for a neutral gene as for a gene under modest balancing selection (S = 0.03). The important difference, however, between neutral genes and genes under balancing selection is the high level of heterozygosity of genes under balancing selection in the ancestral population prior to the bottleneck. In large ancestral populations, such genes tend to possess several alleles present in intermediate allele frequencies, and it takes a considerable amount of drift to lose this amount of polymorphism. In other words, polymorphism in post-bottlenecked populations is 'a ghost of selection past', not 'evidence of selection present'.

Previous studies have also found that drift often overrides the effect of balancing selection during bottlenecks (e.g. Bollmer *et al.,* 2011; Strand *et al.,* 2012; Gonzalez-Quevedo *et al.,* 2015). Our forecast modelling suggests that genetic variation might continue to decline due to a 'drift debt' in which the post-bottlenecked population reaches a new and considerably lower equilibrium level of polymorphism. This is of concern for the conservation genetics of endangered species given that bottlenecked populations are reported to lose a component of their parasite fauna (Bergstrom *et al.,* 1999) and thus the pathogen-mediated selection they face. If the intensity of parasite-mediated balancing selection in the post-bottlenecked Seychelles warbler population remains similar to that in the ancestral population, TLR variation in the contemporary gene pool will have reached its new equilibrium value and it is not expected to decline any further. For this reason, a comparative quantitative analysis of parasite load and diversity between mainland and island populations, or between pre- and post-bottlenecked population samples, would be insightful, as this may help determine the future trajectory of decline in immunogenetic variation and the 'drift debt'.

Considering the genetic background of the Seychelles warbler, there is considerably more variation within the TLR gene-group than was observed in another innate immune gene group of arguably equal importance, avian beta-defensins (AvBDs). Our previous study showed high levels of conservation and monomorphism across AvBD loci (Table S1; Gilroy *et al.,* 2016b), which emphasises the significance of our findings particularly at *TLR15.* As the most polymorphic TLR locus in the Seychelles warbler, *TLR15* is predicted to maintain a considerable amount of heterozygosity with a relatively small selection coefficient (*S* = 0.03). Interestingly, there is still much debate around which PAMPs bind to the *TLR15* receptor, originally it was thought to be exclusively fungi (Boyd *et al.,* 2012) but other studies suggest a wider suite of pathogens including bacteria (Oven *et al.,* 2013; Hu *et al.,* 2016). While it is clear that *TLR15* has a unique role in defence against pathogens, poor understanding of its structure limits understanding of its evolution. Previous studies on other phylogenetically-distant avian species also identified *TLR15* as the most polymorphic locus (Alcaide *et*

al., 2007; Brownlie & Allan, 2011; Boyd *et al.,* 2012). The house finch (*Carpodacus mexicanus*) shows high levels of polymorphism with at least 16 alleles at the *TLR15* locus, which probably reflects the species' large effective population size as well as the effect of balancing selection (Alcaide & Edwards, 2011). More similar to the Seychelles warbler is the New Zealand Stewart Island robin (*Petroica australis raikura*), another island endemic that has undergone a recent bottleneck. In this species, *TLR15* was found to possess two functional variants and was inferred to be under balancing selection (Grueber *et al.,* 2012). Whether or not *TLR15* is currently still under balancing selection can, however, only be determined through an analysis comparing the fitness of individuals that are homozygous and heterozygous at this locus. Given that such analysis requires large sample sizes to obtain sufficient statistical power (Hedrick *et al.,* 2001), this may require an elaborate analysis of (pedigreed) birds collected over multiple generations.

Why did the previous analysis of TLR sequence variation in the Seychelles warbler fail to detect positive selection (also in the historic population)? There has been much criticism on the relatively poor power underlying the use of genetic markers in molecular ecology to detect selection within populations (Waples & Gaggiotti, 2006; Vasemagi & Primmer, 2005; Sutton *et al.,* 2011). Indeed, sequence-based tests of selection come with several caveats such as low statistical power and restrictive assumptions (for review, see Ford, 2002). Sharp changes in demography and population size, as well as the limited number of samples available for analysis, are issues that are particularly problematic in studies of endangered species. For this reason, agent-based models might be a better alternative to understand the evolutionary forces that have shaped genetic variation within endangered populations (see also Carvajal-Rodríguez, 2010). Conservation genetic studies that failed to detect selection in their study species despite observing (moderately) polymorphic loci may have suffered from insufficient statistical power.

