Adaptation and differentiation across island populations of Berthelot's pipit (*Anthus berthelotii*)

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Abstract

The aim of this thesis was to examine how neutral and adaptive processes shape patterns of genetic diversity across populations of Berthelot's pipit (*Anthus berthelotii*), a passerine bird endemic to the Canary Islands, Selvagens and Madeira archipelagos. To achieve this, I examined variation in pathogen infection, neutral microsatellites and functional major histocompatibility complex (MHC) genes. I first reviewed previous evidence for pathogen-mediated selection at the MHC, and showed that differentiating between specific mechanisms of balancing selection may not be possible, and that many studies that have attempted to do so have not fully considered alternative explanations. In Berthelot's pipit, I found marked differences in prevalence of avian malaria and pox across populations, and showed that these differences were stable over time, largely because they were determined by biogeographic features. This, combined with an observed effect on host body condition, suggests that populations face differential selection pressures from pathogens. Microsatellite analyses indicated that the pipit colonised northwards across its range, resulting in genetic bottlenecks in the Selvagens and Madeira archipelagos. I then used the pipit system to assess how population genetic analyses were influenced by microsatellite markers with different levels of variability; lower variability loci appear to more accurately reflect population divergence, whereas higher variability loci better reflect past changes in population size. I also found that two commonly used measures of differentiation (G_{ST} and Jost's D) are both strongly affected by marker variability, but in different ways. Finally, I found that just 11-15 MHC variants persisted through the initial colonisation event. However, since the bottleneck, at least 26 functional MHC alleles have been generated *in situ* across the different populations, all but two by gene conversion. Taken together, my results provide an interesting example of how founder events, mutation, drift and selection can interact to drive differentiation across natural populations.

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Table of Contents

Chapter contributions

Versions of three chapters of this thesis have been published, and two are manuscripts in preparation. All chapters have involved collaboration, I am the lead author on all manuscripts, and have made the largest contribution to all. Below I include the full citation for each chapter, and highlight my contribution.

Chapter 2. Spurgin, L. G., and D. S. Richardson 2010. *Proc. R. Soc. Lond. B. Biol. Sci.* 277, 979-988.

- LGS conceived the review and drafted the manuscript. (80%)

Chapter 3. Spurgin, L. G., J. C. Illera, D. P. Padilla, and D. S. Richardson 2012. *Oecologia* 168, 691-701.

- LGS collected half the samples (from 2009), performed the lab work, analysed the data and drafted the manuscript. (70%)

Chapter 4. Spurgin, L. G., T. H. Jorgensen, J.C. Illera, D. A. Dawson and D. S. Richardson. (In Prep)

- LGS performed the lab work, analysed the data and drafted the manuscript. (70%)

Chapter 5. Spurgin, L. G., J.C. Illera and D. S. Richardson. (In Prep)

- LGS performed the lab work, analysed the data and drafted the manuscript. (80%)

Chapter 6. Spurgin, L. G., C. Van Oosterhout, J. C. Illera, S. Bridgett, K. Gharbi, B. C. Emerson, and D. S. Richardson 2011. *Mol. Ecol.* 20, 5213-5225.

- LGS performed the lab work (with the exception of 454 sequencing), lead the data analysis and drafted the manuscript. (65%)

Chapter 1: Introduction

Jacob with the flock of Laban, by Jusepe de Ribera

1.1 A (very) brief history of genetic variation

For centuries, humans have been aware that variation in exists in natural populations, and that traits can be passed on to subsequent generations. Families have long been recognised to possess distinctive features, which pass from parent to offspring. In Ancient Greece and Egypt, the belief that variation existed and was heritable was so strong that rulers often married within families to keep the blood pure and strengthen the line of succession. Similarly, the importance of variation and inheritance in animal husbandry has been understood since Biblical times. In Genesis (30:25-43), Jacob, a shepherd, and his father-in-law Laban agree that all of the white sheep born into their flock would be Laban's, and all of the black and streaked sheep would be Jacob's. Jacob shrewdly mated his black sheep with Laban's white sheep to produce predominantly streaked sheep. After a few generations, virtually no white animals were left for Laban. Jacob, then, had an understanding of variation, dominance and inheritance, long before the age of genetics.

Although inheritance in a broad sense has been understood for centuries, the mechanism by which traits are passed onto subsequent generations was not understood until Mendel formed his theories of inheritance in 1866 (in fact not until much later, as Mendel's work was largely ignored at the time). Most scientists in the 19th century, including Darwin, accepted a theory of inheritance by 'blending' (Charlesworth and Charlesworth 2009). However, others noted at the time, and afterwards, that inheritance by blending was impossible, as traits would become homogenised over time (Jenkin 1867; Fisher 1930). Mendelian inheritance solves this problem, as under particulate inheritance variation is maintained over time (Charlesworth and Charlesworth 2009). Unfortunately, however, Darwin remained unaware of Mendel's work, and never came to an understanding of the extent and evolutionary role of genetic variation in natural populations. Indeed, this was not addressed in detail by scientists until the 1930s, when R.A Fisher (1930), Sewall Wright (1931) and J.B.S. Haldane (1932) consolidated Mendelian genetics with Darwin's theory of natural selection, thereby laying the foundations of population genetic theory. Two schools of thought developed, often termed the "classical" and "balance" views (see Lewontin 1974; Avise 2004 for an overview). The classical school believed that genetic variation in natural populations was low because purifying selection generally operated to eliminate deleterious mutants; high variation would incur too much "genetic load"

(Kimura and Crow 1964). The balance school, on the other hand, believed that variation within populations was high, and maintained by balancing selection (Dobzhansky 1955). The degree of difference between the two views was stark, with proponents of the classical and balance hypothesis suggesting that the proportion of genes that are polymorphic in a typical populations was 0.1% and "towards 100%", respectively (Muller 1950; Wallace 1958). The debate, however, reached a stalemate, unable to progress until population-level genetic variation could be visualised directly.

In 1966, three landmark papers were published which showed, thanks to the first application of electrophoresis to population-level data, that levels of protein (and by inference genetic) diversity in natural populations were far higher than previously expected (Harris 1966; Hubby and Lewontin 1966; Lewontin and Hubby 1966). Lewontin and Hubby (1966) showed that across five wild populations of *Drosophila pseudoobscura,* 9 out of 18 randomly chosen allozyme loci were polymorphic in at least one population; a surprising finding which the authors struggled to explain. Similarly, Harris (1966) showed that in a single human population, 2 out of 10 randomly chosen enzyme loci were polymorphic, leading Harris to posit that "unless we have been excessively lucky in our choice of enzymes... polymorphism to a similar degree may a fairly common phenomenon among the very large number of enzymes that occur in the human organism".

The work of Lewontin, Hubby and Harris provided both the impetus and the methodology for other researchers to directly observe levels of enzyme polymorphism in wild animal and plant populations. Many such studies were carried out in a wide range of taxa, showing that, in almost all cases, extensive variation existed (reviewed in Lewontin 1973; Lewontin 1985). However, some authors argued that the enzymes chosen for most of these population-level studies were not representative of other protein coding genes, and that polymorphism levels had been overestimated (Brown and Langley 1979; Racine and Langley 1980). Further technical innovation enabled population biologists to visualise variation at the DNA level; including restriction fragment length polymorphism (RFLP) analyses in the late 1970s (Avise et al. 1979; Moritz et al. 1987), polymerase chain reaction (PCR) mediated DNA sequencing in the late 1980s (Saiki et al. 1986; 1988; Kocher et al. 1989), and massively parallel sequencing in the 2000s (Allendorf et al. 2010; Stapley

et al. 2010). Studies using these methods quickly dispelled any remaining doubts over whether appreciable genetic diversity existed in natural populations (Avise 2004), and fuelled extensive study and debate about the relative role of neutral and adaptive processes in shaping genetic variation in natural populations.

1.2 Neutral and adaptive genetic variation

In the 1960s and 1970s, some proponents of the "balance school" maintained that the discovery of extensive genetic variation in natural populations validated their hypothesis; this variation, they argued, could only be explained by genome-wide balancing selection (Wallace 1968). However, it was still generally assumed that all genetic variation was functional, and so proponents of the classical view were still troubled by the problem of genetic load. With this in mind, Motoo Kimura posited that most of the variation observed at the molecular level was the product of random mutations and genetic drift, rather than natural selection; the so-called "neutral theory" of molecular evolution (Kimura 1968a, b; King and Jukes 1969). The neutral theory did not challenge the premise of evolution by natural selection, but suggested that *most* evolutionary change, at least at the genetic level, was non-adaptive. Richard Dawkins, probably the most famous populariser of natural selection in recent times, was customarily succinct when he wrote the following, in *The Blind Watchmaker* (Dawkins 1986)*:*

> "... the neutralists think — rightly, in my opinion — that adaptations are the tip of the iceberg: probably most evolutionary change, when seen at the molecular level, is non-functional."

The "selectionism vs. neutralism" debate that ensued after Kimura's theory was published has been one of the most publicised in the history of evolutionary biology (Lewontin 1974; Nei 2005). While there is still heated debate (e.g. Hahn 2008; Nei et al. 2010), some consensuses have emerged. Perhaps most importantly, it is clear that some DNA sequences have, or continue to be, subject to some form of natural selection, while others appear to have been neutral throughout their evolutionary history (Ford 2002). The assumption of selective neutrality at certain regions of the genome has enabled researchers to use molecular markers as 'windows' into the myriad of past and present

ecological and evolutionary processes occurring at the organism, population and species levels (Sunnucks 2000). Furthermore, great progress has been made in identifying the processes contributing to the distribution of molecular variation. In particular, large amounts of research, facilitated by access to genome-scale sequence data, have been devoted to identifying the regions of the genome that are (or have been) subject to natural selection (Bustamante et al. 2005; Andrés et al. 2009; Hohenlohe et al. 2010). In some cases, these approaches have been enabled researchers to identify *how and why* selection has operated to produce the patterns of variation observed in wild populations (e.g. Gratten et al. 2008; Linnen et al. 2009; Hohenlohe et al. 2010; Nadeau et al. 2012), although this remains an ongoing and exciting area.

1.2.1 Neutral markers as windows into whole-organism processes

Molecular markers can be used to estimate historical and present-day processes that occur at the level of the individual, population and organism. At the individual level, genetic markers can be used to infer patterns of parentage, or kinship within populations, enabling the construction of pedigrees (Queller and Goodnight 1989; Marshall et al. 1998). Within and across populations within a species, molecular markers can be used to estimate divergence times, migration rates, effective population size and, to some extent, past changes in population size (Cornuet and Luikart 1996; Beerli and Felsenstein 2001; Garza and Williamson 2001). Across recently separated species, molecular markers can be used to infer phylogenetic relationships, date divergence events and infer patterns of hybridisation (Swofford 2003; Drummond and Rambaut 2007). Finally, molecular markers have been used to identify the major lineages and patterns of relationship across distantly related groups of organisms (Shimamura et al. 1997; Murphy et al. 2001). Thus, studies that use molecular markers as windows into whole-organism processes can best be viewed as inferring patterns of relatedness at some point along an evolutionary continuum (Avise 2004), and have contributed enormously to our understanding of both ancient and contemporary evolution.

In order to gain clear insight into evolutionary processes through the use of molecular markers, a number of factors need to be considered. First, the markers should be selectively neutral. Selective neutrality is generally assumed in population genetic and

phylogenetic models and analyses, mostly for simplicity (Avise 2004). Selection can distort allele frequencies and DNA sequences in unexpected ways, making it difficult to interpret patterns of population history and demography. Second, depending on the ecological or evolutionary question being asked, researchers should choose the molecular markers with the most appropriate manner and rate of mutation (Parker et al. 1998; Zhang and Hewitt 2003). For example, using very rapidly mutating markers to infer relationships among distantly related groups of organisms will result in problems with homoplasy (Garza and Freimer 1996; Estoup et al. 2002). On the other hand, if the marker chosen has too slow a mutation rate for the organisms being studied, too few mutations will have accumulated, and there will be insufficient power for analyses. Generally, studies of closely related groups of individuals or populations have used microsatellites, due to their extremely rapid mutation rate (c. 10^{-4} substitutions per site per year) (Ellegren 2004). Studies of more distantly related populations or closely related species often use mitochondrial DNA (c. 10^{-6}) (Hasegawa et al. 1985; Moritz et al. 1987), and phylogenetic studies of distantly related species use nuclear (c. 10^{-9}) or ribosomal (10^{-12}) genes, or retroposons (Hillis and Dixon 1991; Shimamura et al. 1997). Increasingly, studies are using multiple markers, with different mutation rates, across the same set of samples to infer multiple evolutionary processes within individual systems (Zhang and Hewitt 2003; Brito and Edwards 2009).

1.2.2 Adaptive genetic variation: selection at the molecular level

Identifying how selection operates at the molecular level is a complex task, as it is usually extremely difficult to identify the genetic basis of a given trait of interest. Consequently, up until the last few years most studies investigating adaptive genetic variation have focused on the few model organisms for which detailed genetic information was available. More recently, population genomic and quantitative trait loci (QTL)-mapping approaches have increased in popularity, predominantly due to developments in molecular and analytical tools. These “top down” approaches usually involve scanning for polymorphisms that associate with variation at a trait of interest, and studies using them have achieved considerable success (see Slate 2005; Stinchcombe and Hoekstra 2007 for reviews). However, there are still practical issues with such approaches. First, resolving genome-wide variation is still costly, especially across large numbers of individuals or

populations. Second, QTL-mapping and population genomic approaches often use large numbers of statistical tests, making it difficult to differentiate between genuine effects and false positives. Third, for QTL-mapping, pedigrees and phenotype data are required, which are often not available for non-model organisms. Finally, without enormous sample sizes it is often only possible to pinpoint a trait of interest down to a large region of the genome, rather than to a specific gene or mutation (Stinchcombe and Hoekstra 2007).

Candidate genes of known functional significance offer a reliable and rapid way of studying adaptation in the wild. Various candidate genes with clear links between genotype, phenotype and fitness have been identified in model systems and, due to being evolutionary conserved, many of these can be characterised and genotyped in non-model organisms with relative ease (see Piertney and Webster 2010 for a review). A wide range of fundamental questions in ecology and evolutionary biology can be addressed using candidate gene approaches, including: i) what are the relative roles of selection and drift at functional genes? ii) What mechanisms of selection drive genetic diversity? iii) What are the (interacting) effects of genes and the environment on phenotypic variation and fitness? Even in the age of genomics, "bottom up" approaches remain a useful tool for evolutionary biologists, and may prove to be even more powerful when used in combination with QTL-mapping and population genomics.

1.3 The Major Histocompatibility Complex – a model gene for studying selection

Genes of the vertebrate major histocompatibility complex (MHC) are among the most well-studied candidate genes in non-model organisms. Due to their importance in human health, their structure and function has been fully characterised in humans and mice (Meyer and Thomson 2001). From an evolutionary perspective, MHC genes are especially interesting due to their extraordinary levels of polymorphism and clear evidence of balancing selection, which has been demonstrated in a wide range of organisms (Bernatchez and Landry 2003; Piertney and Oliver 2005).

1.3.1 Structure and function

MHC genes code for molecules that bind to both self and foreign peptide antigens and present them to T-cells, resulting in a cascade of appropriate immune responses if foreign

antigen-MHC molecules are detected (Klein 1986). MHC molecules are polymorphic integral membrane proteins, consisting of an immunoglobulin 'stalk' that attaches the molecule to the cell membrane, and a peptide-binding region which is responsible for the recognition of one or more antigens. Binding at these antigen recognition sites is determined by the presence of a particular sequence of amino acids at specialised anchor point on the protein. Thus, whilst specificity exists, each MHC molecule can bind several peptides (Klein 1986).

MHC genes exist in a multigene family, which includes two main subfamilies; class I and class II genes. The pathways involved in antigen presentation differ with MHC class I and II. The MHC class I antigen presentation pathway is present in almost all cells, and provides a mechanism for displaying samples of peptides from proteins that are present within the cell at any given time. Endogenous proteins are broken down in the cytoplasm by proteasomes and other enzymes, and the resulting peptides are actively transported into the endoplasmic reticulum before being bound to a peptide loading complex containing the MHC class I molecule. The peptide is trimmed before being released, still bound to the peptide-binding region of the MHC class 1 molecule. The MHC class I-bound peptide is then transported to the cell surface, where it displays the peptide antigen to $CD8^+$ T cells, which have the ability to detect and kill cells expressing viral proteins or tumour antigens (Jensen 2007). The MHC class II presentation pathway is only found in specialist antigen presenting cells, namely thymic epithelial cells, dendritic cells, B cells and macrophages (Jensen 2007). MHC class II molecules bind primarily with proteins found in the endocytic pathway; usually these are exogenous protein sources that have entered the cell by a phagocytosis, endocytosis or similar mechanisms (Germain and Margulies 1993). Peptides from these proteins are then displayed to $CD4^+$ T helper-cells. Thus, the MHC class II pathway facilitates the recognition and immune response primarily to extracellular pathogens.

1.3.2 Polymorphism and selection

MHC genes are the most polymorphic known in vertebrates; in humans, hundreds of alleles have been found in individual populations at both class I and class II loci (Garrigan and Hedrick 2003). In wild animal populations, allelic variation has been detected at the

MHC even when variation at other markers has been absent or extremely low (Richardson and Westerdahl 2003; Aguilar et al. 2004; van Oosterhout et al. 2006). MHC genes also display high levels of polymorphism at the nucleotide level; in some regions of the human MHC (often called human leukocyte antigen, or HLA), nucleotide diversity is several orders of magnitude higher than the genomic average (Garrigan and Hedrick 2003).

New MHC alleles are generated by a combination of point mutation and gene conversion (see chapter 6). There is strong evidence for selection on MHC genes at the sequence level. Balancing selection (like positive selection) is expected to incorporate new adaptive mutations into populations at a higher rate than expected under neutrality. Additionally, under balancing selection, adaptive mutations are expected to be retained for longer periods of time than under neutrality. Both these phenomena can be clearly observed at the MHC. In one of the first studies of selection at MHC genes, Hughes and Nei (1988) showed that the rate of protein changing substitutions in HLA sequences greatly exceeded that of silent substitutions; something subsequently demonstrated within and across many other taxa (Bernatchez and Landry 2003). Evidence for retention of adaptive MHC alleles can be best observed by examining MHC variation across species in a phylogenetic context. By doing this, Jan Klein showed extensive 'trans-species polymorphism' at MHC genes; a phenomenon where MHC sequences from one species clusters with sequences from another species, suggesting long-term retention of lineages (Klein 1980, 1987). More recently many studies have attempted to characterise the nature of selection at the MHC in more detail, with particular focus on the role of pathogens in driving MHC diversity. These studies are reviewed in detail in chapter 2.

1.4 Study system

Islands have been used as model systems for evolutionary research ever since Darwin's fateful voyage on the Beagle (1831-1836) to, amongst other places, the Galapagos Islands, the fauna of which helped shape his ideas about natural selection. There are numerous reasons why island archipelagos are attractive places to study evolutionary biology (see Emerson 2002 for an in-depth review). For example, islands represent geographically distinct units, enabling clear delimitation of populations for analyses. Second, oceans act as barriers to gene flow between islands. Third, because of their small

size, islands are relatively simplistic in terms of species diversity and ecosystem complexity. Fourth within archipelagos there is often variation in the geological and ecological characteristics of individual islands.

Macaronesia, which translates from Greek as “the fortunate islands”, is the collective name for five archipelagos in the North Atlantic: the Azores, Madeira, The Selvagens (or Savage Islands), the Canary Islands, and Cape Verde. All five archipelagos are volcanic in origin, formed between 0.8 (El Hierro, Canary Islands) and 21 million years ago (Fuerteventura, Canary Islands). The islands vary in size (between approximately 2 and 2,000 km^2), isolation (between approximately 100 and 1,000 km from the nearest mainland) and altitude (between approximately 100 and 3,000 metres above sea level). An amazingly wide range of habitats can be found on these islands, including semi-arid coastal scrub, pine forest, laurel forest and dry subalpine scrub. The Macaronesian archipelagos are characterised by distinctive flora and fauna, with large numbers of endemic species and subspecies (Silvertown 2004; Emerson and Kolm 2005). As a result, they have been the subject of many evolutionary and ecological studies (Juan et al. 2000).

Berthelot’s pipit (*Anthus berthelotii*) is a small passerine bird, endemic to three of the Macaronesian archipelagos; Madeira (three island populations) Selvagens (one island population) and the Canary Islands (nine populations on eight islands; Fig. 1.1). On Tenerife in the Canary Islands, a population occurs on an alpine plateau on the mountain of Teide more than 2,000 m above sea level. This population is separated from the rest of the Tenerife population by dense pine and laurel forests on the mountainsides, which the pipit does not inhabit. For this reason, Teide is considered as a separate, thirteenth population throughout this thesis. The Berthelot’s pipit’s closest relative is the tawny pipit (*Anthus campestris*), a common passerine which breeds across Europe, Asia and North-West Africa, from which Berthelot’s pipit has been separated for approximately two million years (Arctander et al. 1996). Berthelot’s pipit is presently split into two sub-species: *A. berthelotii berthelotii* on the Canary Islands and the Selvagens, and *A.berthelotii madeiriensis* on Madeira (Clarke 2006; but see Illera et al. 2007). The pipit is one of the most abundant passerines throughout its range, where it occupies semi-arid habitats from coastal scrub to high subalpine regions (up to 2,500 metres on El Teide,

Tenerife) (Illera 2007; Fig. 1.2). The relative population size across islands is therefore largely a product of island size (Illera 2007), though nothing is known about historical population sizes. Berthelot's pipits, like most pipits, have a generation time of approximately three years, and lay clutches of between two and four eggs per year (Garcia-Del-Rey and Cresswell 2007).

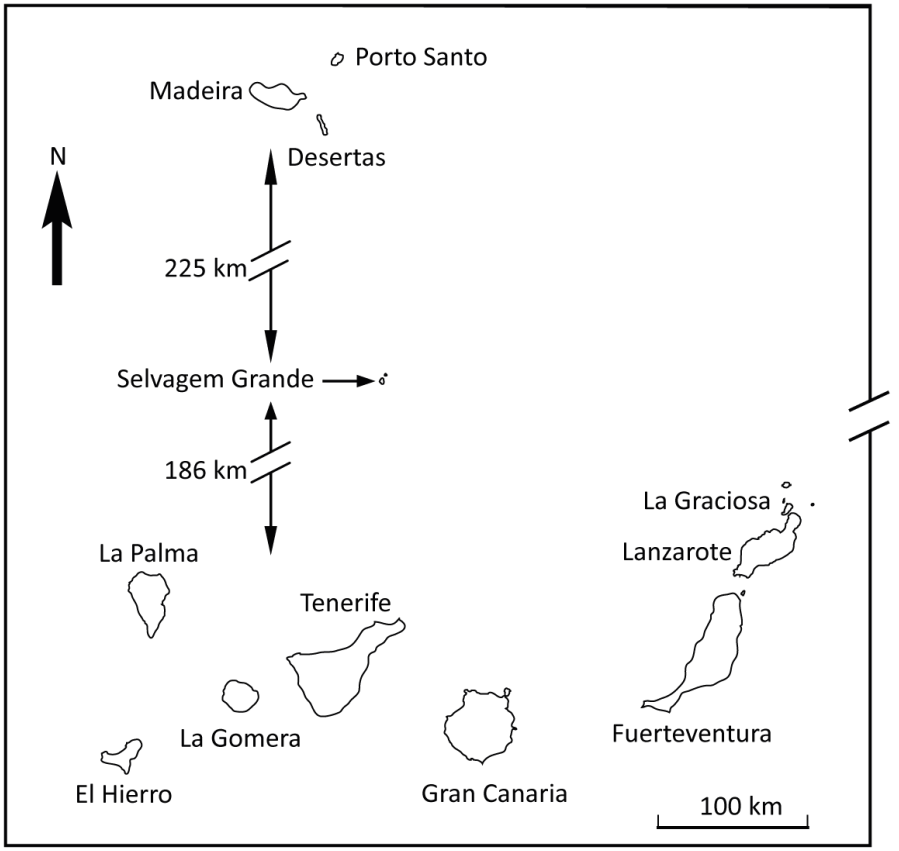

Figure 1.1 Distribution and sampling locations of Berthelot's pipits in the North Atlantic. Note that two populations were sampled from Tenerife; the coastal population, and the high mountain of Teide.

Previous work on Berthelot's pipit suggests that this species is an ideal model for population and evolutionary genetics research. The pipit has extremely low levels of genetic variation, with just one and four haplotypes at the mitochondrial control region and cytochrome *b* genes respectively, across all 13 populations (Illera et al. 2007). The pipit was also found to have lower levels of genetic variation compared to other passerine species at a set of conserved microsatellite markers (Dawson et al. 2010). Screening at five microsatellite loci revealed significant genetic structure across the 13 populations,

particularly across, but also within archipelagos, and significant isolation-by-distance was detected (Illera et al. 2007). These data are consistent with a small founding population of pipits having recently colonized these islands, with limited subsequent dispersal (Illera et al. 2007). This sets the pipit apart from many other island bird systems, which have either been separated for very long periods of time (making it difficult to tease apart the complex array of processes affecting genetic diversity) or are too recently separated (with not enough time for differences to accumulate across populations). A preliminary screening of avian malaria and pox across the pipit populations suggests that there are marked differences in pathogen prevalence (Illera et al, 2008), although it is not clear whether these differences are stable over time. Thus, the Berthelot's pipit provides an interesting opportunity to explore how neutral (demography) and adaptive (parasites) shape genetic diversity in the early stages of differentiation.

1.5 Aims of thesis

In this thesis, I examine how neutral and adaptive processes shape patterns of genetic diversity across natural populations. In chapter 2, I review the logic and evidence supporting the main hypothesised mechanisms proposed to explain how pathogen-mediated balancing selection maintains genetic diversity at MHC genes. Then in chapter 3 I explore the potential for pathogen-mediated selection in the Berthelot's pipit, by screening spatio-temporal variation in pathogen prevalence across the 13 island populations. In chapter 4 I use microsatellites, derived both anonymously and from expressed regions of the genome, to explore how demographic processes have promoted divergence across the pipit populations, with particular focus on the importance of marker variability when using neutral markers to infer population history. In chapter 5, I expand upon the microsatellite analyses, and explore specifically how measures of genetic differentiation (G_{ST} and D) respond to marker variability. Finally, in chapter 6, I screen the pipit populations at a set of duplicated MHC loci, and explore how allelic variation at these functional genes is partitioned across the pipit populations, and discuss the roles of demography, mutation and natural selection in driving genetic diversity at these functional genes. Finally, in chapter 7 I discuss the overall findings from chapters 2-6, and suggest some directions for future research.

Figure 1.2 Berthelot's pipit (*Anthus berthelotii*; top), and typical Berthelot's pipit habitat in coastal (middle) and alpine (bottom) regions.

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Chapter 2: How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings

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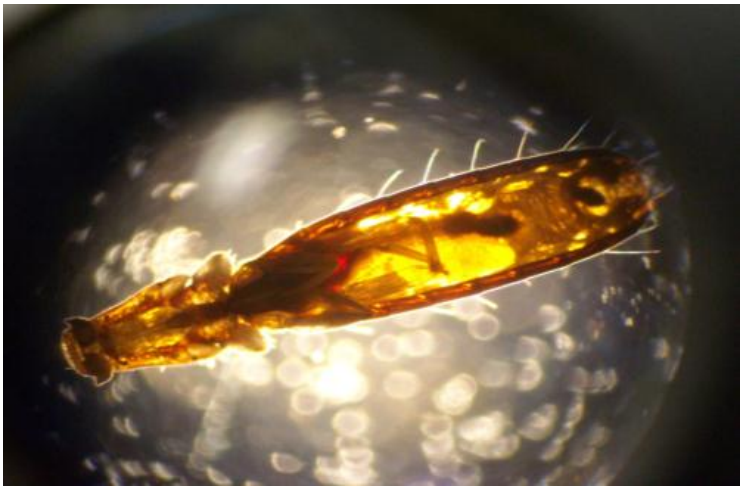

Feather louse, found on a canary (*Serinus canaria*)

2.1 Abstract

Major histocompatibility complex (MHC) genes have been put forward as a model for studying how genetic diversity is maintained in wild populations. Pathogen-mediated selection (PMS) is believed to generate the extraordinary levels of MHC diversity observed. However, establishing the relative importance of the three proposed mechanisms of PMS (heterozygote advantage, rare-allele advantage and fluctuating selection) has proved extremely difficult. Studies have attempted to differentiate between mechanisms of PMS using two approaches: (i) comparing MHC diversity with that expected under neutrality and (ii) relating MHC diversity to pathogen regime. Here, we show that in many cases the same predictions arise from the different mechanisms under these approaches, and that most studies that have inferred one mechanism of selection have not fully considered the alternative explanations. We argue that, while it may be possible to demonstrate that particular mechanisms of PMS are occurring, resolving their relative importance within a system is probably impossible. A more realistic target is to continue to demonstrate when and where the different mechanisms of PMS occur, with the aim of determining their relative importance across systems. We put forward what we believe to be the most promising approaches that will allow us to progress towards achieving this.

2.2 Introduction

Explaining how genetic variation is maintained in wild populations has long been a central question in evolutionary biology. Since previously unpredicted numbers of alleles were detected in populations of humans and Drosophila (Harris 1966; Lewontin & Hubby 1966), biologists have debated the relative roles of balancing selection and neutral processes in maintaining the diversity observed in wild populations (reviewed in Nei 2005; see also section 1.1). This debate has now matured, and the parsimonious appeal of neutral theory has led to it being accepted as the null hypothesis against which selection can be tested (Kreitman 1996). A number of genes believed to be subject to balancing selection (a bracket term encompassing a number of forms of selection that act to maintain multiple alleles within a population) have now been identified (Ford 2002). However, in the majority of cases, the exact causes and mechanisms behind the selection remain unclear. A major reason for this is that while DNA data have been relatively easy to collect, it has proved more difficult to identify gene function and more difficult still to show how variation in function is influenced by selection (Ford 2002). In wild-living organisms, finding suitable candidate genes for studying balancing selection is an especially difficult task, as the genetic basis of traits of interest is usually poorly understood.

Genes of the vertebrate major histocompatibility complex (MHC) arguably provide the most promising opportunity for studying how balancing selection operates to maintain genetic variation in populations. The extensive population-level allelic richness (hereafter referred to as 'diversity') observed at these genes, alongside their central role in the vertebrate immune system, makes them ideal candidates for studying selection (Hedrick 1994; Meyer & Thomson 2001). The structure and function of MHC genes is now well understood in a range of organisms (e.g. Bjorkman et al. 1987; Sato et al. 1998; Kaufman et al. 1999; Hess & Edwards 2002), allowing testable hypotheses to be formed concerning the nature of selection operating on these genes.

It has long been suggested that pathogen-mediated selection (PMS) is the driving force maintaining diversity at MHC loci (Doherty & Zinkernagel 1975; Jeffery & Bangham 2000; Bernatchez & Landry 2003). Gene conversion and recombination, sexual selection and

maternal–foetal interactions may also play a role, though these factors are outside the scope of this chapter (for extensive reviews, see Edwards & Hedrick 1998; Martinsohn et al. 1999; Penn & Potts 1999). Three main hypotheses of PMS have been proposed, which are all forms of balancing selection: heterozygote advantage (Doherty & Zinkernagel 1975), rare-allele advantage (Slade & McCallum 1992) and fluctuating selection (Hill 1991). A strong theoretical framework has been established supporting the idea that any of the three mechanisms, or any combination of the three, could drive MHC diversity (Hughes & Nei 1988; Takahata & Nei 1990; Apanius et al. 1997). However, it has proved much more difficult to identify and differentiate between them empirically in wild populations, and so the key aim of determining the relative importance of the different mechanisms has remained elusive (Bernatchez & Landry 2003; Piertney & Oliver 2005). Early MHC studies focused on detecting selection operating over macro-evolutionary time scales, using methods based on coalescence theory or differences in synonymous and non-synonymous substitution rates (Hughes & Nei 1988; Takahata & Nei 1990). These methods proved useful for detecting the historical presence of selection on genes, but tell us little about the timing or nature of selection (Garrigan & Hedrick 2003). More recently, there has been increasing interest in examining selection at the MHC either within a single generation or over ecological time scales. Doing so allows for the detection of recent selection events and has led people to attempt to differentiate between mechanisms of PMS in non-model organisms. Indeed, over the last few years, the number of studies examining contemporary selection at the MHC has been overwhelming. Yet how much these studies have delivered, whether they are being conducted appropriately and how our understanding of MHC evolution is developing as a result have yet to be questioned.

A number of excellent reviews on MHC evolution have been published (Edwards & Hedrick 1998; Meyer & Thomson 2001; Bernatchez & Landry 2003; Garrigan & Hedrick 2003; Piertney & Oliver 2005; Sommer 2005); however, none has explicitly addressed the question of if and how we can differentiate between mechanisms of PMS. Our aim here is to assess the extent to which empirical studies have been able to do this. First, after a brief introduction to the MHC, we describe in detail the three mechanisms of PMS. This is important as some confusion appears to exist in the literature with regard to these

hypotheses. Second, we provide a critique of the approaches used to detect selection in contemporary populations. We then describe how the outcomes of each approach relate to the different mechanisms of PMS and review the current MHC literature in this context. Finally, we highlight the major problems with current approaches to MHC research and discuss whether we are ever likely to be able to determine the relative roles of different mechanisms of PMS in maintaining MHC diversity in the wild.

2.3 MHC structure and function

MHC genes code for molecules that bind to both self-peptide and non-self-peptide antigens, and present them to T-cells, thereby triggering a cascade of immune responses (Klein 1986; Potts & Wakeland 1990; Edwards & Hedrick 1998; Meyer & Thomson 2001; for a full overview see section 1.3.1). An important feature of the MHC for studies using non-model organisms is that different taxa exhibit different rates of gene conversion and recombination in this region. This has led to many MHC genes being found in multiple, tightly linked copies, making it extremely difficult to identify and isolate independent loci, with the consequence that studies are often only able to amplify multiple loci (e.g. Binz et al. 2001; Richardson & Westerdahl 2003; Babik et al. 2009).

2.4 Mechanisms of selection at MHC loci

The *heterozygote advantage* hypothesis proposes that individuals heterozygous at MHC loci are able to respond to a greater range of pathogen peptides than homozygotes and, consequently, benefit from increased resistance to pathogens. Heterozygotes are, therefore, more likely to have higher relative fitness and, as a result, on average more MHC alleles will persist in the population (Doherty & Zinkernagel 1975; Hughes & Nei 1988).

Heterozygote advantage can be, and has been, a confusing concept for a number of reasons. First, heterozygote advantage can occur through both dominant and overdominant selection. If pathogen resistance is dominant, the heterozygous genotype exhibits the same level of fitness as the fittest homozygote (but not higher) and so achieves higher levels of fitness than the average for all homozygotes. If it is overdominant then the combined, synergistic effect of two alleles at a locus will result in

the MHC heterozygote being fitter than the fittest homozygous genotype (Hughes & Nei 1988). This distinction is important because dominance alone cannot maintain diversity within individual populations, whereas overdominance can (Takahata & Nei 1990; McClelland et al. 2003), although both may operate to maintain diversity across metapopulations (see fluctuating selection hypothesis below). Second, because infection with a given pathogen will result in more than one non-self-peptide being present in the host, the heterozygote advantage gained by being able to detect a wider range of peptides can operate in response to both single (Kurtz et al. 2004) and multiple (McClelland et al. 2003; Wegner et al. 2003b) pathogens. In a population subject to heterozygote advantage, one may therefore expect to observe associations between MHC heterozygosity and both pathogen load and diversity. Nonetheless, heterozygote advantage may be best understood when considered in the context of multiple pathogens, as MHC alleles conferring resistance to one pathogen can increase susceptibility to another (Penn & Potts 1999). Finally, it has been argued that, at the individual level, it is not maximized but rather optimized heterozygosity that provides maximum fitness benefits; having too high MHC diversity may have diminishing returns on T-cell diversity, owing to the deletion of T-cells that react with self-peptide–MHC combinations during development (Nowak et al. 1992). This 'optimal' theory has received considerable empirical support (Wegner et al. 2003a; Kalbe et al. 2009). Therefore, studies examining maintenance of population-level MHC diversity should consider the immunological constraints on intra-individual diversity in order to fully understand the processes underlying selection at these genes.

The *rare-allele advantage* (also called negative frequency-dependence) hypothesis proposes that there is strong selection on pathogens to overcome the resistance of the most common host MHC alleles. Therefore, new alleles that arise within the population are likely to offer greater protection to pathogens than common alleles, and so have a selective advantage (Takahata & Nei 1990). Old, rare alleles may also be selected for; an allele may decrease in frequency within a population owing to pathogens evolving resistance, but once the allele becomes rare, the pathogen adaptation may decrease or disappear, causing the selective advantage of the allele to increase again. The result of this process is a cyclical, coevolutionary arms race in which pathogens and MHC alleles

fluctuate in frequency, thus maintaining diversity via a dynamic process (Slade & McCallum 1992).

Finally, the *fluctuating selection* hypothesis proposes that spatial and temporal heterogeneity in the type and abundance of pathogens may maintain diversity at the MHC (Hill 1991). If the pathogen regime faced by an organism fluctuates spatio-temporally, the intensity of directional selection at MHC genes will also fluctuate. This will lead to different subsets of MHC alleles being selected for at different points in space and/or time, thus maintaining genetic diversity across subpopulations. Key to this model is (i) that selection is directional, rather than cyclical and (ii) that pathogen fluctuations are determined externally—by the biotic or abiotic environment or chance dispersal and extinction events—rather than by coevolution of host and pathogen. Hedrick (2002) showed that, theoretically, diversity at the MHC could be maintained via fluctuating selection, even in the absence of heterozygote and rare-allele advantage.

Defining the mechanisms of PMS is, however, the easy part. Determining their relative roles in maintaining MHC diversity is another matter as they are by no means mutually exclusive and may operate in concert with other selective and neutral forces (Apanius et al. 1997). Moreover, the mechanisms may interact with one another. For example, there is an inherent frequency-dependent component in the heterozygote advantage model, and vice versa; because individuals are unlikely to inherit two copies of a rare allele, such alleles will occur disproportionately in heterozygous individuals. The intensity of selection on an allele under heterozygote or rare-allele advantage may also vary in space and time, owing to fluctuations in pathogens.

2.5 Empirical evidence for the nature of selection on MHC genes

Selection upon genes in contemporary populations is expected to produce detectable effects on the distribution of alleles within those populations (Meyer & Thomson 2001; Hedrick 2002). Therefore, by comparing patterns of variation at MHC genes with those expected under neutrality, one can make inferences about the nature of selection. Contemporary selection can also be revealed by examining pathogen load in relation to

MHC characteristics. These approaches have been used not only to detect the presence of PMS, but also to try to differentiate between the models. However, it is often unclear exactly how the different results that arise from these approaches relate to different mechanisms of PMS, and so conclusions are often ambiguous. In an attempt to clarify predictions, in Table 2.1 we summarize the ways in which selection at MHC loci can be detected in contemporary populations, alongside the possible observations that arise from each of these approaches. We also state the mechanisms of PMS that we predict to be compatible with each observation. In what follows, these predictions are discussed in light of the empirical studies that have attempted to differentiate between mechanisms of PMS.

2.5.1 Proportions of genotypes within populations

Many studies have attempted to detect selection at MHC genes over a single generation by comparing the distribution of MHC genotypes with a theoretical neutral distribution. Balancing selection is expected to result in a surplus of heterozygotes, and this was originally interpreted as evidence of heterozygote advantage (Doherty & Zinkernagel 1975). However, a simple excess of heterozygotes at MHC loci may also be compatible with rare-allele advantage, as heterozygotes may be selected for because they carry rare, resistant alleles, rather than because they are heterozygous per se (Penn 2002; Table 2.1). Nonetheless, a number of studies have used this method, though few have detected a heterozygote excess (Paterson et al. 1998; Gutierrez-Espeleta et al. 2001; Penn et al. 2002; Seddon & Ellegren 2004; Oliver et al. 2009a). This may be because in many cases selection is not strong enough to be detected within a single generation.

An alternative approach is to examine the frequency of alleles in populations, which can provide a 'snapshot' of ongoing evolutionary processes. Alleles are unlikely to occur at equal frequencies. Instead, under neutrality, we expect a few common alleles, with the rest occurring at a relatively low frequency. By calculating a theoretical neutral distribution and contrasting it with empirical observations, it is therefore possible to detect selection (Ewens–Watterson test; Ewens 1972; Watterson 1978). However, because the Ewens–Watterson test is based on patterns of heterozygosity, it is still not possible to confidently differentiate between heterozygote advantage and rare-allele

advantage using this method alone (Table 2.1). Studies that have used the Ewens–Watterson test to assess selection at the MHC have found that the presence and/or strength of selection varies across subpopulations, with selection being detected in 15 to 50 per cent of subpopulations (Garrigan & Hedrick 2003). Such findings could represent fluctuating selection, spatially heterogeneous patterns of heterozygote advantage or snapshots of ongoing rare-allele advantage at varying points in evolutionary time (Table 2.1). Furthermore, the Ewens–Watterson test assumes constant population size, so departures from neutrality calculated using this method may arise owing to historical demographic processes (Nei 1987). The varying results from the studies that have used this test may, therefore, be due to differential demographic histories across populations and not because of fluctuations in selective forces. One way to circumvent the problems associated with a theoretically derived neutral allelic distribution has been to assess this distribution directly using neutral markers (Boyce et al. 1997). While demographic processes affect all loci, selection targets specific genes; therefore, by contrasting patterns of variation at MHC and neutral loci, the effects of demography can be controlled for.