A computer simulation approach also offers a further important advantage over population genetic statistics in that it enables researchers to estimate the future loss of genetic variation that may occur in endangered species. Such information allows conservation managers to make informed decisions by anticipating deleterious changes in genepools and to strategically plan interventions such as genetic supplementation (Lynch & Hely, 2001; Miller *et al.,* 2003; van Oosterhout *et al.,* 2007; Frankham, 2008). Forecast modelling of the Cousin island population of Seychelles warbler indicated that the genetic variation at *TLR15* might continue to decline depending on the presence or absence of parasite-mediated selection. The further loss, however, is small, with less than 5% further decline in variation predicted in the next 80 years, assuming no change in *Ne*. In more recently bottlenecked populations, the continued decline in genetic variation will be more severe because the allele frequencies in such gene pools will be further from their equilibrium values than the Seychelles Warbler. In this species (and our simulations), the bottleneck commenced 34 generations ago, during which time most of the loss of variation has already occurred; for example, according to the simulations 8.2% of the gene diversity of *TLR15* was lost within the first 10 generations after the bottleneck. Without contemporary parasite-mediated selection, the observed loss of genetic variation at immune genes in recently bottlenecked populations is likely to significantly exceed the level expected in a genepool that is in a mutation-drift-selection equilibrium. This emphasises the importance

in also considering the conservation of pathogens, particularly when translocating individuals to new islands and wanting to optimise genetic capture and long-term viability (Fairfield *et al.,* 2016). Analogous to the 'extinction debt' (Kuussaari *et al.,* 2009), genetic variation is expected to be lost under a 'no change' scenario. We have referred to this as the 'drift debt', and we believe this is likely to affect many recently bottlenecked populations and needs to be considered in conservation management strategies.

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Table 1. Posterior distributions of mutation rate (μ; per base pair per generation) for five TLR loci derived from analysis with DIYABC v2.1. For each distribution, the median and inter-quartile ranges are presented, and whether the estimated mutation rate exceeds typical values at each locus. The programme was unable to estimate posteriors for the two monomorphic loci (*TLR4* and *TLR21*).

Table 2. Polymorphism statistics for TLR loci in the Seychelles warbler. Adapted from Gilroy *et al*. 2016.

Table 3. Selection coefficient (S) estimates based on plotted simulated TLR heterozygosity (H) following specific demographic scenario after 100 000 generations under a constant selective pressure. P-values indicate any significant difference between observed and simulated *H* based on the percentage of simulations (n = 1000) where $H_{sim} < H_{obs}$ and if less than 5%, the selection coefficient was rejected as being too weak.

Figure 1. Simulated heterozygosity of a gene subject to overdominance selection and which consists of 528 base pairs in a population with an effective population size Ne = 6900 and a mutation rate $\mu = 10^{-9}$ across a range of selection coefficients (S = 0.00, 0.01, 0.02, 0.03 and 0.05). The equilibrium heterozygosity is reached after ca 100 000 generations.

Figure 2. Mean (5 - 95% CI) simulated heterozygosity (H_{sim}) across a range of selection coefficients (S) in a contemporary population of Seychelles warblers. The observed heterozygosity H_{obs} is indicated by the dashed line for each locus.

Figure 3. Heterozygosity in simulations with a constant balancing selection S = 0.03 both before and after the bottleneck event (solid) and balancing selection in the ancestral population (Sb = 0.03) followed by neutral evolution during / after the bottleneck (Sn = 0) (open symbols). Dotted horizontal line indicate actual heterozygosity (*He* = 0.682) observed in the population sample and the contemporary sample was collected at generation 34 (dashed vertical line). There is little difference between simulations with and without balancing selection, indicating that the loss in polymorphism is governed by drift, not selection. However, without contemporary balancing selection, the population continues to lose genetic diversity due to the 'drift debt'.