Table 2.1 Predictions arising from different models of pathogen-mediated selection. Note that selection is assumed here - the effects of drift must be controlled for by measuring neutral variation empirically (see text).

	Possible Observations	**Possible PMS Explanations**
Proportions of genotypes within populations	An excess of heterozygotes	Dominance, overdominance, rare-allele advantage (as rare alleles will normally exist in heterozygotes)
	An excess of homozygotes	Underdominance
	An excess of heterozygotes in a subset of populations studied	Fluctuating selection, Fluctuations in any mechanism
	No deviation from neutral expectations	Neutrality
Distribution of allelic frequencies within populations	More even than expected under neutrality	Dominance, overdominance, rare-allele advantage
	Less even than expected under neutrality	Directional selection, fluctuating selection if variable across multiple populations
	More even than expected under neutrality in a subset of populations studied	Fluctuating selection, fluctuating dominance, fluctuating overdominance, rare-allele advantage
	No deviation from neutral expectations	Neutrality
Levels of population structure relative to neutral loci across populations	Higher population structure at MHC compared to neutral loci	Fluctuating selection, rare-allele advantage maintaining old alleles
	Lower population structure at MHC compared to neutral loci	Dominance, overdominance, rare-allele advantage selecting new alleles
	Equal population structure at MHC compared to neutral loci	Neutrality
Associations with pathogen regime	Associations between individual pathogen load and MHC alleles	Rare-allele advantage, fluctuating selection
	Association between individual pathogen load and MHC heterozygosity	Heterozygote advantage (to a single pathogen). Overdominance if heterozygote associated with lower pathogen load than both homozygotes.
	Association between individual pathogen load and number of MHC alleles (across multiple duplicated loci)	Heterozygote advantage, rare-allele advantage, fluctuating selection
	Associations between pathogen diversity and individual MHC alleles	None

Table 2.1 (cont.)

	Possible Observations	Possible PMS Explanations
Associations with pathogen regime	Association between pathogen diversity and MHC heterozygosity	Heterozygote advantage (to multiple pathogens). Overdominance if heterozygote associated with fewer pathogens than both homozygotes.
	Associations between pathogen diversity and number of MHC alleles (across multiple duplicated loci)	Heterozygote advantage, rare-allele advantage, fluctuating selection
	No association between MHC structure and pathogen regime	Neutrality

Incorporating neutral variation into tests of selection has highlighted that migration and drift may sometimes be more important than selection in shaping MHC diversity over micro-evolutionary time scales. Indeed, many studies have reported that neither MHC nor neutral variation differ from random expectations (Bernatchez & Landry 2003). However, in any system where multiple forces act on allelic distributions, there is the possibility that they may cancel each other out to produce patterns that do not deviate from neutral expectations. On the other hand, a few studies have reported selection at MHC loci after controlling for demographic processes, though only in subsets of populations. Studies on salmonids have reported elevated heterozygosity at MHC compared with neutral loci in 16 to 43 per cent of populations (Landry & Bernatchez 2001; Miller et al. 2001; Aguilar & Garza 2006). Recently, Oliver et al. (2009a) reported elevated heterozygosity in metapopulations of water voles (*Arvicola terrestris*), but only in 3 out of 10 metapopulation-years. Authors tend to attribute these findings to differential selection pressures arising from spatio-temporal variation in pathogen abundance, though due to its dynamic nature, rare-allele advantage cannot be ruled out. Westerdahl et al. (2004) adopted a temporal approach, comparing variation in nine successive cohorts of great reed warblers (*Acrocephalus arundinaceus*). Overall, variation in the frequency of MHC alleles was significantly greater than for microsatellite alleles. Moreover, the frequency of two MHC alleles, but no microsatellite alleles, varied more between cohorts than expected, suggesting that selection favours different MHC alleles in different years—findings consistent with both the rare-allele advantage and fluctuating selection hypotheses. Hess et al. (2007) also observed temporal shifts in MHC allelic frequencies in

populations of house finches (*Carpodacus mexicanus*), though their results were less pronounced.

Studies on bottlenecked populations have found higher levels of MHC compared with neutral diversity, indicating that balancing selection can act to counter demographic processes. For example, the San Nicolas Island fox (*Urocyon littoralis dickeyi*) is reportedly the most genetically monomorphic sexually reproducing animal population, having gone through a bottleneck of less than 10 individuals (Aguilar et al. 2004). Despite this, high levels of diversity were observed at three MHC-linked microsatellites and one class II gene (Aguilar et al. 2004). Similar, though less extreme, elevated MHC diversity relative to neutral loci has been observed in bottlenecked populations of several other species (e.g. Richardson & Westerdahl 2003; Jarvi et al. 2004; Hansson & Richardson 2005; van Oosterhout et al. 2006).

2.5.2 Patterns of population structure

Selection can also be detected by contrasting population structure at MHC and neutral loci across multiple populations (Table 2.1). In populations subject to heterozygote advantage, within-population MHC diversity is predicted to be high compared with total diversity, resulting in a lower population structure at MHC than at neutral loci (Schierup et al. 2000). Conversely, in populations subject to fluctuating selection, different subsets of MHC alleles will arise, and higher population structure will be observed at MHC relative to neutral loci (Charlesworth et al. 1997). The rare-allele advantage model adds confusion to this picture, as we know little about the nature of the rare alleles themselves. Obviously, only rare alleles that currently confer resistance to pathogens are selected. However, whether these alleles are predominantly old (i.e. ones that have previously been common within the population) or newly arisen will have different consequences for patterns of population structure. If they are old, population structure will be lower at MHC than neutral loci (Schierup et al. 2000). If newly arisen alleles are selected for, then different subsets of alleles will arise in different populations, and consequently structure will be relatively higher at the MHC.

Studies comparing population structure at MHC and neutral loci have yielded mixed results. Many studies have found no difference between MHC and neutral population structure (e.g. Boyce et al. 1997; Gutierrez-Espeleta et al. 2001; Babik et al. 2008; Biedrzycka & Radwan 2008), suggesting that little or no selection is operating across populations, or that multiple selective forces are operating and masking any overall effects. On the other hand, differentiation at MHC relative to neutral loci across populations has been reported, for example, in various fish species (Landry & Bernatchez 2001; Miller et al. 2001; Aguilar & Garza 2006) and in great snipe (*Gallinago media*; Ekblom et al. 2007). In all these studies, the authors conclude that MHC diversity is maintained by different patterns of PMS across differing ecological environments. Alcaide et al. (2008) also reported elevated MHC differentiation across populations of lesser kestrel (*Falco naumanni*). In this case, as the kestrel populations inhabit similar habitats—with presumably similar pathogen communities—the authors conclude that this pattern has arisen from geographically varying coevolution, supporting the rare-allele advantage model of selection. However, none of these studies demonstrate explicitly that the pathogen fauna does, or does not, vary across populations. Such studies are badly needed; without them attempts at differentiating between rare-allele advantage and fluctuating selection are speculative at best. Very few studies have detected lower levels of divergence at MHC than at neutral loci. van Oosterhout et al. (2006) found lower differentiation at MHC class II DAB genes than at microsatellite loci across two populations of Trinidadian guppies (*Poecilia reticulata*). These results suggest that MHC diversity may be maintained either by heterozygote advantage or by rare-allele advantage in which old, rare alleles are maintained. Two other studies have compared MHC and mitochondrial genes and found lower F_{ST} values at MHC loci (Sommer 2003; Mona et al. 2008); however, the use of mitochondrial genes as a neutral marker in this manner is questionable (William et al. 1995).

This brings us to a major problem intrinsic to studies that compare MHC and neutral variation; namely, which markers are most appropriate for assessing neutral diversity. Most studies have used microsatellites, presumably because they allow an efficient, low-cost assessment of neutral diversity. However, these studies are confounded by the fact that the manner and rate in which microsatellites and MHC sequences mutate are very

different. Specific statistical tests are often required for the different markers, and the outcomes of tests that can be used with both markers may be affected by their differential mutation rates (Hedrick 2005; Brito & Edwards 2009). A logical solution to this problem would be to use nuclear sequence polymorphisms, which are likely to evolve in a fashion more similar to MHC genes (Brito & Edwards 2009), though this has rarely been done.

2.5.3 Associations with pathogens

Contemporary selection at MHC genes may also be detected by examining associations between the MHC and pathogen load of an organism (Table 2.1). Associations between specific pathogens and particular MHC alleles suggest a role for either rare-allele advantage or fluctuating selection. Associations between MHC heterozygosity and pathogen abundance and/or richness suggest a role for heterozygote advantage, though other mechanisms cannot be ruled out from this observation alone. Studies of this kind should also measure neutral variation to control for potential confounding effects of demographic processes on MHC structure. Those that have done so, described below, constitute the most detailed examples of how PMS can act to maintain MHC diversity.

In house sparrows (*Passer domesticus*), Bonneaud et al. (2006) found that two different MHC alleles were associated with resistance to the same malarial strain in different populations. Both alleles exist in both populations, indicating that local adaptation to malarial infection occurs. This study provides the strongest available evidence supporting rare-allele advantage, though a temporal component to the association between alleles and the pathogen would need to be incorporated to rule out other mechanisms completely. In great reed warblers, Westerdahl et al. (2005) found that the number of MHC class I alleles in an individual—a measure suggested to reflect heterozygosity across multiple loci—was associated with the presence of a particular malarial lineage, whereas heterozygosity measures derived from microsatellite data did not show this relationship. A single MHC allele was also associated with presence of the malarial lineage. While these findings indicate that PMS is important in the great reed warbler, they are compatible with all three mechanisms of selection. Other studies have screened for multiple pathogens. In three-spined sticklebacks (*Gasterosteus aculeatus*), Wegner et al. (2003b)

detected a positive correlation between the number of alleles across MHC loci (but not at microsatellites) within a population and population-level pathogen diversity, suggesting a role for heterozygote advantage, though other mechanisms could not be ruled out. Oliver et al. (2009b) contrasted MHC class II variation and pathogen regime in a population of water voles in which only two alleles, and thus three genotypes, are found. The heterozygous MHC genotype was associated with lower numbers, and fewer types, of ectoparasites. Importantly, the heterozygotes were more resistant than both homozygotes, indicating heterozygote advantage through overdominance. An association between infection with a specific parasite and an MHC allele was also detected, suggesting that rare-allele advantage and/or fluctuating selection may also operate. Finally, in populations of montane water voles (*Arvicola scherman*), Tollenaere et al. (2008) found an association between specific MHC alleles and pathogens, but no relationship between pathogen diversity and MHC diversity. This may suggest a greater role for rare-allele advantage and/or fluctuating selection and a lesser role for heterozygote advantage in this system. Overall, these studies indicate that all three mechanisms of PMS may operate to maintain MHC diversity in wild populations, but they do not allow us to rule out alternative mechanisms nor determine their relative importance.

2.6 Can we differentiate between mechanisms of PMS in wild populations?

The number of studies examining selection at the MHC over a micro-evolutionary time scale has increased rapidly over the last few years. This work has highlighted that while selection at MHC genes is almost always detected over macro-evolutionary time scales, in contemporary populations MHC diversity is shaped by a range of neutral and selective forces, any combination of which may be operating at particular points in space and time. The research has confirmed the role of pathogens in MHC evolution and highlighted that pathogens and MHC genes interact closely in a number of ways, and that these interactions vary spatio-temporally. Some progress has been made in studying the mechanisms of PMS and the evidence, though circumstantial, now suggests that all three mechanisms may operate in natural systems.

Collating studies that have attempted to differentiate between mechanisms of PMS have shown that in many situations, the three mechanisms can produce the same effects on MHC diversity. Indeed, as Table 2.1 shows, in the majority of cases, there are multiple explanations for any observation in a given test, and no one approach yields observations that allow the mechanisms to be differentiated between. To complicate matters further, the mechanisms are likely to interact, and other evolutionary processes, such as sexual selection, are also likely to contribute towards shaping MHC diversity (reviewed in Penn & Potts 1999). For example, MHC-based mate choice may often serve as an 'amplifier' to PMS, helping to achieve an optimal or maximal number of MHC alleles (Richardson et al. 2005; Jager et al. 2007; Eizaguirre et al. 2009). However, if PMS within a population is directional (and so reduces diversity) and mate choice operates to maintain diversity, the two may effectively cancel each other out (Apanius et al. 1997).

This leads us to ask: what can we resolve, and how can we best move forward? By contrasting MHC variation with pathogen load and/or survival, it should be possible to detect the presence of heterozygote advantage, though few studies have done this convincingly in wild populations (but see Oliver et al. 2009b). Doing so requires examining associations between pathogens and both genotypes (heterozygosity) and specific alleles. It also requires a sufficient range of pathogens and an appropriate MHC screening method (i.e. single-locus amplification). Therefore, though the lack of heterozygote advantage observed so far may be because this mechanism is relatively unimportant in maintaining MHC diversity, it may also be because appropriate study systems have proved difficult to find.

A more serious challenge lies in separating rare-allele advantage and fluctuating selection. None of the approaches listed in Table 2.1 enables us to tease apart these mechanisms as their effects upon MHC allelic frequencies in populations and on associations between MHC and pathogen structure are likely to be the same. A number of studies infer the importance of one mechanism even though their results cannot rule out alternatives. In particular, studies claim evidence of fluctuating selection after finding different levels of balancing selection across populations, or higher levels of population structure at MHC than at neutral genes (Landry & Bernatchez 2001; Miller et al. 2001;

Aguilar & Garza 2006; Ekblom et al. 2007; Alcaide et al. 2008; Oliver et al. 2009a). However, as we have explained (Table 2.1), such patterns could be due to different intensities of heterozygote advantage, or specific forms of rare-allele advantage. Combining observations of allelic frequencies with the genealogy of MHC alleles may go somewhere towards disentangling these effects, as new, rare alleles are expected to be less divergent than older ones.

The best way to differentiate between rare-allele advantage and fluctuating selection would be to study MHC and neutral variation in relation to pathogen load over periods of evolutionary time in multiple replicate populations. Under rare-allele advantage, one would expect to see different alleles conferring resistance to the same pathogen in different populations, and for resistance to change with time, so that different alleles become associated with resistance. Under fluctuating selection, one would expect to observe external biotic and/or abiotic forces driving spatio-temporal variation in pathogen abundance, leading to distinct subsets of alleles being selected for in different populations and/or different time periods. Of course, such long-term, multiple-population studies are difficult and costly to carry out, and appropriate study systems are difficult to find.

As well as the theoretical problems with teasing apart mechanisms of PMS, there are technical issues that also need to be resolved if we are to progress towards a fuller understanding of MHC evolution. First, although much of the variation in findings from MHC studies may have arisen because MHC evolution is indeed sporadic, it is highly likely that poor quality control in the studies themselves has also confused matters. In particular, an inability to assign alleles to individual loci means that it is often not possible to identify true genotypes, making it difficult to employ many of the analyses used to separate mechanisms of PMS. The only way to circumvent this problem will be to use single-locus systems that allow for identification of true genotypes (e.g. Worley et al. 2008; Oliver et al. 2009b). An equally serious problem is that it is often unknown whether the MHC loci being studied are actually expressed, or whether diversity at these loci is being fully characterized. Detailed molecular groundwork is required to ensure that the full complement of expressed MHC variation is accurately assessed. Real-time PCR can be

used to assess patterns of MHC expression in non-model organisms, though few studies have done so thus far (but see Wegner et al. 2006). The use of next-generation sequencing for MHC screening (Babik et al. 2009) is likely to be a great help in terms of more accurately characterizing MHC diversity, and we expect to see an increase in both the efficiency and resolution of MHC genotyping in non-model organisms in the near future.

Even when all the variation at a particular MHC locus is screened, we are faced with the problem of what equates to a functionally important MHC allele. Clearly, two alleles that differ by multiple amino acids are going to be able to detect a broader spectrum of antigens than two that differ by a single substitution. Yet allelic divergence is rarely taken into account in MHC studies, and so it is questionable whether these studies have classified biologically meaningful alleles. One way to obviate this problem could be to group sequences into functionally important 'supertypes' (Doytchinova & Flower 2005; Naugler & Liwski 2008). Another may be to quantify levels of amino acid divergence and use this in analyses.

A final 'technical' issue to consider is that MHC study systems are usually confounded by the very thing that makes the MHC variation an attractive subject to study—namely complexity. Most organisms are faced with enormous numbers of pathogens and are, in turn, characterized by a highly complex MHC. Is it any wonder therefore that studies contrasting a single exon of an MHC locus with individual pathogens produce mixed results? Fully characterizing the MHC and pathogen load is unlikely to be possible in most study systems, and even if it were, statistical analysis may be intractable. One rewarding approach could be to focus on highly simplified study systems (e.g. Richardson & Westerdahl 2003; Oliver et al. 2009b), though whether results from such studies are applicable to more complex systems is questionable. The best study systems will probably be characterized by intermediate levels of pathogen diversity and simple, well-characterized MHC structures, thus avoiding oversimplification while retaining statistical tractability.

Experimental infection studies may also help alleviate some of the problems associated with differentiating between mechanisms of PMS. A number of experimental infection studies with MHC congenic mice have shown that heterozygote advantage, through both dominance and overdominance, can operate to combat pathogenic infection (reviewed in Penn 2002). Experiments that differentiate between rare-allele advantage and fluctuating selection have yet to be designed, perhaps because doing so would require long-term, controlled selection lines, with more generations than would be realistically possible in a vertebrate (though see Conover & Van Voorhees 1990). Even so, if this problem can be circumvented, the advantage of being able to control for demographic processes and external fluctuations in pathogen load may be extremely useful in differentiating between these two mechanisms.

Given that in the majority of cases the three mechanisms of PMS produce similar final effects on MHC dynamics, and that they are likely to interact, is differentiating between them in wild populations actually important? We believe so: understanding how diversity is maintained in gene regions such as the MHC is fundamental to our understanding of natural selection and antagonistic coevolution. Experimental infections, though more controlled, are limited in the information they can provide about the dynamics of PMS. Moreover, balancing selection clearly operates in different ways across ecologically and evolutionarily differing environments. Only by assessing MHC diversity in the wild will we be able to further understand these patterns and processes. Furthermore, there are applied implications for understanding how PMS operates to maintain MHC diversity in the wild, such as in conservation. For example, different mechanisms of PMS are likely to produce different phenotypic and population-level effects in response to the introduction of novel pathogens—a phenomenon that is likely to increase with a warming global climate (Smith et al. 2009). Reduction and fragmentation of populations will change pathogen dynamics and are likely to affect the nature of selection acting upon MHC genes (van Oosterhout et al. 2006). Understanding how organisms are likely to respond evolutionarily to factors such as genetic bottlenecks requires knowledge of how PMS operates to maintain diversity. Such understanding may also help us determine how best to conserve diversity at the MHC—or, for that matter, at any immunologically important

genes—in wild populations, or how to maximize diversity through selective breeding in captive populations.

2.7 Conclusions

Owing to the non-exclusivity of the mechanisms of PMS, alongside the likely interference of other selective and neutral forces, we do not believe that it is possible to convincingly elucidate the relative roles of mechanisms of PMS within specific wild populations. A more realistic approach is to attempt to demonstrate whether a particular mechanism of PMS is occurring within a specific system. Doing so will be extremely difficult, not least because appropriate long-term study systems are vital for this. Researchers of contemporary MHC evolution therefore need to carefully consider which mechanisms of PMS can be detected within their study system and which can be ruled out, and interpret their findings accordingly. Technical issues concerning the characterization of expressed MHC variation also need to be carefully considered, as they have not been in much of the MHC literature to date. Nonetheless, with enough appropriate studies, we may be able to determine how often different mechanisms of PMS occur, as well as the different spatial and temporal scales at which they prevail. The accumulation of such knowledge across systems will allow us to evaluate the general importance of the different PMS mechanisms across vertebrates as a whole. Advances in our understanding of what maintains MHC diversity will also feed into our general understanding of host–pathogen coevolution and the maintenance of genetic diversity.

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Chapter 3: Biogeographical patterns and co-occurrence of pathogenic infection in island populations of Berthelot's pipit

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Berthelot's pipit infected with avian pox

3.1 Abstract

Pathogens can exert strong selective forces upon host populations. However, before we can make any predictions about the consequences of pathogen-mediated selection, we first need to determine whether patterns of pathogen distribution are consistent over spatio-temporal scales. We used molecular techniques to screen for a variety of blood pathogens (avian malaria, pox and trypanosomes) over a three-year time period across 13 island populations of the Berthelot's pipit. We screened 832 individuals, and identified two strains of *Plasmodium*, four strains of *Leucocytozoon*, and one pox strain. We found strong differences in pathogen prevalence across populations, ranging from 0 to 65%, and while some fluctuations in prevalence occurred, these differences were largely stable over the time period studied. Smaller, more isolated islands harboured fewer pathogen strains than larger, less isolated islands, indicating that at the population level, colonization and extinction play an important role in determining pathogen distribution. Individual-level analyses confirmed the island effect, and also revealed a positive association between *Plasmodium* and pox infection, which could have arisen due to dual transmission of the pathogens by the same vectors, or because one pathogen lowers resistance to the other. Our findings, combined with an effect of infection on host body condition, suggest that Berthelot's pipits are subject to different levels of pathogen-mediated selection both across and within populations, and that these selective pressures are consistent over time.

3.2 Introduction

Pathogens—disease-causing organisms—play a vital role in the ecology and evolution of their hosts. In wild animal populations, pathogens can affect individual fitness in a number of ways, such as increasing predation risk, reducing survival and reducing reproductive output (Anderson and May 1979; Gulland 1995; Johnson et al. 2008; Møller and Nielsen 2007). These effects can be observed at higher organizational levels, with pathogens playing a decisive role in host population dynamics and range distributions (Anderson and May 1981; Hudson et al. 1998; Ricklefs 2010), driving genetic variation (Acevedo-Whitehouse et al. 2003; Ortego et al. 2007; see chapter 2) and sexual selection (Hamilton and Zuk 1982). Understanding how patterns of pathogen-mediated selection vary across populations may therefore provide new insights into the mechanistic processes behind adaptation and natural selection. However, before we can investigate how patterns of pathogen-mediated selection operate across populations, we first need to establish how and why pathogen regimes vary over spatio-temporal scales. Yet, determining the causes and consequences of pathogen distribution is likely to be extremely difficult in most cases, as in any given system an enormous array of pathogens may be present, and many different environmental, ecological and physiological variables may all influence pathogen distribution.

Island archipelagos have been described as “natural laboratories” for ecological and evolutionary research, as they contain multiple populations in geographically discrete yet ecologically variable locations (Whittaker 1998). The simplified nature of island systems has meant that they have been particularly useful for host–pathogen association studies, as the pathogen fauna on islands is generally less diverse than on mainland systems (Alcaide et al. 2010; Dobson 1988), simplifying analyses. Moreover, island archipelagos provide an opportunity to tease apart the different factors governing pathogen distribution across populations. In a scenario where host–pathogen associations are replicated across islands, “island effects” may arise due to differing ecological conditions, which may affect pathogen success due to differences in the availability of a specific habitat or vector for the pathogen (Apanius et al. 2000). Alternatively, differences in pathogen community composition between islands may occur as a result of temporal patterns and fluctuations in pathogen colonization and extinction, independent of island

ecology (Fallon et al. 2004). For vector-borne pathogens, colonization and extinction are expected to play an especially important role, as the concurrent presence of both pathogen and vector is required for transmission. In addition to island effects, pathogen distribution may be constrained by factors related directly to the host. If the distribution of pathogens was determined solely by that of the host, one would expect the pathogen distribution to be homogeneous over the host's range, even across islands (Apanius et al. 2000). Within-host factors such as age, sex, host behaviour or immune competence (McCurdy et al. 1998; Mougeot and Redpath 2004; Sol et al. 2003; Sorci 1996; Tompkins et al. 2010; van Oers et al. 2010) may also affect the observed patterns of infection. In reality, the most likely scenario is that the effects of hosts and islands will interact, resulting in unique outcomes of host–pathogen relationships, and therefore different selection regimes, across populations (Apanius et al. 2000; Fallon et al. 2003).

Spatio-temporal scale is a key factor to consider for host–pathogen association studies. For example, fine-scale ecological variations can result in marked differences in pathogen distribution within populations (Wood et al. 2007), meaning that effects of different biotic and abiotic variables on pathogen distribution may be obscured if the sampling regime is too coarse. Temporal variation in pathogen regimes, both seasonally and across longer time periods, also needs to be accounted for (Bensch and Åkesson 2003; Cosgrove et al. 2008; Fallon et al. 2004; Marghoob 1995). Without sampling over more than one time period, it is not possible to tell whether any observed patterns of spatial variation in the pathogen distribution represent consistent differences across populations, or whether they represent a "snapshot" of a rapidly changing pathogen community. This distinction is particularly important in the context of pathogen-mediated selection, as selection is only likely to produce observable differences among host populations if the pathogen regime is consistent within populations. Studies conducted over a range of spatio-temporal scales will provide the most comprehensive overview of what governs the pathogen distribution, and therefore variation in pathogen-mediated selection, in wild populations. However, such studies are, at present, few and far between.

In wild birds, the most widely studied pathogens are malarial species of the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon* (Bensch et al. 2004; Eggert et al. 2008;

Ishtiaq et al. 2008; Pérez-Tris et al. 2005; Ricklefs et al. 2008; van Riper et al. 1986; Vögeli et al. 2011). Avian malaria is a vector borne disease, transmitted by several genera of mosquito and biting midges (Martinez-de la Puente et al. 2011; Njabo et al. 2011). Malarial infection has been shown to have implications for host mate choice (Dale et al. 1996), parental investment (Wiehn et al. 1999), reproductive success (Dufva 1996), immune gene variability (Bonneaud et al. 2006; Westerdahl et al. 2005) and population or species persistence (van Riper et al. 1986). Other avian pathogens have received less attention in the ecological literature. For example, trypanosomes (*Trypanosoma* spp.) are also vector-transmitted (by simuliid and hippoboscid flies) blood pathogens that infect avian hosts worldwide, and are known to be detrimental to host growth and fitness (Apanius 1991). Yet the factors affecting trypanosome distribution within and across avian host populations have rarely been studied. Avian pox is a viral disease comprising numerous species in the genus *Avipoxvirus*. This pathogen is often fatal, and can be transmitted by vectors (*Culex* mosquitoes), directly by contact, or indirectly through contact with contaminated water (Ritchie 1995; Smits et al. 2005). Avian pox is being reported in an increasingly large number of wild bird species (Mondal et al. 2008; Saito et al. 2009; Smits et al. 2005; Tarello 2008; Van Riper and Forrester 2007), and has been highlighted as a threat to island bird populations (Kleindorfer and Dudaniec 2006; van Riper et al. 2002). Again, this pathogen has so far been largely overlooked in an ecological and evolutionary context (but see Carrete et al. 2009). Less well explored still is how these pathogens interact in wild populations. For example, avian malaria and pox have recently been shown to be positively associated in Hawaiian birds (Atkinson et al. 2005), yet the extent to which this occurs in other systems is not known.

In this study, we use molecular techniques to screen for avian malaria, pox and trypanosomes in all 13 Berthelot's pipit populations (Fig. 1.1) over a 3 year time-period. We test two main hypotheses: first, that spatio-temporal variation in pathogen distribution can be explained by biogeographical factors (i.e., island size and isolation); and second, that there are significant associations between infection with different pathogens. We also explore whether, within an individual island, geographic structuring of pathogen infection occurs across subpopulations. The implications of our findings for host ecology and evolution are discussed.

3.3 Materials and methods

3.3.1 Study species and sampling

Representative samples (ca. 30 individuals) were obtained from each of the 12 main island populations (Fig. 1.1). Samples were obtained during two field seasons: the first was carried out by J.C. Illera between April 2005 (Selvagens), January–March 2006 (Canary Islands) and September 2006 (Madeira), and the second was carried out by the author between January and April 2009 (for all islands). The three-year time period between screenings is likely to exceed the average lifespan of pipits (Coulson 1956), and thus the period over which selection can be expected to operate. Individuals were captured at multiple localities across each island to obtain a representative sample of the population as a whole. Nonetheless, fine-scale structuring of avian pathogens has been shown to occur (Wood et al. 2007). To explore this, in April 2010, one of the largest populations, Tenerife, was sampled more extensively, obtaining at least 30 individuals from three distinct subpopulations in the northwest, south, and east of the island, as well as from the top of Teide, which is located in the centre of Tenerife. Note that the pipits are less common in the wetter north-eastern peninsula of the island.

Birds were captured using spring traps baited with *Tenebrio molitor* larvae. Each bird was fitted with a unique numbered aluminium ring from the relevant Spanish or Portuguese ministries, or with a coloured plastic ring. Individuals were aged on the basis of feather moult pattern (Cramp 1985), and seven morphological measurements (wing length, tarsus length, bill length, height and width, head length and mass) were taken. Individuals were examined for pox lesions, which usually consist of growths on the feet, legs or face (Smits et al. 2005); where possible, small samples were taken with a sterile scalpel, diluted in 800 µL of absolute ethanol in screw-cap microfuge tubes, and stored at room temperature. Blood samples (c. 40 µL) were collected by brachial venipuncture, and likewise preserved in absolute ethanol.

3.3.2 Molecular procedures

Genomic DNA was extracted from blood using a salt extraction technique (Richardson et al. 2001). DNA extraction techniques do not appear to affect the accuracy of malarial identification (Freed and Cann 2006). However, amplifying pox DNA from blood and lesions could potentially be problematic. In order to minimize the possibility of the DNA extraction technique affecting the amplification of pox DNA, we extracted DNA from lesions and from blood samples of birds on which lesions were found using both the salt extraction method and DNeasy blood and tissue kits (Qiagen), following the manufacturer's instructions. The quality of genomic DNA was visualized on 1.2% agarose gel after electrophoresis. Prior to pathogen screening, the extracted DNA was used to determine the sex of the birds using the molecular protocol described in Griffiths et al. (1998). Samples that did not produce strong amplicons for this sexing procedure were re-extracted or discarded. This ensured that only samples which contained amplifiable DNA went on to be used in the pathogen screening procedures.

Molecular methods were used to detect and characterize the strains of each pathogen. For avian malaria, a nested polymerase chain reaction (PCR) was used that amplifies a 422 bp fragment of the mitochondrial cytochrome *b* gene (Waldenstrom et al. 2004). For avian pox, primers developed by Lee and Lee (1997) were used, which amplify a 578 bp fragment of the 4b gene. For both malaria and pox PCR reactions, the reagents and conditions described in Illera et al. (2008) were used. For trypanosomes, primers developed by Maslov et al. (1996) were used as well as the nested PCR reaction described in Sehgal et al. (2001), which amplifies a 326 bp fragment of the small subunit ribosomal RNA gene. To ensure the accuracy of the results, all samples were screened twice, and where results from two reactions were not concordant, samples were screened a third time. Given the low level of discrepancy between repeated PCRs (see section 3.4), this was deemed to be a sufficient number of replicates. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced on a PerkinElmer ABI PRISM 3700 automated sequencer. Only positive results that amplified twice and gave good sequences were counted as genuine infections. The quality of sequences was checked using FinchTV (http://www.geospiza.com/finchtv/), and sequences were aligned using BIOEDIT version 5.0.6 (Hall 1999), against homologous sequences published in the

National Centre for Biotechnology Information (NCBI) GenBank database. Malarial sequences were also searched for in the MalAvi public database for avian malaria sequences (Bensch et al. 2009) in order to identify if, when, and where strains had previously been found.

3.3.3 Statistical analyses

At the island level, linear regression was used to test whether larger, less isolated islands harboured more pathogen species than smaller or more isolated ones. For the purpose of this analysis, individual pathogen strains were counted as "species" (Bensch et al. 2004), and thus pathogen "species richness" is, for our purposes, the number of pathogen strains found on an island. A common problem with this kind of analysis is that sampling effort might correlate with both island size and pathogen species richness (Walther et al. 1995). This is unlikely to be an issue in the present study, as sample size was roughly equal across all populations. Nonetheless, path analysis (Sokal and Rohlf 1995) was used to assess the direct and indirect effects of sampling effort (for details of methods, see Guégan and Kennedy 1996; Ishtiaq et al. 2010). Island isolation was calculated as both the total land area within a 100 km radius of the coastline of the focal island, and the distance to the nearest continental mainland (Europe or Africa), using Google Earth (http://earth.google.com). Island size was obtained from the Island Directory website (http://islands.unep.ch/isldir.htm). In all cases, least-squares regression was used on log-transformed variables. As some islands had no pathogens, n + 1 was used for pathogen species richness (Cornell 1986; Hockin 1981).

Generalized linear models (GLMs) were used to test the factors affecting infection at the individual level. First, to test whether pathogen prevalence varied across space and time, GLMs were constructed for each pathogen using all individuals, with pathogen presence/absence as the response variable and island identity and year as explanatory variables. A second set of GLMs were then carried out to test for associations between pathogens while controlling for potentially confounding factors. For these models, only islands where pathogens were found in more than two individuals were included, as the presence of individuals from islands where pathogens are very rare or absent may confound results. Again, a separate GLM was carried out for each pathogen, this time

including island, year, age and sex as explanatory variables. Presence/absence of infection with other pathogens was subsequently added as explanatory variables in order to test their independent explanatory power on likelihood of infection (Crawley 2007). For all GLMs, a quasi-binomial error structure was used, with a logit link function. To explore the effect of infection on body condition, mass was entered as the dependent variable in a general linear model (LM) with body size as a covariate—a preferable approach to using mass/length residuals (Green 2001). As an indicator of overall body size, the first component from a principal component analysis of the six morphometric measurements (excluding mass) was used (Freeman and Jackson 1990; Green 2001). Age, sex, island, year and infection with each pathogen were entered into the LM as additional explanatory variables. All statistical tests were carried out in R version 2.12.2 (R Development Core Team 2008), and *P* values are two-tailed unless indicated otherwise.

3.4 Results

3.4.1 Molecular characterization and prevalence levels

In total, 832 individuals were screened for pathogenic infection. We found 27 instances of non-concordance between the two PCRs. In all but five cases, infection was confirmed by a third PCR. Those five cases were counted as negatives. In all cases, positive controls successfully amplified while negative controls did not. For avian malaria, no *Haemoproteus* was detected, but two *Plasmodium* strains were identified. These same strains were detected in Berthelot's pipits by Illera et al. (2008). In the present study, the most common *Plasmodium* strain, TF413, was found in all but two of the individuals infected with *Plasmodium*. The other strain, PAL282, was detected in two individuals—one from La Palma and one from El Hierro—in 2006, but was not found in any individuals in 2009. *Leucocytozoon* infection was rare (see below), though four different strains were detected. Three were identical in mitochondrial sequence to the previously described sequences RS4, REB11 and SYAT22 (Bensch et al. 2009), while the fourth strain, which we named ANBE1, has not previously been detected and appears to be unique to Berthelot's pipit. This strain has been submitted to GenBank (accession number JF803824.1). In the 2006 samples, *Leucocytozoon* infection was detected on three islands, with three strains on Porto Santo (REB11, RS4 and SYAT22), two (REB11 and ANBE1) on Gran Canaria, and one (REB11) on Tenerife. REB11 was the most common strain. In 2009, only REB11 was

found, and only on Porto Santo. No evidence for trypanosome infection was found in any of our samples, despite the successful amplification of trypanosome DNA from positive controls. For avian pox, successful amplification was achieved in seven samples from 2006 (six from Porto Santo and one from Lanzarote), all of which gave identical sequences, apparently unique to Berthelot's pipit (Illera et al. 2008). We were unable to achieve amplifications from any 2009 samples (discussed later).

Considering all samples, *Plasmodium* prevalence was 19.2% in 2006 and 17.1% in 2009, *Leucocytozoon* prevalence was 0.02% in 2006 and 0.01% in 2009, and pox prevalence (determined from the presence of lesions) was 9.2% in 2006 and 11.2% in 2009. The low overall prevalence of *Leucocytozoon* was due to it being very rare or absent from all populations other than Porto Santo, where it was abundant in both years (Table 3.1). Indeed, the prevalence of all pathogens differed markedly across populations, ranging from 0 to 65% (Table 3.1). Temporal stability in pathogen abundance was observed: considering all populations, there was a strong correlation between population-level prevalence across the two sampling years for both malaria and pox (Pearson correlation: malaria, $R = 0.71$, d.f. = 11, $P = 0.007$; pox, $R = 0.73$, d.f. = 11, $P = 0.005$, Fig. 3.1). The central and eastern Canary Islands, as well as Porto Santo, had consistently moderate to high levels of pathogens in both years. Other islands had consistently low prevalence levels, while three islands (Madeira, Deserta Grande and Selvagem Grande) remained free of all screened pathogens in both years (Table 3.1). After removing populations with no parasites, this relationship was no longer significant (malaria, $R = -0.22$, d.f. = 8, $P = 0.69$; pox, $R = 40$, d.f. = 8, $P = 0.43$), suggesting that a degree of temporal fluctuation in prevalence occurred in populations with moderate to high pathogen levels (Fig. 3.1).

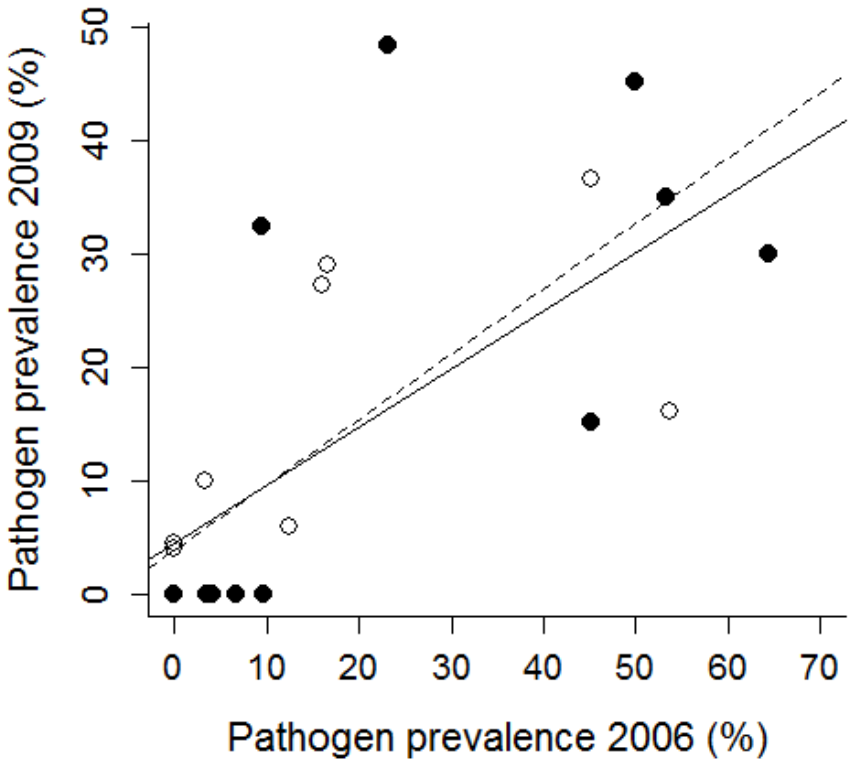

Figure 3.1 Temporal patterns of pathogen prevalence (percentage of individuals infected) across island populations of Berthelot's pipit (see table 3.1 for sample sizes). The filled circles and solid line represent malaria, and the open circles and dashed line represent pox.

Pathogen species richness was positively related to island size (linear regression, R^2 = 0.35, d.f. = 11, P = 0.034; Fig. 3.2A), as well as to the total land area within a 100 km radius of the coastline (R^2= 0.45, d.f. = 11, P = 0.012; Fig. 3.2B), suggesting that smaller and more isolated islands harbour fewer pathogens. It is possible that the latter of these two relationships was driven by a single point, Selvagem Grande, which is highly isolated (Fig. 1.1; bottom-left point in Fig. 3.2B). The regression was therefore performed again while excluding this population, and the relationship remained significant (R^2 = 0.36, d.f. = 10, P = 0.039). There was no significant relationship between pathogen species richness and distance to the nearest continental mainland (R^2= 0.15, d.f. = 11, P = 0.19). Path analysis revealed no direct or indirect effect of sampling effort on pathogen species

richness ($P > 0.05$). There was no relationship between population level prevalence of Plasmodium or pox and island size or isolation (all $P > 0.05$).

On Tenerife, a total of 217 samples were collected from the three coastal subpopulations and the mountain population of Teide, of which 62 were from 2006, 59 from 2009 and 97 from 2010. Moderate levels of *Plasmodium* infection were observed in the southern and eastern subpopulations, though this pathogen was rare on Teide and absent from the north-western subpopulation (Table 3.2). There was even more pronounced geographic structuring of pox infection; high pox prevalence was observed in the southern subpopulation, but no pox infection was detected anywhere else other than in one individual on Teide (Table 3.2). The only individual found to be infected with *Leucocytozoon* was from the eastern subpopulation.

3.4.2 Individual-level analyses

As *Leucocytozoon* infection was largely restricted to a single population, GLMs including all individuals were only carried out for pox and *Plasmodium*. For both pathogens, there was a significant effect of island identity on infection, but not one of year (Table 3.3A). There was a significant island by year interaction for *Plasmodium*, and a near-significant interaction for pox (Table 3.3A). The second set of more detailed models revealed that infection with *Plasmodium* had a significant effect on pox infection, while controlling for age, sex, island, and year (Table 3.3B). This association was positive; pox prevalence was 30% in individuals with *Plasmodium*, compared to 17% in those individuals without *Plasmodium*. Similarly, infection with pox was associated with an increased likelihood of *Plasmodium* infection (Table 3.3B); prevalence of *Plasmodium* in individuals with pox was 52%, compared to 33% in individuals without pox. For *Leucocytozoon*, the individual-level analysis was restricted to individuals from Porto Santo (n = 60), the only island where it was found at anything but very low levels. Here, we found no effect of *Plasmodium* or pox on infection while controlling for other variables (Table 3.3B). A GLM restricted to individuals from Tenerife confirmed the intra-island variation, with a highly significant effect of region on infection with both pox and malaria. In this analysis, there was a less strong but nonetheless significant region × year interaction for pox, and an effect of year for *Plasmodium* (Table 3.4).