Table 1.

Locus	Median μ	IQR	95% range	Reject posterior estimate
TLR1LA	5.27E-08	2.40×10^{-08} - 1.10×10^{-07}	4.38×10^{-09} - 3.91×10^{-07}	Accept
TLR1LB	8.33E-08	4.92×10^{-08} - 1.42×10^{-07}	1.92×10^{-08} - 4.49×10^{-07}	Accept
TLR3	1.12E-07	6.96×10^{-08} - 1.92×10^{-07}	2.96×10^{-08} - 5.60×10^{-07}	Reject
TLR4	*Not estimated*	*Not estimated*	*Not estimated*	*Not estimated*
TLR5	8.63E-08	4.93×10^{-08} - 1.50×10^{-07}	1.72×10^{-08} - 4.80×10^{-07}	Accept
TLR15	1.63E-07	9.79×10^{-08} - 2.73×10^{-07}	3.83×10^{-08} - 6.93×10^{-07}	Reject
TLR21	*Not estimated*	*Not estimated*	*Not estimated*	*Not estimated*

Table 2.

Locus	Number of sequences	Fragment size (bp)	Number of unique alleles	Number of functional alleles*	Heterozygosity (H_{obs})
TLR1LA	44	531	2	1	0.35
TLR1LB	66	750	4	2	0.63
TLR3	56	801	5	3	0.53
TLR4	60	648	1	0	0.00
TLR5	46	741	3	1	0.12
TLR15	60	528	4	3	0.68
TLR21	60	453	1	0	0.00
*alleles resulting in different translated amino acids					

Table 3.

Locus	S based on *H*	S based on number of haplotypes	Percentage of $H_{obs} < H_{sim}$ when S = 0	Reject or accept H_{obs}
TLR1LA	0.010	0.010	0.991	Accept
TLR1LB	0.025	0.045	0.000	Reject
TLR3	0.015	0.075	0.000	Reject
TLR4	0.000	0.000	0.929	Accept
TLR5	0.005	0.010	0.964	Accept
TLR15	0.030	0.085	0.000	Reject
TLR21	0.000	0.000	0.986	Accept

Figure 1.

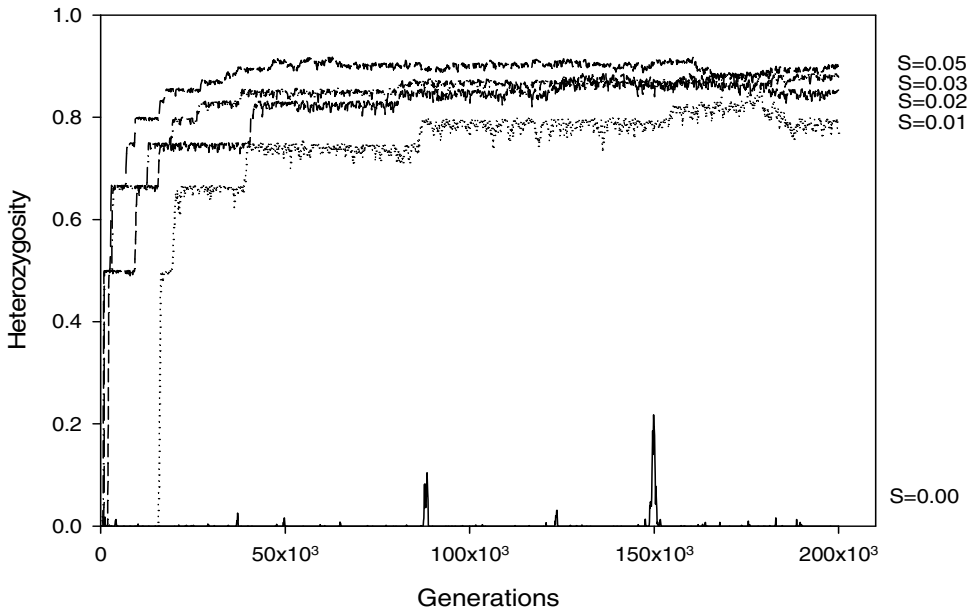

Figure 2.