Table 3.1 Prevalence (percentage of individuals infected) of blood pathogen infection in 13 populations of Berthelot's pipit across Macaronesia.

Island	*Plasmodium*		*Leucocytozoon*		Pox		Sample size	
	2006	2009	2006	2009	2006	2009	2006	2009
Deserta Grande	0	0	0	0	0	0	31	4
Madeira	0	0	0	0	0	0	33	29
Porto Santo	64.5	30	25.78	13.33	45.25	36.77	31	30
Selvagem Grande	0	0	0	0	0	0	34	42
La Graciosa	4.20	0	0	0	0	0	24	26
Lanzarote	23.13	48.38	0	0	53.83	16.13	13	31
Fuerteventura	50	45.25	0	0	16.67	29	12	31
Gran Canaria	45.25	15.25	6.50	0	16.13	27.36	31	33
Teide	6.75	0	0	0	0	4	30	25
Tenerife	9.42	32.37	3.13	0	12.50	5.91	32	34
La Gomera	53.33	35	0	0	3.33	10	30	20
La Palma	3.67	0	0	0	0	4.50	28	22
El Hierro	9.73	0	0	0	0	0	31	30

Table 3.2 Prevalence (percentage of individuals infected) of blood pathogen infection in four sub-populations of Berthelot's pipit on Tenerife.

Sub-population	*Plasmodium*	*Leucocytozoon*	Pox	Sample size
North-West	0	0	0	46
South	34.76	0	21.75	69
East	33.27	2.08	0	48
Teide	3.63	0	1.81	55

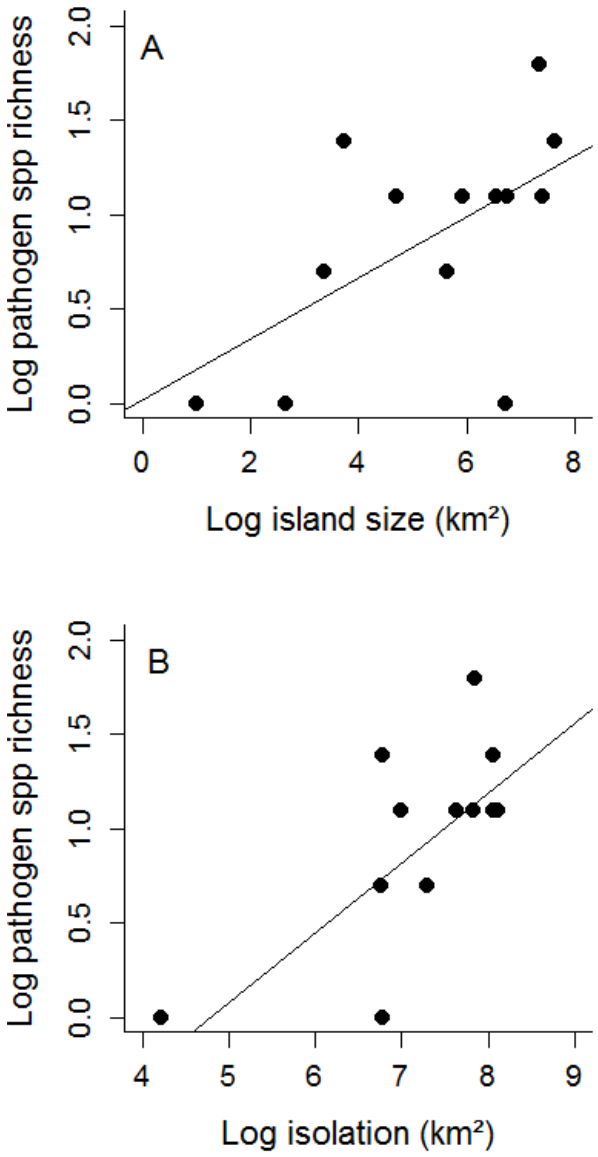

Figure 3.2 Pathogen species richness in island populations of Berthelot's pipit in relation to **A** island area and **B** land area within 100 km of the coast of each island (isolation). See table 3.1 for sample sizes

Table 3.3 Results of generalized linear models showing, **A** across all populations of Berthelot's pipit, the effect of island identity and sampling year on pathogen load and, **B** within infected islands, the effect of other blood pathogens on likelihood of infection after controlling for island, year, sex and age. Significant results ($P < 0.05$) are highlighted in bold.

	d.f.	b	s.e.	Deviance	Residual Deviance	*P*
A. All Islands						
Pox						
Null					531.21	
Island	12, 819	-	-	148.09	383.12	**< 0.001**
Year (2009)	1, 818	-0.001	0.001	0.11	383.01	0.66
Island*Year	12, 806	-	-	11.1	371.9	0.07
Plasmodium						
Null					796.7	
Island	12, 818	-	-	206.88	589.83	**< 0.001**
Year (2009)	1, 817	-0.001	0.001	0.78	589.04	0.28
Island*Year	12, 805	-	-	28.93	560.12	**< 0.001**
B. Infected Islands only						
Pox						
Null					405.26	
Island	5, 412	-	-	35.89	369.37	**< 0.001**
Year (2009)	1, 411	-0.3	0.25	0.17	369.2	0.68
Sex (Male)	1, 410	0.03	0.27	0 .003	369.2	0.96
Age (Juvenile)	1, 409	-0.12	0.26	0.76	368.43	0.39
Plasmodium (Infected)	1, 408	0.64	0.3	10.71	357.72	**< 0.001**
Plasmodium						
Null					537.06	
Island	5, 412	-	-	16.51	520.55	**0.01**
Year (2009)	1, 411	-0.11	0.31	0.14	520.41	0.71
Sex (Male)	1, 410	-0.15	0.32	0.15	520.27	0.71
Age (Juvenile)	1, 409	0.08	0.31	1.57	518.7	0.22
Pox (Infected)	1, 408	0.65	0.3	10.85	507.85	**0.001**
Leucocytozoon						
Null					56.76	
Year (2009)	1, 57	-2.05	0.88	3.09	53.67	0.07
Age (Juvenile)	1, 56	-2.14	0.93	5.58	48.09	**0.01**
Sex (Male)	1, 55	1.05	0.84	0.91	47.18	0.33
Plasmodium (Infected)	1, 54	0.7	0.85	1.23	45.95	0.253
Pox (Infected)	1, 53	0.94	0.8	1.37	44.58	0.23

Table 3.4 Results of generalized linear model showing the effect of intra-island variation on pathogen load in a single population (Tenerife) of Berthelot's pipit. Significant results ($P < 0.05$) are highlighted in bold.

	d.f.	b	s.e.	Deviance	Residual Deviance	*P*
Pox						
Null					114.38	
Region	3, 214	-	-	27.47	86.91	**< 0.001**
Year (2009)	1, 213	-	-	0.68	86.23	0.23
Region*Year	3, 210	-	-	6.03	80.21	**0.005**
Plasmodium						
Null					213.24	
Region	3, 213	-	-	47.03	166.21	**< 0.001**
Year (2009)	1, 212	-	-	3.66	162.54	**0.02**
Region*Year	3, 209	-	-	5.01	157.54	0.07

3.4.3 Effects on body condition

Analyses of body condition were restricted to the six islands where pathogens were present in more than two individuals. There was a significant association between both pox and Plasmodium infection with mass, while controlling for body size, age, sex, island and year (Table 3.5). Infected individuals were, on average, heavier than uninfected individuals: mean ± S.D. mass for infected and uninfected individuals, respectively, was 16.9 ± 0.8 and 16.2 ± 0.7 g for pox, and 16.7 ± 0.3 and 16.2 ± 0.7 g for malaria.

3.5 Discussion

Our study is one of the first to examine the distributions of multiple pathogens over a range of spatio-temporal scales across populations of a wild animal. The evidence indicates that, in Berthelot's pipit, there are strong population-level differences in pathogen distribution, and that pathogen species richness is related to island size and isolation. These broad differences in distribution were stable over the three-year time period of this study. However, across some of the islands where the pathogens were present, prevalence levels varied considerably over the two sampling periods. Within a single population, we observed marked differences in pathogen presence and prevalence across subpopulations. Analysis at the individual level further supported the island effect,

and we also detected a positive association between pathogens. Finally, pathogenic infection was associated with the body condition of Berthelot's pipits.

Table 3.5 Results of general linear model, showing the effects of malaria and pox on mass in Berthelot's pipit, while controlling for body size, age, sex, island and year. For body size, the first principal component from a principal components analysis of seven morphological measurements was used.

Explanatory variable	b	s.e.	F	d.f.	*P*
Body Size	0.11	0.03	8.22	1	0.004
Island	-	-	10.25	5	<0.001
Year (2009)	0.06	0.11	0.17	1	0.68
Sex (Male)	-0.45	0.16	6.35	1	0.01
Age (Juvenile)	-0.23	0.11	3.31	1	0.07
Malaria (Infected)	**0.26**	**0.11**	**6.35**	**1**	**0.01**
Pox (Infected)	**0.27**	**0.13**	**5.12**	**1**	**0.02**

Over the three-year time period of our study, which roughly corresponds to the lifespan of pipits (Coulson 1956), we observed a high degree of temporal stability in pathogen presence at the population level (Table 3.1). In all populations where a pathogen was observed in one year but not another, the pathogen occurred in less than three individuals in the infected year (Table 3.1). This suggests that our failure to detect them in both years may have been due to them being very rare and not picked up in our sample, rather than absent. In other words, pathogen load on some islands is consistently low (or zero), and consistently moderate to high on others. Little work has been done on the long-term temporal stability of avian pathogens, though recent evidence from Hawaii suggests that avian pox variants have been maintained in populations for over 100 years (Jarvi et al. 2008). Similarly, the presence of avian malarial lineages has been shown to be relatively stable within populations over periods of up to a decade (Fallon et al. 2004). Over these sorts of time periods, however, marked fluctuations in the prevalence of these pathogens are expected to occur (Fallon et al. 2004). This was the case in our study, where temporal shifts in prevalence did occur within a few of the populations where pathogens were present at moderate to high levels (Fig. 3.1). However, with only two sampling periods, we have to be cautious in interpreting the extent to which pathogen load varies over time. In order to do so more fully, long-term datasets, ideally from multiple populations, are now needed.

Island biogeography theory predicts that smaller, more isolated islands will exhibit lower species richness than larger, less isolated islands due to lower rates of colonization and higher rates of extinction (MacArthur and Wilson 1967). Biogeographic studies of pathogens have mostly considered hosts as the "islands" (Dritschilo et al. 1975; Kuris et al. 1980). However, island size itself may also affect patterns of pathogen distribution, though evidence for this in the literature is currently limited, and has yielded mixed results. In a recent study, Ishtiaq et al. (2010) examined species–area relationships in Plasmodium and Haemoproteus lineages infecting white-eyes (*Zosterops* spp.) in 16 southwest Pacific islands. Significant species–area relationships were found for Plasmodium, but not for Haemoproteus. In Darwin's finches (*Geospiza fuliginosa*), a positive relationship between pathogen (pox and ectoparasite) abundance, but not diversity, was observed (Lindström et al. 2004). In *Anolis* lizards, no relationship was found between island size, elevation or rainfall and the presence of malaria (Staats and Schall 1996). In our study, we observed significant effects of both island size and isolation on pathogen species richness across islands. One would predict island size and isolation to be especially important for vector-borne pathogens, as screened for here, as transmission to the host requires both the pathogen and vector to be present at a given point in time. Nonetheless, our population-level data suggest that colonization and extinction may have roles to play in determining pathogen distribution in our study system, and provide an explanation for why patterns of pathogen distribution are temporally stable across populations.

Within a single island, Tenerife, we observed a high degree of structuring in pathogen distribution, suggesting that in addition to the observed island-level effects, intra-island level factors also play an important role. Recent evidence from blue tits (*Cyanistes caeruleus*) has shown that pathogen lineages can be restricted to defined spatial regions, and that changes of up to 50% in malarial prevalence can occur at distances of less than 1 km (Wood et al. 2007). Our study confirms that local spatial variation in host–pathogen systems can occur. It is difficult to speculate about how variations at the inter- and intra-island levels may interact. One possibility is that larger islands are more likely to contain within-population variation in the pathogen distribution, and higher pathogen species richness as a result. However, more fine-scale sampling is now needed to determine the

factors underlying within-population spatio-temporal variation, the scale at which it occurs, and its effects on population-level distribution.

At the individual level, we detected a positive association between avian pox and Plasmodium. This is somewhat surprising, as one may expect to find a low number of individuals with multiple infections either because of competitive interactions between pathogens, or due to the potential fitness costs incurred to the host (Balmer et al. 2009; Beadell et al. 2004; Haukisalmi and Henttonen 1993). However, positive associations between pathogens can occur, and associations between avian malaria and pox have recently been detected in birds from Hawaii (Atkinson et al. 2005). There are a number of possible explanations for such findings. First, it could be that infection with one pathogen reduces host resistance and makes birds more susceptible to the other, or that a third, unknown pathogen makes the birds more susceptible to both malaria and pox. A number of pathogens, including malaria, are well known to have immunosuppressive effects, and this can often lead to positive associations between multiple pathogens (Cox 2001). Alternatively, the two pathogens could be transmitted by the same vector. This is also possible; for example, *Culex* mosquitoes have been demonstrated to transmit both pox and malaria to wild birds (Akey et al. 1981). Unfortunately, however, little is known about the distribution of invertebrate hosts across the North Atlantic archipelagos, and less still is known about the relationships between pathogens and invertebrate hosts across this region. More research in this area is now needed (see, for example, Hellgren et al. 2008; Njabo et al. 2011). Finally, it could be that the two pathogens are restricted to the same areas, and that the observed effect has arisen from sampling over multiple subpopulations (i.e., some with both pathogens and some with neither). Our data from Tenerife suggest that the latter of these explanations is unlikely to be the case in Berthelot's pipits, as we found a subpopulation with only one of the two pathogens (Table 3.2). Such a finding would, if anything, obscure positive associations. In contrast, there was evidence of a positive association between the two pathogens in the southern subpopulation, the only one in which both pathogens occurred ($\chi^2 = 5.37$, $P = 0.02$), suggesting that Plasmodium and pox co-occur on a very local scale.

Research into the impact of avian diseases on host body condition has generally shown that, as one may expect, infected individuals present poorer body conditions than uninfected individuals (Marzal et al. 2008; Valkiunas et al. 2006). However, in our study, we found the opposite: infected individuals had better body conditions than uninfected individuals. One possible explanation for this is that there is variation in both size and immunocompetence within populations (i.e. larger subpopulations are less immunocompetent due to higher investment in growth). Another possible explanation is that infection kills low-quality individuals, and that our sample consisted of the high-quality individuals that have been able to cope with infection. This is in line with the fact that we were, for the most part, unable to amplify pox DNA from the pox lesions we sampled, nor from the corresponding blood samples. Scars from pox lesions can last on birds for months (Ritchie 1995), making it possible that individuals in 2009 had retained lesions from a previous infection but were no longer infected. Alternatively, it may be that some of the pox-like lesions were caused by a different, unknown pathogen, although this seems highly unlikely given the similarity in appearance to the pox lesions observed in pipits by ourselves and others (Smits et al. 2005). Similarly, avian malaria can remain in bird blood at chronic levels for long periods of time after an initial, acute infection (Atkinson et al. 2001; Kilpatrick et al. 2006; Valkiunas 2005). If infection with pox and malaria does kill low-quality individuals, this implies that infection with the pathogens studied here confers severe fitness costs to the hosts. However, we cannot rule out the possibility that individuals with good body conditions are more susceptible to infection due to decreased immunocompetence, rather than the survivors of infection. An assessment of infection levels using qPCR (e.g. Knowles et al. 2010), as well as data on the effects of pathogenic infection on survival and reproduction, would help to confirm fitness costs.

As Berthelot's pipit has only recently dispersed across its range, with little subsequent migration between populations (Illera et al. 2007), the differential levels of pathogenic infection observed are likely to constitute an important selective force for promoting differentiation across populations. Moreover, because spatial variation in the pathogen regime appears to be constrained, at least in part, by biogeographical factors, these differential selective pressures are consistent over time, at least at scales comparable to

the lifespan of this species. This is an important point, as spatial variation in selection is only likely to produce detectable effects upon host populations if it is consistent over time. Thus, our findings provide a foundation for further research into the genetic, physiological and behavioural consequences of these differential selective pressures.

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Chapter 4: Microsatellites with high and low variability provide different insights into the population history of an island bird

A version of this chapter has been submitted for publication in *Molecular Ecology*

Ilheu Chao, viewed from Deserta Grande, Madeira

4.1 Abstract

Genomic resources are facilitating the rapid discovery of microsatellite markers for non-model species. However, the characteristics of marker sets designed using different methods can vary greatly, which may have significant consequences for the analyses undertaken. We test how microsatellite variability can be used to inform population history in Berthelot's pipit. We analysed patterns of variation at 10 highly conserved loci that have low levels of variability across bird species ("LV loci"), and compared this to 12 higher variability (HV) loci. We show that founder events resulted in dramatic reductions in allelic richness at HV, but not LV loci; a pattern reflected in tests for genetic bottlenecks, which yielded contrasting results depending on the marker set used. Similarly, pairwise analyses of population structure revealed inflated levels of differentiation in comparisons involving bottlenecked populations, but only when HV loci were used. We suggest that because LV loci are less sensitive to loss of variation during bottlenecks, they more accurately reflect population divergence. Meanwhile, HV microsatellites better reflect past changes in population size. Utilising differences in microsatellite variability to inform population genetic analyses more fully may therefore be a rewarding approach.

4.2 Introduction

Genetic markers are powerful tools for making inferences about historical and contemporary processes that have occurred within and among populations, such as migration, changes in population size and divergence times. To provide the best resolution, genetic markers should have the appropriate mutation rates and levels of polymorphism to address the specific questions at hand (Parker *et al.* 1998; Sunnucks 2000). The combined use of different markers with different properties (e.g. nuclear sequences, mitochondrial genes, microsatellites), may help maximise the information obtained and the accuracy of eventual conclusions, and reveal how evolutionary change operates over a range of temporal scales (Palumbi and Baker 1994; Ross et al. 1999; Wang 2010).

Microsatellites are perhaps the most widely used genetic markers for investigating how genetic diversity is partitioned within and among closely related populations (Jarne and Lagoda 1996; Balloux and Lugon Moulin 2002). They are uniquely suited to this purpose, owing to their co-dominance, high mutation rates, selective neutrality and abundance across the genome. While microsatellites remain an invaluable source for population genetic studies (Guichoux *et al.* 2011), care needs to be taken when using microsatellites to infer population history. The manner and rapid rate by which microsatellites mutate can result in problems with homoplasy, potentially confounding estimates of differentiation (Garza and Freimer 1996; Estoup et al. 2002). The high variability observed at microsatellites also makes them particularly sensitive to reductions in population size. This can elevate population structure in bottlenecked populations, making it difficult to distinguish whether the levels of structure observed represent true divergence between populations, or whether they are a function of past changes in population size (Chakraborty and Nei 1977; Hedrick 1999; Hedrick et al. 2001; Whitehouse and Harley 2001).

Methodological developments, including the arrival of high-throughput sequencing, mean that microsatellites can now be isolated relatively quickly for most taxonomic groups (for an overview see Guichoux *et al.* 2011). Some loci have been successfully developed into markers with extensive cross-species utility (Dawson *et al.* 2010; Jan *et al.* 2012) and as a

result increasing numbers of markers are readily available for non-model organisms. Moreover, by multiplexing – combining multiple markers into single PCRs – large numbers of individuals can be genotyped at increasingly low cost (Chamberlain *et al.* 1988; Kenta *et al.* 2008). A consequence of the increased availability of microsatellites is that a greater degree of variability in marker characteristics is expected. Microsatellites vary in repeat length, repeat number and proximity to functional genes, all of which have consequences for mutation rate (Ellegren 2004). These factors, and thus marker variability, may differ depending on the criteria used when markers are developed (Dawson *et al.* 2010; Dawson et al. unpublished data). Where systematic differences in levels of variability occur across sets of microsatellite markers, some interesting and novel opportunities may arise for population genetic studies. For example, using microsatellite markers with lower mutation rates may result in reduced instances of homoplasy and less sensitivity to population bottlenecks, providing a clearer assessment of divergence between populations (O'Reilly *et al.* 2004; Woodhead *et al.* 2005). On the other hand, high levels of marker variability are required for some analyses, including the detection of bottlenecks (e.g. Primmer *et al.* 1995; Cornuet and Luikart 1996; Garza and Williamson 2001). To our knowledge, however, no studies to date have utilised systematic differences in microsatellite variability to better understand population history.

Here we examine how differences in marker variability can inform population history in Berthelot's pipit. We first use microsatellite data to infer the colonisation pathway of this species across its island distribution. Then, using a panel of 12 'high variability' and 10 'low variability' microsatellite loci, we test how genetic diversity has been affected by founder events, and whether these patterns are consistent for the two sets of markers. We then use two different approaches to test whether founder events resulted in detectable bottlenecks at the different marker sets. Finally, we examine the consequences of founder events for patterns of genetic differentiation across the two sets of markers. We discuss how utilising differences in marker variability can enable clearer interpretation of some commonly employed population genetic analyses.

4.3 Materials and Methods

Representative samples (*c.* 30 individuals) were obtained from all 13 Berthelot's pipit populations (see section 3.3 for full details). For this chapter, only samples from 2006 were used (see chapter 7 for discussion).

4.3.1 Marker selection and molecular procedures

Genomic DNA was extracted using a modified salt extraction method (Bruford *et al.* 1992; Richardson *et al.* 2001) and diluted to a concentration of 10-50 ng/µL. For population genetic analyses we used the four loci genotyped by Illera *et al.* (2007) that conformed to Hardy-Weinberg expectations. In addition, we tested three sets of conserved microsatellite markers (80 loci in total) for variability in the pipit. Thirty-four of the loci tested were developed on the basis of having a high degree of sequence homology between the zebra finch (*Taeniopygia guttatta*), expressed sequence tag (EST) database and the chicken (*Gallus gallus*) genome. These '*TG*' loci are therefore highly conserved across passerine birds (Dawson *et al.* 2010). In addition, we tested 24 loci isolated from the zebra finch and chicken genomes (Conserved Avian Microsatellite, or *CAM* loci; Dawson *et al.* unpublished data), and 22 house sparrow (*Passer domesticus*) loci of utility in other passerines (Dawson *et al.* 2012). On average, the *TG* loci have fewer repeat units compared to other conserved markers, and as a result fewer of these loci are polymorphic across bird species (Dawson *et al.* Unpublished data). We therefore classified the *TG* loci *a priori* as 'low variability' (LV), and the remaining loci as 'high variability' (HV).

All loci were initially tested in four individuals from across the three archipelagos. Loci that amplified with clear, scorable products and at which at least two alleles were found were tested in 24 individuals from a single population (Tenerife) and checked for Hardy-Weinberg equilibrium (HWE) and null alleles (see below for details). After excluding monomorphic loci, and loci that deviated from HWE/ showed evidence of null alleles, 22 markers remained, which were arranged into five multiplex reactions (Table 4.1). PCRs were carried out in 2 µL reactions using a method based on Kenta *et al.* (2008). Briefly, 1 µL of genomic DNA solution was added to each PCR well and the liquid evaporated. To this, 1 µL primer mix (containing all forward and reverse primers in the multiplex reaction at 0.2 µM) and 1 µL 2x QIAGEN Multiplex PCR Master Mix was added. Samples were

overlaid with mineral oil before PCR. An initial denaturing phase of 95ºC for 15 minutes was followed by 40 cycles of 94˚C for 30 seconds, 56˚C for 90 seconds and 72˚C for 60 seconds. A final hold of 60˚C for 30 minutes completed the reaction. PCR products were diluted 1 in 400 and separated on an ABI 3730 DNA analyzer. Allele sizes were determined using GeneMapper version 3.7 (Applied Biosystems). To estimate the rate of scoring error, we random selected a subset of samples (30 individuals) to be scored by two different individuals (LGS and D.P. Padilla). The maximum number of mismatches at a locus was one, suggesting an error rate of less than 3%.

4.3.2 Analyses

Unless stated otherwise, all statistics and plots were generated in R version 2.12.2 (R Development Core Team 2008). For all variables used in parametric tests, normality was tested using Shapiro-Wilks tests and transformed where appropriate.

For each microsatellite locus and population, HWE and linkage disequilibrium were tested using Genepop version 4.1 (Raymond and Rousset 1995) and null allele frequencies were estimated using CERVUS version 3.0.3 (Marshall *et al.* 1998). Allele frequencies and Nei's gene diversity (Nei 1987) were calculated using Fstat version 2.9 (Goudet 1995). Summary statistics of genetic diversity (gene diversity, allelic richness and number of private alleles) within populations were calculated for each locus and averaged for each population and archipelago across HV and LV loci separately. Because significant differences occurred across, but not within archipelagos (see results), we focussed on differences at the archipelago level. Allelic richness and numbers of private alleles per archipelago were calculated after controlling for differences in sample size, using a rarefaction approach implemented in HP rare (Kalinowski 2004, 2005). Differences in genetic diversity across marker types and archipelagos were tested using general linear models. An interaction term was included in models (marker type*archipelago) to test whether archipelago-level differences in genetic diversity were consistent across the two marker types.

The presence of positive selection at microsatellites, particularly those derived from EST databases, is a potential concern for population genetic studies (Ellis & Burke 2007). Thus, an outlier approach was used to test for selection at individual loci, plotting F_{ST} against

heterozygosity, and comparing this to a simulated null distribution (Beaumont and Nichols 1996). The null distribution was generated using 20,000 simulations and a finite island model, implemented in Arlequin version 3.5 (Excoffier and Lischer 2010). All loci were used to generate the original simulations, then loci with significantly high or low F_{ST} values were removed, and the simulations were run again. All loci were included in the final plot. Loci with significantly high overall F_{ST} values relative to heterozygosity are considered to be subject to diversifying selection, whereas those with low values are considered to be under balancing selection (Beaumont and Nichols 1996).

Table 4.1 22 microsatellite loci amplified in the Berthelot's pipit. Loci classified *a priori* as 'high variability' (see text for details) are in italics.

Multiplex	Locus	Reference
1	PCA7	(Dawson *et al.* 2000)
	TG03-002	(Dawson *et al.* 2010)
	TG13-009	(Dawson *et al.* 2010)
2	*TG01-047*	(Dawson *et al.* 2010)
	TG02-088	(Dawson *et al.* 2010)
	CAM13	(Dawson *et al.* unpublished data)
3	PDO46	(Dawson *et al.* 2012)
	TG03-098	(Dawson *et al.* 2010)
	CAM2	(Dawson *et al.* unpublished data)
	CAM23	(Dawson *et al.* unpublished data)
	CAM4	(Dawson *et al.* unpublished data)
	CAM8	(Dawson *et al.* unpublished data)
4	PDO47	(Dawson *et al.* 2012)
	PPI2	(Martinez *et al.* 1999)
	TG04-004	(Dawson *et al.* 2010)
	TG09-018	(Dawson *et al.* 2010)
	CAM18	(Dawson *et al.* unpublished data)
	HRU5	(Primmer *et al.* 1995)
5	PDO5	(Dawson *et al.* 2012)
	TG01-024	(Dawson *et al.* 2010)
	TG05-053	(Dawson *et al.* 2010)
	TG06-009	(Dawson *et al.* 2010)

Population-level pairwise differentiation was calculated as $F_{ST}/(1-F_{ST})$ (Slatkin 1995) in Arlequin, with *P* values obtained from 1000 bootstraps. Relationships between pairwise genetic and geographical distance matrices were tested using Mantel tests, implemented in the Ecodist package in R (Goslee and Urban 2007). To control for archipelago-level founder effects, which may be misinterpreted as genuine isolation-by-distance relationships (Slatkin 1993), partial Mantel tests were performed, including a coding variable that indicated which archipelagos were involved in each pairwise comparison (e.g. Canary Islands-Canary Islands, Canary Islands-Madeira, etc.).

A Bayesian approach was used to identify the number of distinct genetic clusters (*K*), without using *a priori* information about the origin of individuals, implemented in the program STRUCTURE (Pritchard *et al.* 2000). A model allowing admixture and correlated gene frequencies was used. For each run a burn-in of 10,000 steps was followed by a run of 500,000 steps. Four independent runs were carried out for each value of *K*, and the value with the highest average 'log probability of data' was used. A statistic based on second order changes in likelihood (ΔK) was also calculated, as it may be a better representative of the true value of *K* than the log probability (Evanno *et al.* 2005).

Two methods were used to detect whether populations had undergone genetic bottlenecks. First, a method that tests for heterozygosity excess, which occurs due to the loss of rare alleles shortly after bottlenecks (Cornuet and Luikart 1996), was implemented in the program BOTTLENECK (Piry *et al.* 1999). A two phase mutation model was used, with 95% stepwise and 5% non-stepwise mutations. The probability of heterozygosity excess was then calculated using Wilcoxon tests. Second, Garza and Williamson's index (*M*) was calculated by dividing the number of alleles in a population (*k*) by the range in allele size (*r*) (Garza and Williamson 2001). This was modified to $M = k/(r+1)$ to avoid dividing by zero in monomorphic populations (Excoffier *et al.* 2005). Following a reduction in population size, *M* is expected to be reduced, and the authors have suggested that values below *c.* 0.68 are indicative of a bottleneck (Garza and Williamson 2001).

4.4 Results

In total, 371 individuals were genotyped at the 22 microsatellite loci. None of these loci were found to be in HW disequilibrium in more than two populations, and none were found to be in linkage disequilibrium after correction for multiple tests. Allele frequencies for each marker and population are shown in Appendix 1.

4.4.1 F_{ST} outlier analyses

One LV marker, *TG01-124*, had significantly high genetic structure relative to heterozygosity, indicating positive selection (Fig. 4.2). This marker was variable in Madeira, but had very low levels of heterozygosity across the Canary Islands (Appendix 1), the opposite pattern to that observed at other loci (Appendix 1, also see below). A BLAST search against the zebra finch genome found locus *TG01-124 t*o be closely linked (0.01 kb) to the *GABRB3* gene (Olano-Marin *et al.* 2011), which is associated with autism and other neurological disorders in humans and mice (Samaco *et al.* 2005). Due to being under selection in this species, *TG01-124* was not included in the subsequent analyses presented here, though it should be noted that its presence did not significantly change results (data not shown).

4.4.2 Genetic diversity across marker types and archipelagos

Within archipelagos, there were no significant differences between populations in any measure of genetic diversity (all $P > 0.6$). There were, however, significant differences in genetic diversity and number of private alleles across archipelagos, as well as between HV and LV markers (Table 4.2). Both measures of genetic diversity (allelic richness and gene diversity) were highest in the Canary Islands (Table 4.2), and almost all variation in Madeira and Selvagens was a subset of this (Fig. 4.2).

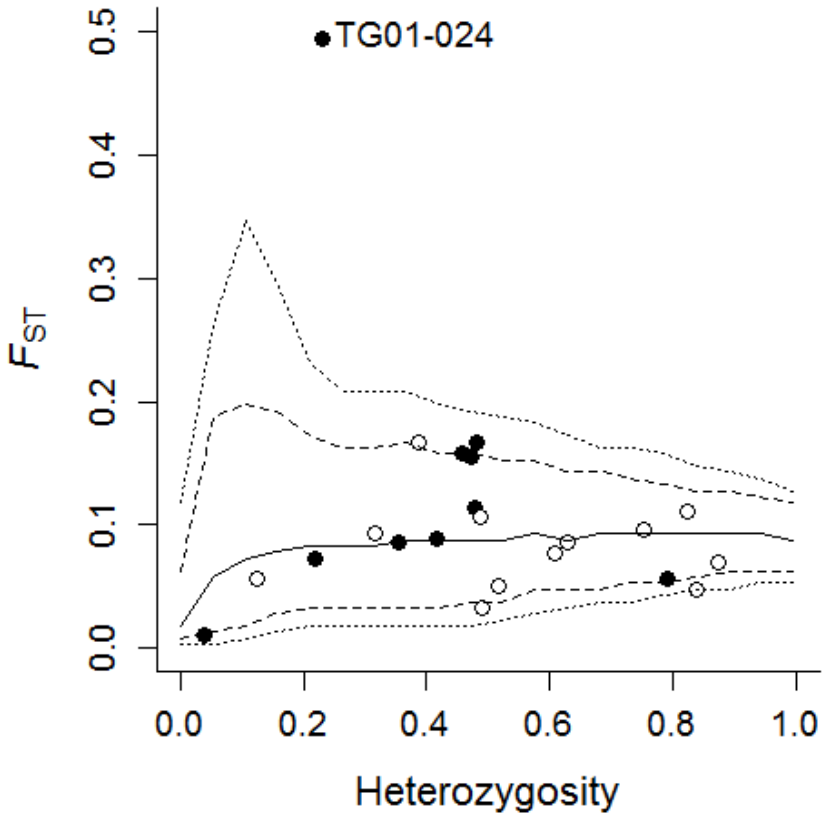

Figure 4.1 Population structure in relation to heterozygosity at 10 low variability (filled circles) and 12 high variability loci (open circles) in 371 Berthelot's pipit individuals. A finite island model was used, comparing patterns of differentiation among populations. Solid, dashed and dotted lines represent the expected median, 95% and 99% confidence intervals, respectively, from null distributions generated using 20,000 simulations in Arlequin.

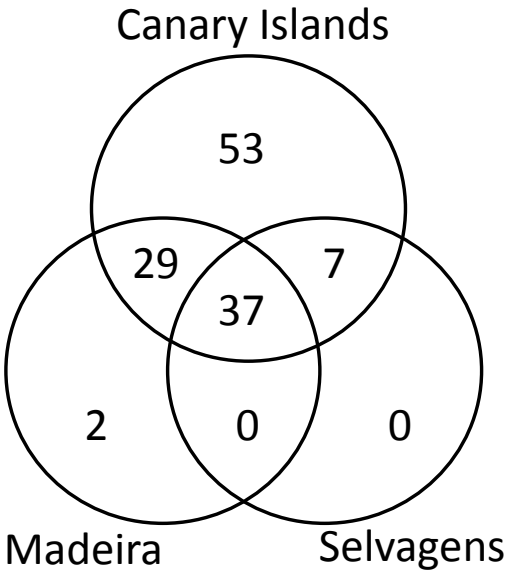

Figure 4.2 Distribution of allelic variation from 371 Berthelot's pipit individuals at 22 microsatellite loci. Numbers represent the total number of alleles found within, or shared across, archipelagos.

All measures of genetic diversity were significantly higher at loci selected *a priori* as HV, compared to LV loci (Table 4.2). Importantly, there was an interactive effect of archipelago and marker type on allelic richness and on the number of private alleles (Table 4.2), suggesting that differences across archipelagos are not consistent between HV and LV loci. Indeed, at the HV loci, allelic richness in the Canary Islands was more than twice that in Madeira and Selvagens, whereas at the LV loci this difference was much less pronounced, especially between the Canary Islands and Madeira, where there was no significant difference (Fig. 4.3). No significant interaction between archipelago and marker type was found for gene diversity, suggesting a consistent pattern across archipelagos between the two marker types for this measure (Table 4.2).

Table 4.2 Genetic diversity at low and high variability microsatellites in three archipelagos of Berthelot's pipit (371 individuals), and results from a general linear model testing for differences across archipelagos and marker type.

	Mean ± SE						General linear model					
	Canary Islands		Madeira		Selvagens		Archipelago		Marker type		Interaction	
	LV	HV	LV	HV	LV	HV	F	P	F	P	F	P
Allelic richness	2.92 ± 0.16	6.02 ± 0.41	2.43 ± 0.23	2.97 ± 0.27	1.6 ± 0.31	2.36 ± 0.39	15.4	< 0.001	40.7	< 0.001	5.1	0.007
Private alleles	0.76 ± 0.09	2.68 ± 0.28	0.18 ± 0.09	0.21 ± 0.06	0.07 ± 0.07	0.08 ± 0.06	22.7	< 0.001	34.5	< 0.001	7.4	< 0.001
Gene Diversity	0.36 ± 0.02	0.58 ± 0.03	0.35 ± 0.05	0.42 ± 0.04	0.16 ± 0.07	0.33 ± 0.08	9.9	< 0.001	40.5	< 0.001	1.5	0.22

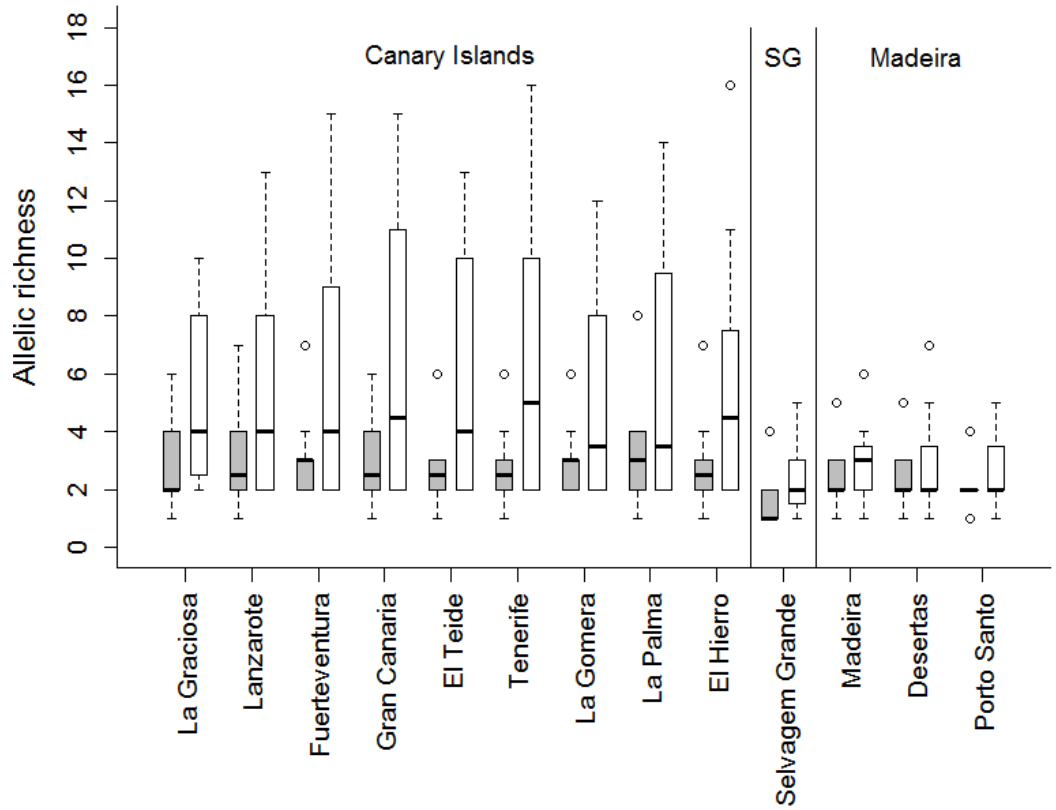

Figure 4.3. Boxplots of allelic richness at low variability (grey) and high variability (white) microsatellite markers in Berthelot's pipit populations. Solid lines represent medians, the edges of the boxes the inter-quartile range, and the bars represent the range (excluding outliers). Outliers are values more than 1.5 times the inter-quartile range outside the upper or lower quartiles. Sample sizes are given in brackets.

4.4.3 Genetic structure and isolation-by-distance

Genetic clustering analysis in STRUCTURE yielded an optimum value of $K = 3$, regardless of which markers were used (Fig 4.4), and these clusters corresponded to the three archipelagos (Fig. 4.5). Geographic structuring was strongest when all 22 loci were combined into a single analysis, and stronger structuring was observed at HV compared to LV loci (Fig. 4.5). To test whether this difference in geographical structuring was significant, each of the $K = 3$ clusters was assigned to the best matching archipelago (i.e. in Fig. 4.5, dark grey bars for Canary Islands, light grey bars Madeira and white bars Selvagens). The mean proportion of genetic variation within individuals attributed to their 'archipelago cluster' was significantly higher when all 22 loci were used (mean ± s.e. = 0.96 ± 0.003) compared to HV loci (mean ± s.e. = 0.93 ± 0.006; paired Wilcoxon test, $P < 0.001$). Both of these values far exceeded the level of geographic structuring at LV loci, (mean ± s.e. = 0.65 ± 0.015; $P < 0.001$).

Population differentiation (measured as $F_{ST}/(1-F_{ST})$) was significantly greater than zero in most pairwise comparisons at both LV (79% of comparisons with $P < 0.05$) and HV loci (86% of comparisons with $P < 0.05$; Table 4.3). Pairwise patterns of differentiation at the two marker types were positively related (Mantel test, R = 0.69, $P < 0.001$). Across all populations, there was an apparent signal of isolation-by-distance at both marker sets, and this pattern was strongest when LV loci were used (Mantel tests, LV loci: R = 0.52, $P = 0.002$; HV loci: R = 0.26, $P = 0$ .024; all loci: R = 0.39, $P = 0.002$; Fig. 4.6). However, after controlling for archipelago-level effects these patterns were no longer significant (Partial Mantel tests, LV loci: R = 0.02, $P = 0.89$; HV loci: R = -0.03, $P = 0$ .78; all loci: R = -0.02, $P = 0.86$; Fig. 4.6). Pairwise structuring at the archipelago-level differed substantially between the HV and LV markers. At the HV loci, there were elevated levels of differentiation in pairwise comparisons involving the Selvagens, with pairwise values reaching as high as 0.75 (Fig. 4.6; Table 4.3).