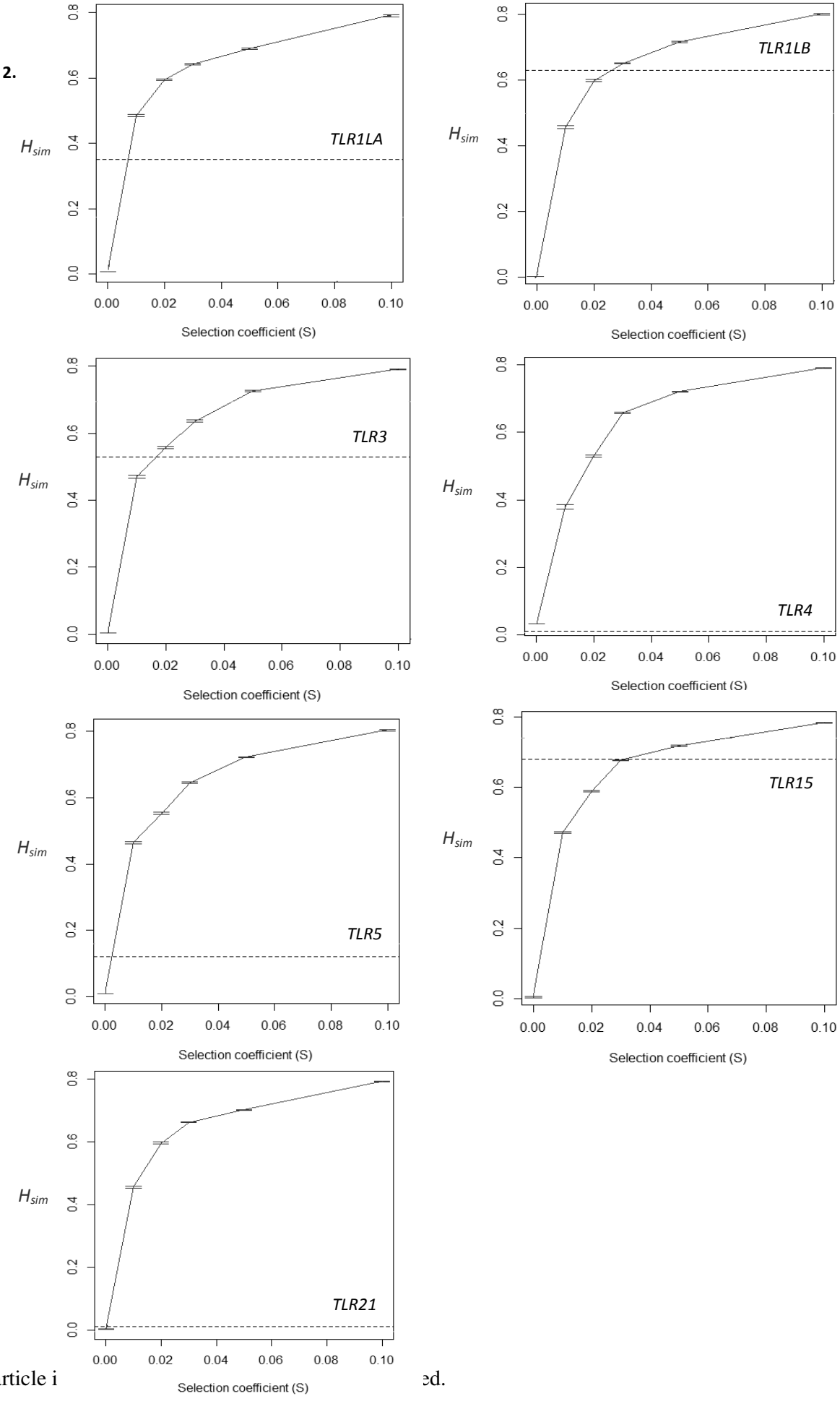

Figure 3.