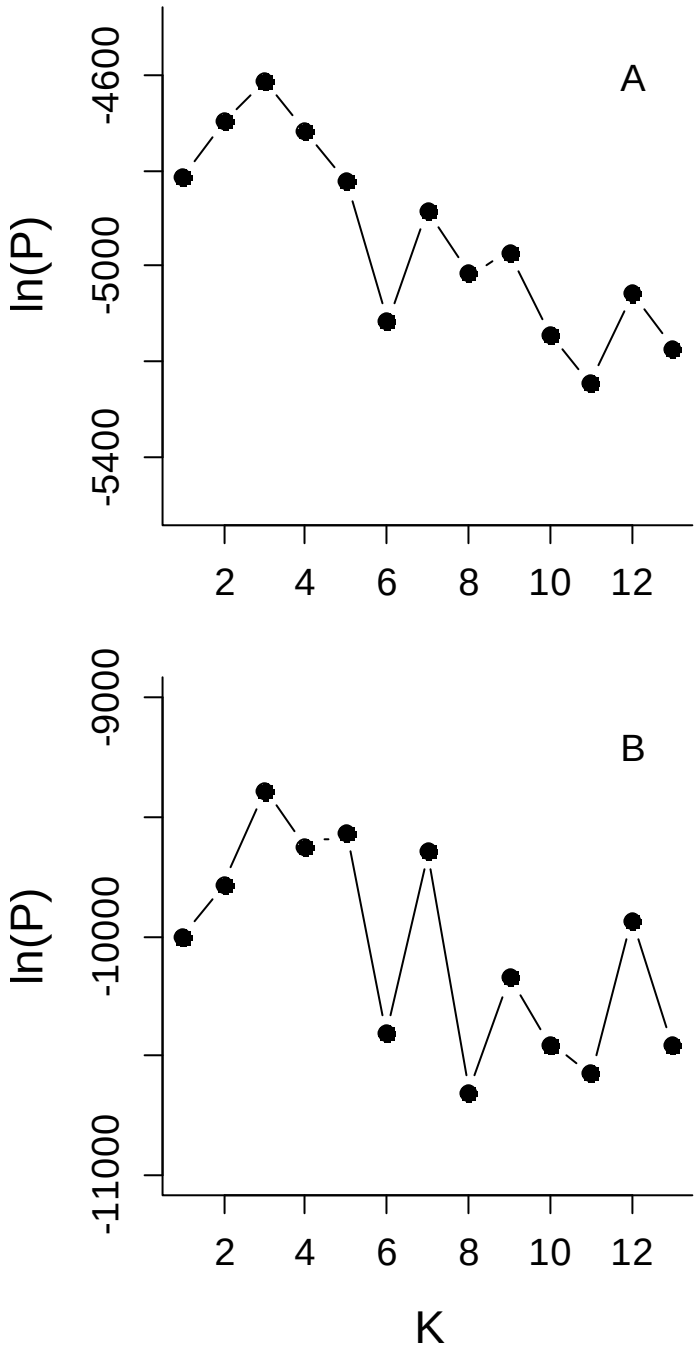

Figure 4.4 Output from STRUCTURE analysis on 371 Berthelot's pipit individuals, at low variability (**A**) and high variability (**B**) microsatellite loci. Graphs show the log likelihood of the microsatellite data being partitioned into K = 1 to K = 13 clusters (see text for details).

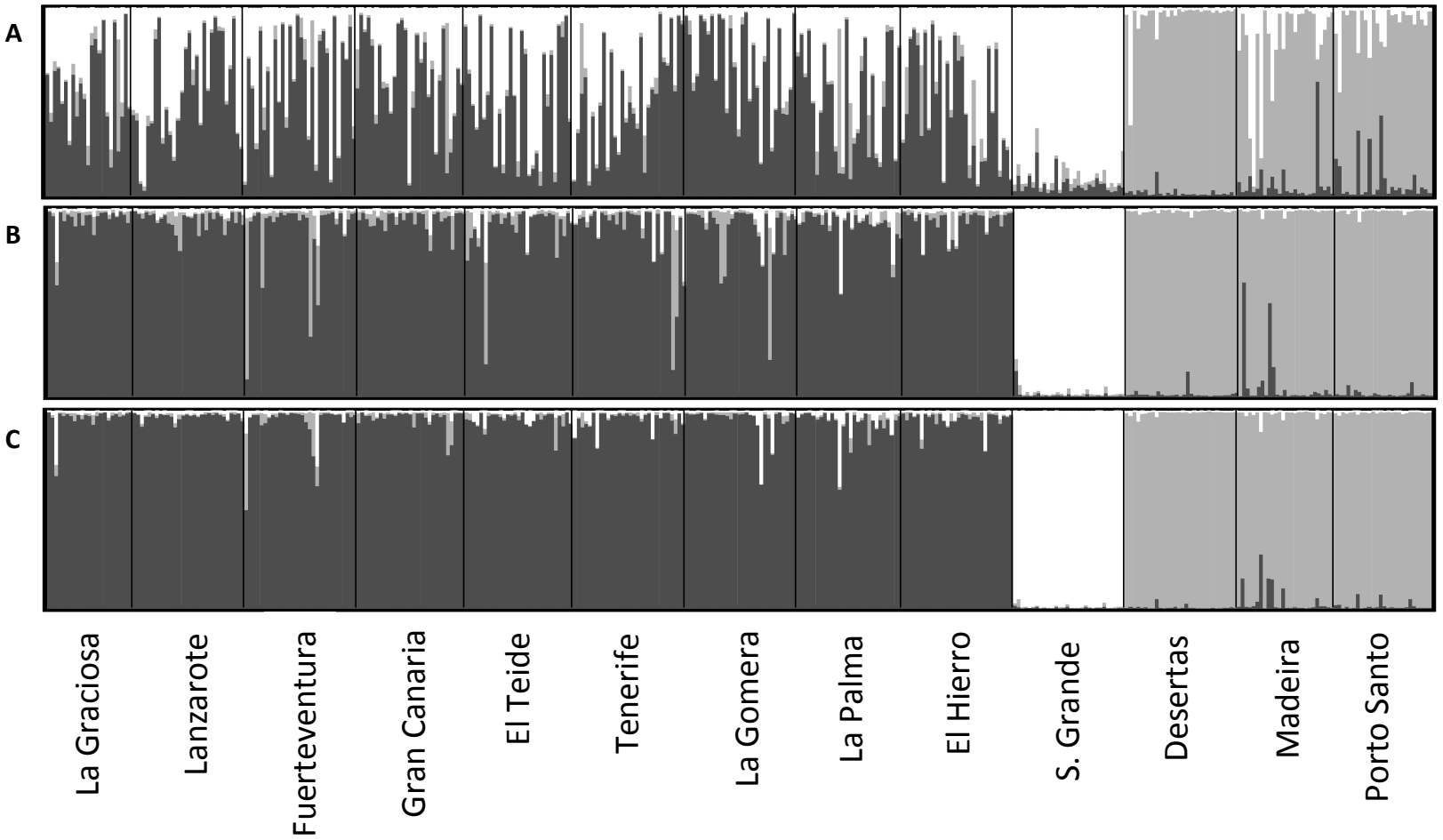

Figure 4.5 Output from a Bayesian clustering algorithm using microsatellite markers with **A** LV, **B** HV and **C** all loci combined, on 371 Berthelot's pipit individuals, implemented in STRUCTURE. Each individual is represented by a single vertical bar, which is divided into *K* = 3 shaded segments, which represent the proportion of variation in each individual attributable to each of the K clusters

Table 4.3 Pairwise $F_{ST}/(1-F_{ST})$ at low variability (below diagonal) and high variability (above diagonal) microsatellite loci in Berthelot's pipit populations. Non-significant comparisons ($P > 0.05$) are highlighted in bold.

	GRA	LZ	FV	GC	TEID	TF	GOM	PAL	EH	SG	M	DG	PS
La Graciosa	-	0.022	0.025	0.026	0.023	0.022	0.022	0.047	0.054	0.390	0.117	0.170	0.182
Lanzarote	0.057	-	0.011	**0**	**0.004**	**0**	**0.006**	**0.003**	0.013	0.319	0.097	0.167	0.130
Fuerteventura	0.042	**0.002**	-	**0.003**	**0.001**	0.014	**0.002**	0.013	0.020	0.316	0.077	0.123	0.130
Gran Canaria	0.088	**0**	0.036	-	**0.003**	0.012	**0**	0.015	0.011	0.338	0.084	0.147	0.119
El Teide	0.043	**0**	**0.012**	**0**	-	**0.006**	**0.007**	0.021	0.025	0.321	0.097	0.163	0.136
Tenerife	0.056	**0.006**	0.020	**0.008**	**0.006**	-	0.011	0.025	0.015	0.242	0.097	0.170	0.125
La Gomera	0.059	0.019	0.045	0.018	**0.011**	0.030	-	0.014	0.022	0.310	0.088	0.130	0.134
La Palma	0.058	**0**	**0.008**	**0.002**	**0**	**0**	0.026	-	0.022	0.241	0.109	0.161	0.135
El Hierro	0.069	0.012	0.029	0.019	0.020	**0**	0.032	**0.012**	-	0.303	0.092	0.182	0.120
Selvagem Grande	0.236	0.224	0.251	0.240	0.216	0.142	0.284	0.184	0.135	-	0.619	0.745	0.633
Madeira	0.204	0.207	0.188	0.231	0.219	0.147	0.247	0.168	0.109	0.197	-	0.054	0.021
Deserta Grande	0.3542	0.335	0.281	0.372	0.360	0.293	0.398	0.280	0.248	0.430	0.057	-	0.103
Porto Santo	0.264	0.254	0.181	0.284	0.276	0.185	0.298	0.194	0.158	0.363	0.051	0.067	-

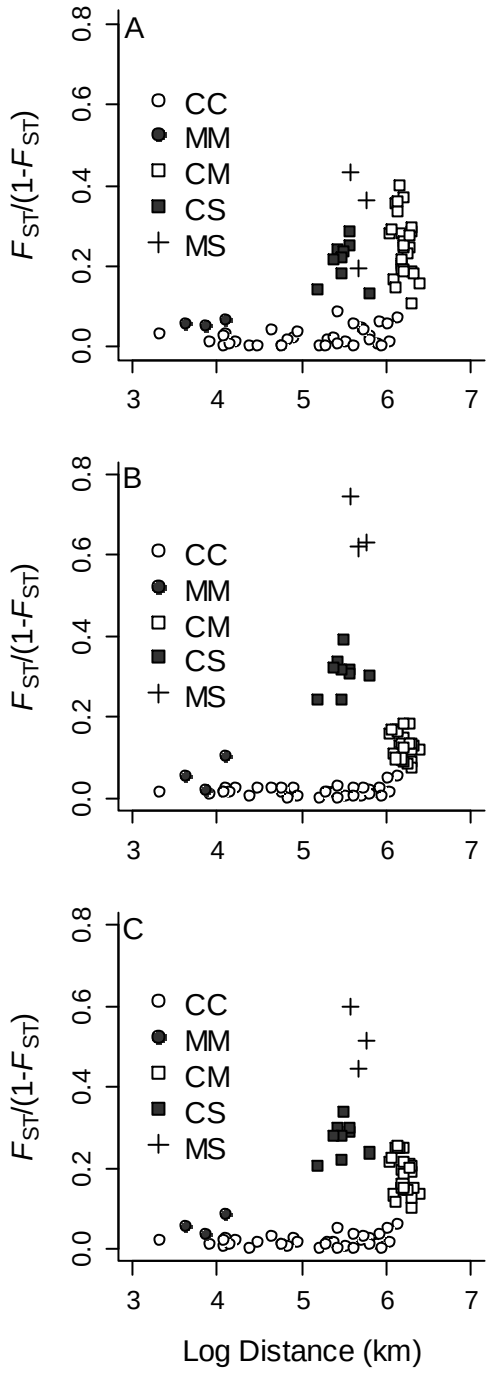

Figure 4.6 Pairwise genetic structure in relation to geographic distance at microsatellite markers in 371 Berthelot's pipit individuals, using; **A** low variability loci, **B** high variability loci and, **C** all loci combined. Different shapes represent pairwise comparisons within and across archipelagos (C = Canary Islands, M = Madeira, S = Selvagens).

4.4.4 Bottleneck tests

There was no evidence for a bottleneck in any of the Canary Island populations, regardless of the test or marker set used (Table 4.4). Tests for heterozygosity excess (Piry *et al.* 1999) strongly suggested a recent reduction in population size in Selvagem Grande at both HV and LV loci (Table 4.4). Similarly, both marker sets suggested evidence of a bottleneck in Deserta Grande, the smallest Madeiran island (Table 4.4). In the other two Madeiran islands there was no significant heterozygosity excess when either marker set was used in isolation, but when all loci were combined there was evidence of a bottleneck in both populations (Table 4.4). Garza and Williamson's (2001) *M* ratio was lowest in Selvagem Grande (Table 4.4). In the Madeiran islands, *M* varied strongly across marker types. There was evidence for a bottleneck in all three populations when the HV loci were used, but in none at the LV loci (Table 4.4). Averaging across all loci resulted in intermediate values of *M* (Table 4.4).

4.5 Discussion

The data from the present study strongly support the previous hypothesis that Berthelot's pipit dispersed northwards from the Canary Islands to Madeira and Selvagens to form its current range (Illera *et al.* 2007). Highest levels of allelic variation were found in the Canary Islands, and almost all allelic variation found in the other two archipelagos was a subset of this (Fig. 4.2). Moreover, of the allelic variation shared between the Canary Islands and the other two archipelagos, most is shared between all three archipelagos (Fig. 4.2), suggesting that Selvagens and Madeira were colonised at similar points in time, perhaps in a single dispersal event. The apparent pattern of isolation-by-distance previously detected in this species (Illera *et al.* 2007) was not detected after controlling for archipelago effects, suggesting that patterns of genetic diversity observed across the pipit populations are the product of very recent founder events and bottlenecks across archipelagos. Thus, contrary to the suggestion from our previous study (Illera *et al.* 2007), the pipit populations do not appear to be in mutation-drift equilibrium. Importantly we found that the effects of the founder events on levels of genetic diversity were not consistent across the two sets of microsatellite markers studied here, with significant implications for the population genetic analyses employed.

Table 4.4 Two tests for genetic bottlenecks in Berthelot's pipit populations: Wilcoxon tests for heterozygote excess (Piry *et al.* 1999) and *M* (Garza and Williamson 2001). Values highlighted in bold are those indicative of a bottleneck ($P \leq 0.05$ for the Wilcoxon tests and $M < 0.68$ for the Garza-William statistic)

	***P* (Heterozygote excess)**			***M***		
	LV	**HV**	**ALL**	**LV**	**HV**	**ALL**
Canary Islands						
La Graciosa	0.98	0.26	0.86	0.78	0.76	0.78
Lanzarote	0.50	0.57	0.54	0.82	0.75	0.78
Fuerteventura	0.65	0.40	0.44	0.89	0.77	0.82
Gran Canaria	0.59	0.72	0.71	0.83	0.82	0.82
El Teide	0.32	0.69	0.58	0.72	0.78	0.75
Tenerife	0.75	0.48	0.58	0.79	0.84	0.82
La Gomera	0.75	0.17	0.33	0.86	0.72	0.78
La Palma	0.90	0.72	0.90	0.87	0.76	0.81
El Hierro	0.27	0.45	0.31	0.75	0.76	0.75
Selvagens						
Selvagem Grande	**0.05**	**0.02**	**0.01**	**0.49**	**0.45**	**0.47**
Madeira						
Madeira	0.10	0.06	**0.02**	0.72	**0.53**	**0.62**
Deserta Grande	**0.03**	**0.03**	**0.01**	0.73	**0.50**	**0.60**
Porto Santo	0.13	0.16	**0.03**	0.71	**0.47**	**0.57**

We utilised two tests designed explicitly to detect bottlenecks, which produced contrasting results across marker sets. Garza and Williamson's (2001) *M* ratio and Cornuet and Luikart's (1996) test for heterozygosity excess have different responses to the age or severity of the bottleneck, with *M* being more likely to retain a signal when bottlenecks are less recent or severe (Spear *et al.* 2006; Swatdipong *et al.* 2010). However, less often considered, at least in empirical studies, is that the signal of a bottleneck at a particular set of loci is a complex trade-off between the age and severity of the bottleneck and marker characteristics. Simulation studies have shown that detecting heterozygosity excess is strongly affected by the number of markers employed (Cornuet and Luikart 1996), and *M* by marker variability (Williamson-Natesan 2005). Our empirical results confirm this: in a previous study with five loci, no evidence for heterozygosity excess was found (Illera *et al.* 2007), and in the present study heterozygosity excess was detected most often when all 22 loci were combined. Similarly, the strongest signal of a bottleneck using *M* was found when marker variability was high. It is crucial that these effects are taken into account when interpreting tests for

bottlenecks. Moreover, by taking the effects of marker characteristics into account when employing tests for bottlenecks, it is possible to make inferences about the nature of the bottleneck itself. Our combined findings (Table 4.4) suggest that in the pipit, the founder bottleneck in Selvagem Grande was strongest and/or most recent: a bottleneck signal was detected using both tests, regardless of the number or variability of loci used. Likewise, our data suggest that in the Madeiran archipelago (particularly in the larger islands of Madeira and Porto Santo) bottlenecks were older and/or less severe, as both tests produced mixed results depending on marker characteristics (Table 4.4).

Highly variable loci are, by their very nature, susceptible to disproportionate reductions in allelic richness during bottlenecks, which can increase levels of population structure across bottlenecked populations (Hedrick 1999). Accordingly, in our study we found that reductions in allelic richness were less severe at LV than HV loci. Therefore it may be that the patterns of population structure observed at LV loci better represent divergence time between populations, while those at HV loci reflect the history of bottlenecks. In the Berthelot's pipit, similar levels of structure were observed between archipelagos at LV loci, a finding consistent with the hypothesis of the colonisation of Madeira and Selvagens at similar points in time (Fig. 4.6A). In contrast, at the HV loci elevated levels of population structure were observed in comparisons involving Selvagens (Fig. 4.6B), the most severely bottlenecked population (Table 4.4). Moreover, HV loci were considerably more effective at defining genetic groups (Fig. 4.5). It therefore appears that the two marker sets, with their different mutation rates, reflect different aspects of population history, and can be utilised to answer different questions within the same system.

We found that one locus (*TG01-124*), linked to a gene involved with neurological function, was a clear F_{ST} outlier (Fig. 4.1). This marker had extremely low levels of heterozygosity in the Canarian founder populations, but high levels of heterozygosity in the more recently colonised Madeiran archipelago; the opposite pattern to that seen at other markers (Appendix 1). The most likely explanation for this, given that no evidence for null alleles was found, is that *TG01-124* has undergone a selective sweep in the Canary Islands or, alternatively, that purifying selection was relaxed in the Madeiran islands after colonisation. Determining the cause of selection at loci where the function is not

completely understood is difficult (Ford 2002), and beyond the scope of this study. That only one locus was found to be under selection, combined with the lower levels of diversity at the LV loci, is consistent with the prevailing view that purifying, rather than positive selection is the predominant force acting on mutations at EST-linked microsatellites (Ellis and Burke 2007). Nonetheless, if the proportion of loci under selection is similar to the present study, microsatellites mined from EST databases may be a useful source for searching for functional polymorphisms in non-model organisms (see also Vasemägi *et al.* 2005).

4.6 Conclusions

Examining patterns of genetic variation at markers with different mutation rates can provide new understanding into different evolutionary processes. Researchers using sequence data, for example, take full advantage of the different mutation rates in mitochondrial and nuclear genes to uncover both fine-scale and deep evolutionary relationships between groups of organisms (Hillis and Dixon 1991; Zhang and Hewitt 2003).Here we have found that, in recently separated populations, using sets of microsatellites with systematic differences in levels of variability may be useful in a similar manner. Utilising the variation in properties of microsatellites obtained using different methods may therefore prove to be a promising approach. Population genetic studies usually select microsatellites with high levels of heterozygosity (often 0.8 or higher; Meirmans and Hedrick 2011). Our results suggest that markers with lower levels of variability (H_S of 0.3-0.5) may also be informative, albeit in a different manner. At the least, we suggest that if microsatellites are to be used in studies of population history, explicitly accounting for how marker properties are expected to affect analyses will considerably improve the quality of interpretation.

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Chapter 5: Genetic differentiation in non-equilibrium populations: an empirical evaluation of G_{ST} and D

A version of this chapter has been submitted for publication in *Evolution*

A Berthelot's pipit in the hand

5.1 Abstract

Measuring and interpreting genetic differentiation across populations remains a central issue in evolutionary biology. Traditional measures of differentiation (F_{ST}, G_{ST} and their relatives) have recently been criticised, and a new measure, *D,* has been put forward as an alternative. Simulations have shown that G_{ST} and *D* are both affected by marker variability, but respond differently to migration and drift in non-equilibrium conditions. Here we provide the first empirical assessment of G_{ST} and *D.* We screened 13 Berthelot's pipit populations at 21 microsatellite loci with wide-ranging heterozygosity. We found that first, relative levels of G_{ST} and *D* were strongly dependent on marker variability, with G_{ST} higher when heterozygosity was low and *D* higher when heterozygosity was high. Second, pairwise estimates of G_{ST} and *D* both ranked populations in different orders depending on the variability of markers used. Third, relative levels of G_{ST} and *D* revealed a history of recent bottlenecks in Berthelot's pipit. Our results highlight that marker variability must be taken into account when calculating summary statistics of differentiation. If this is done, comparing G_{ST} and *D* across populations may provide greater insight than either measure in isolation.

5.2 Introduction

Genetic differentiation between subpopulations has traditionally been measured as F_{ST} (Wright 1931) or one of a number of related measures (e.g. Nei 1973; Weir and Cockerham 1984; Slatkin 1995). These statistics can be used to estimate migration rates (Wright 1931), infer demographic history (Slatkin 1991), identify genetically distinct subpopulations of conservation interest (Hedrick and Parker 1998) and identify regions of the genome that have been subject to positive selection (Beaumont and Nichols 1996). As a result they are among the most widely used summary statistics in population and evolutionary genetics (see Holsinger and Weir 2009 for an overview).

A criticism of F_{ST} is that because it is constrained by within-population heterozygosity, it is likely to underestimate differentiation (Charlesworth 1998; Hedrick 2005). This problem is most easily visualised by looking at Nei's (1973) $G_{ST,}$ which was developed to account for multiple alleles and is highly similar to F_{ST}:

$$G_{\mathrm{ST}} = \frac{H_{\mathrm{T}} - H_{\mathrm{S}}}{H_{\mathrm{T}}}$$

where H_T is expected heterozygosity across the total population and H_S is average heterozygosity within subpopulations. It is clear that as H_S increases, the maximal value of G_{ST} is reduced. This is a problem for studies empirically evaluating differentiation, especially when highly polymorphic markers such as microsatellites are used, as the true level of differentiation will be underestimated (see for example O'Reilly *et al.* 2004). In fact, G_{ST} can be low even when calculated across two populations with non-overlapping sets of alleles (Charlesworth 1998; Jost 2008). Recently Jost (2008) proposed an alternative measure of allelic differentiation, which can be defined in terms of $H_{T,}$ H_S and the number of subpopulations n:

$$D = \left(\frac{n}{n-1}\right)\left(\frac{H_{\mathrm{T}} - H_{\mathrm{S}}}{1 - H_{\mathrm{S}}}\right)$$

Jost (2008) showed that D, unlike G_{ST}, is mathematically independent from H_S and as a result provides a much better measure of allelic differentiation between populations.

Since the publication of Jost's (2008) paper many empirical studies have assessed population differentiation using D (e.g. White et al. 2010; Alberto et al. 2011; Edelaar et

al. 2012; Kerth and Van Schaik 2012). Meta-analyses have shown that D is generally higher than G_{ST} in natural populations, suggesting that G_{ST} does indeed under-estimate differentiation (Heller and Siegismund 2009). However, theoretical and simulation studies on G_{ST} and D suggest that relationship between the two measures depends on a range of factors, including marker mutation rate, population size, migration rate and time since divergence (Jost 2009; Ryman & Leimar 2009; Whitlock 2010; Leng & Zhang 2011; Meirmans & Hedrick 2011). Importantly, by examining how and where G_{ST} and D differ across a set of populations, it may be possible to make biological inferences that would not be possible based on either measure in isolation (Leng and Zhang 2011). For example, a recent simulation study of G and D in non-equilibrium populations showed that D is generally expected to be higher than $G_{ST,}$ except when mutation rate is low, when populations are in the early stages of differentiation, or when the effective population size is small (Leng and Zhang 2011). To our knowledge, however, no empirical studies to date have explored how differences in G_{ST} and D can inform population history.

The aim of this chapter is to use the pipit system as a model to empirically evaluate how G_{ST} and D behave in non-equilibrium populations. Using a panel of 21 microsatellites with wide-ranging levels of variability, we test how global values of D and G_{ST} at each locus are related to marker variability. We then divide the markers into 'high variability' and 'low variability' subsets (see chapter 4) and test whether average levels of differentiation are consistent across the two marker sets. Finally, we calculate pairwise values of G_{ST} and D at the two marker sets and test how the measure of differentiation used, and its interaction with marker variability, affects estimates of levels of differentiation across populations. We investigate and discuss how differences in G_{ST} and D can be used to inform population genetic studies.

5.3 Materials and Methods

5.3.1 Sampling and molecular procedures

Representative samples (*c.* 30 individuals) were obtained from all 13 Berthelot's pipit populations (see section 3.3 for full details). For this chapter, only samples from 2006 were used (see chapter 7 for discussion). DNA was extracted from blood samples using a modified salt extraction method (Richardson *et al.* 2001) and diluted to 10-50 ng/µl. All

individuals were genotyped at 21 microsatellite markers arranged into five multiplex reactions (see chapter 4).

Nine of the loci used were developed on the basis of having a high degree of sequence homology between the zebra finch, *Taeniopygia guttatta*, expressed sequence tag (EST) database and the chicken *Gallus gallus* genome. These '*TG*' loci are therefore highly conserved across passerine birds (Dawson *et al.* 2010). Compared to other conserved microsatellite markers, on average, the *TG* loci are less polymorphic across bird species, including the Berthelot's pipit (Dawson *et al.* 2010; Dawson *et al.* Unpublished data; see chapter 4). We therefore classified the *TG* loci *a priori* as 'low variability' (LV). The remaining loci come from other, less conserved marker sets (see chapter 4).

5.3.2 Analyses

Unless stated otherwise, all statistics and plots were generated using R version 2.14.1 (R Development Core Team 2008). For each microsatellite locus and population, HWE and linkage disequilibrium were tested using Genepop version 4.1 (Raymond and Rousset 1995) and null allele frequencies were estimated using CERVUS version 3.0.3 (Marshall *et al.* 1998).

Global and pairwise values of G_{ST} and D_{EST} (the unbiased estimator of D; Jost 2008) were calculated using the MMOD package in R (https://github.com/dwinter/mmod). Each measure of differentiation was averaged across all loci, and separately across HV and LV loci. Jost's D was designed as a single locus measure, but has been adapted for multiple loci by taking the harmonic mean of locus-specific values of D_{EST} (Crawford 2010), or by averaging Hs across loci and calculating D_{EST} based on this (Meirmans and Hedrick 2011). We calculated average D_{EST} using both these methods. Confidence intervals were obtained using the bootstrapping approach developed by Chao *et al.* (2008).

We visualised the overall relationship between variability at each microsatellite locus, D_{EST} and G_{ST} by plotting D_{EST}/G_{ST} against H_S (Meirmans and Hedrick 2011). The minimum (H_T = 1) and maximum ($H_T = H_S$) possible values of D/G_{ST} were calculated for $0 \leq H_S \leq 1$ and n = 13 populations (using equations 3 and 11 from Jost 2008). We then tested separately

whether each measure of differentiation was related to marker polymorphism using linear and polynomial regression. Marker polymorphism was measured as both allelic richness and expected heterozygosity across islands. Relationships between pairwise patterns of differentiation were tested using Mantel tests, implemented in the Ecodist package in R (Goslee and Urban 2007).

5.4 Results

In total, 371 individuals were genotyped at the 21 microsatellite loci. None of the loci were in HW disequilibrium in more than two populations, and none were in linkage disequilibrium after correction for multiple tests. The loci exhibited marked variation in levels of variability across populations, with H_S ranging between 0.04 and 0.81, and this was reflected in the relative levels of G_{ST} and $D_{EST,}$ which varied by orders of magnitude (Fig. 5.1A). G_{ST} was higher than D_{EST} at loci with low levels of $H_{S,}$ whereas D_{EST} was higher than G_{ST} at loci with high H_S (Fig. 5.1A).

There was a positive linear relationship between D_{EST} and marker variability, measured as H_S (R^2 = 0.72, d.f. = 19, P < 0.001; Fig. 5.1B). This was not the case for G_{ST} (R^2 = 0.002, d.f. = 19, P = 0.82) which was instead highest at intermediate values of H_S (Fig. 5.1C). Indeed, a significant relationship between G_{ST} and H_S was detected when a second-order polynomial regression was fitted (R^2 = 0.32, d.f. = 18, P = 0.01). D_{EST} was also positively related to allelic richness, though G_{ST} was not (D_{EST}: R^2 = 0.77, d.f. = 19, P < 0.001; G_{ST} (linear): R^2 = 0.02, d.f. = 19, P = 0.53; G_{ST} (polynomial): R^2 = 0.09, d.f. = 18, P = 0.28).

Heterozygosity was lower at *a priori* defined LV than at HV loci (mean H_S: LV loci = 0.37, HV loci = 0.52; Table 5.1). When averaged over all loci, global estimates of G_{ST} and D_{EST} were almost identical (Fig. 5.2A; Table 5.1). However, when averaged across LV and HV separately, the two measures of differentiation differed, and the direction of this difference depended on the class of markers used: at LV loci, G_{ST} was significantly higher than $D_{EST,}$ but at HV loci G_{ST} was significantly lower than D_{EST} (Fig. 5.2A; Table 5.1). These patterns were only observed when D_{EST} was calculated based on average H_S; when harmonic mean was calculated, D_{EST} was always very low (LV loci = 0.006, HV loci = 0.05, all loci combined = 0.012; Table 5.1).

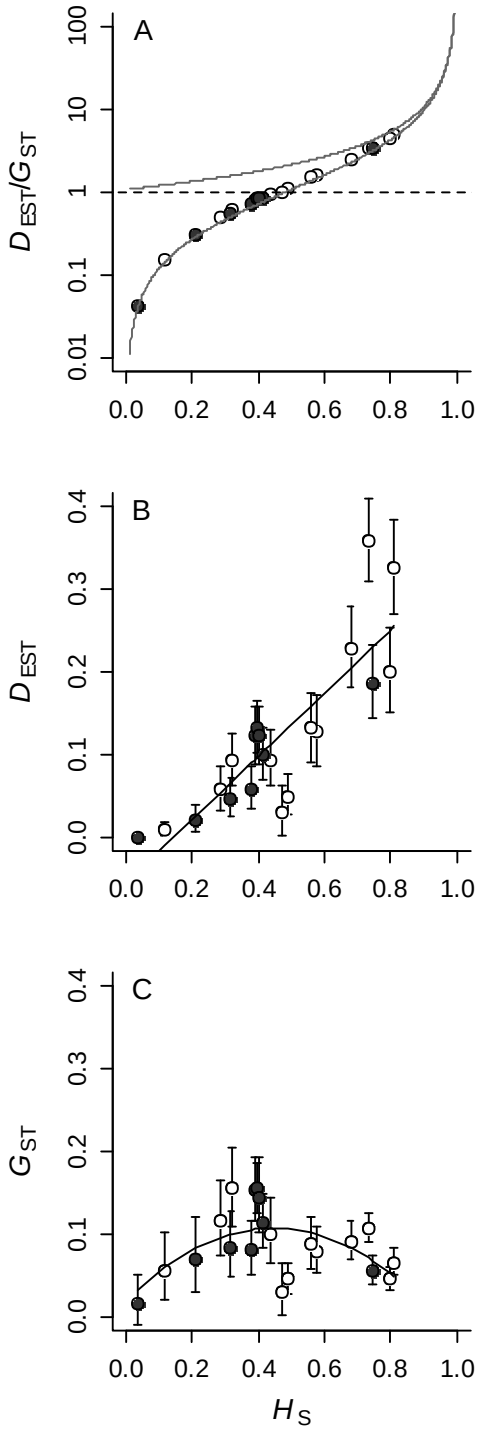

Figure 5.1 Genetic differentiation in relationship to marker variability at low variability (grey dots) and high variability (white dots) microsatellite loci across 13 Berthelot's pipit populations (371 individuals). **A** Observed levels of D_{EST}/G_{ST} in relation to H_S. The solid lines represent lower and upper limits of D/G_{ST} ($H_T = H_S$ and at $H_T = 1$, respectively), when the number of subpopulations (n) = 13. The dotted line represents the relationship $D_{EST} = G_{ST}$. **B** D_{EST} in relation to H_S, with a line fitted from a linear regression. **C** G_{ST} in relation to H_S, with a line fitted from a second-order polynomial regression. Error bars represent 95% confidence intervals.

Table 5.1 Genetic differentiation at low variability (LV) and high variability (HV) microsatellite loci across 13 Berthelot's pipit populations (371 individuals). Values in brackets are 95% confidence intervals. D_{EST} was averaged across loci by averaging within-population heterozygosity (Hz) and by taking the harmonic mean of single-locus estimates of D_{EST} (H-mean; see text for details).

	H_S	G_{ST}	D_{EST} (Hz)	D_{EST} (H-mean)
LV loci	0.366	0.105 (0.094-0.118)	0.074 (0.066-0.083)	0.006 (-0.004-0.02)
HV loci	0.523	0.080 (0.072-0.088)	0.103 (0.090-0.111)	0.050 (0.026-0.072)
All loci	0.456	0.089 (0.082-0.097)	0.088 (0.081-0.095)	0.012 (0.003-0.029)

Pairwise values of D_{EST} and G_{ST} were strongly related at both LV (Mantel test, $R = 0.96$, $P < 0.001$; Fig. 5.2B) and HV loci ($R = 0.98$, $P < 0.001$; Fig. 5.2C). Importantly however, the relative levels of differentiation across archipelagos differed depending on the marker set and measure of differentiation used. At HV loci, D_{EST} was consistently higher than G_{ST}, but the two measures ranked levels of differentiation across archipelagos in the same order, with highest levels of differentiation in comparisons involving the Selvagens (Fig. 5.2B). However at the LV loci D_{EST} was higher than G_{ST} in comparisons within and across the Canary Islands and Madeira, but lower in comparisons involving the Selvagens. As a result the two measures did not rank levels of differentiation across archipelagos in the same order (Fig. 5.2C).

5.5. Discussion

Theoretical and simulation studies examining how G_{ST} and D behave under different scenarios of mutation, drift and gene flow have warned that the two measures are strongly affected by the mutation rate of markers, and in different ways (Ryman and Leimar 2009; Leng and Zhang 2011). We provide the first empirical demonstration of the strong relationships between G_{ST}, D and marker variability within a single system. Our results are consistent with the theory and simulations, and highlight some of the potential pitfalls - and opportunities - associated with investigating differentiation using these measures in wild populations.

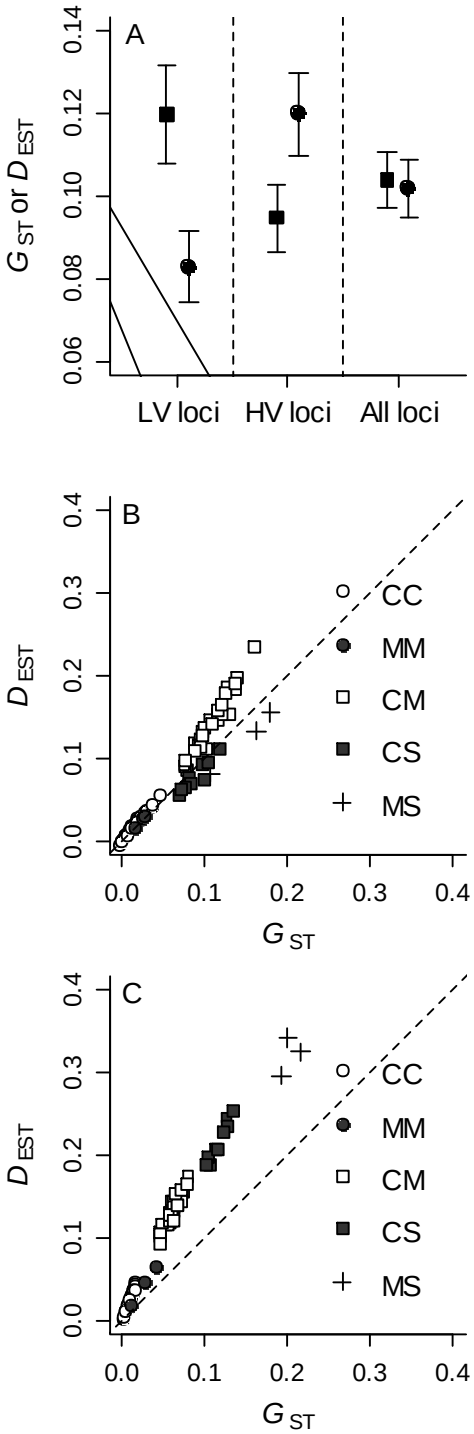

Figure 5.2 Average G_{ST} and D_{EST} across 13 Berthelot's pipit populations (371 individuals) at low (LV) and high variability (HV) microsatellites. **A** Mean G_{ST} (squares) and D_{EST} (circles) across all populations. Error bars represent 95% confidence intervals obtained by booststrapping. The relationship between pairwise G_{ST} and D_{EST} at LV (**B)** and HV (**C)** markers. The dotted line represents the relationship D_{EST} = G_{ST}. Coloured shapes represent comparisons within and across the different archipelagos (C = Canary Islands, M = Madeira, S = Selvagens).

In a recent meta-analysis Heller and Siegismund (2009) showed that D was higher than G_{ST} in all but two of 34 studies, and concluded that G_{ST} underestimates population differentiation. In an extended meta-analysis, Meirmans and Hedrick (2011) showed that D is only higher than G_{ST} when H_S is high, as is usually the case with microsatellites; however when H_S is low, the opposite is true. In our empirical study we also found this to be the case, with relative levels of G_{ST} and D_{EST} at individual loci varying by orders of magnitude, depending on marker variability (Fig. 5.1A). However in contrast to the previous meta-analyses, which found a negative relationship between H_S and both D and G_{ST}, we found that D_{EST} was positively related to H_S and that G_{ST} was highest at intermediate values of H_S (Fig. 5.1B and 5.1C). A decrease in G_{ST} at high H_S is consistent with evidence from theoretical and empirical studies (O'Reilly et al. 2004; Meirmans and Hedrick 2011), and suggests that G_{ST} does indeed underestimate differentiation when marker variability is high. The quadratic relationship we observed between G_{ST} and Hs has most likely arisen because we used a few markers with very low H_S (Fig. 5.1A), something which few other studies have done. Likewise, although D is mathematically independent of H_S, theoretical and simulation studies have shown that D is also expected to be strongly affected by mutation rate, which may lead to a correlation between D and H_S (Ryman and Leimar 2009; Leng and Zhang 2011). To our knowledge, our findings represent the first empirical example of a positive relationship between D and marker variability. Jost (2009) has argued that a positive relationship between D and marker variability is to be expected from a true measure of allelic differentiation, which should be influenced not only by migration and drift, but also mutation. However, as others have pointed out, evolutionary biologists are rarely interested in the mutational properties of markers *per se,* but instead in using neutral markers to infer patterns of demography or population history within and across populations (Whitlock 2010; Meirmans and Hedrick 2011). Thus, the observed relationships between G_{ST}, D_{EST} and Hs pose a significant problem for most population genetic studies. In special cases where allelic differentiation itself is of interest, D will be useful, for example when examining how adaptive genetic variation (e.g. at genes of the vertebrate major histocompatibility complex) is partitioned across populations of conservation concern (Sommer 2005; see also chapter 2).

Because G_{ST} and D are influenced by marker variability, it has been suggested that comparing levels of differentiation across species or marker sets is difficult (Ryman and Leimar 2009; Whitlock 2010). We detected strong differences in both measures of differentiation when used with sets of microsatellites with systematic differences in levels of variability, with D higher at HV markers and G_{ST} higher at LV markers (Fig. 5.2A). If such pronounced differences can arise within a single species and class of marker, directly comparing levels of differentiation across markers (e.g. microsatellites and SNPs) is largely meaningless. Similarly, comparisons of differentiation across species will be extremely difficult to interpret unless the effects of marker variability and ascertainment bias can be ruled out. The method by which multi-locus measures of differentiation are calculated is also important. Jost's D was designed as a single-locus measure (Jost 2008), though a multi-locus measure can be obtained by either taking the harmonic mean of single locus estimate of D (Crawford 2010), or by averaging H_S across loci (Meirmans and Hedrick 2011). Most empirical studies to date have used the harmonic mean (e.g. Portnoy *et al.* 2010; Rutledge *et al.* 2010; Schrey *et al.* 2011); however, our findings suggest that if there is large variation in locus-specific estimates of D, this method will drastically underestimate average differentiation across loci (Table 5.1). We therefore suggest that future studies averaging D across loci do so by calculating both the harmonic mean and heterozygosity-based methods, and interpret differences accordingly.

Within-population heterozygosity at neutral loci is affected by a combination of mutation, migration and drift. As a result of the simple relationship between G_{ST}, D and H_S, comparing relative levels of G_{ST} and D across multiple populations at the same set of loci may shed light on past and/or present evolutionary processes. Recent simulations have shown that G_{ST} is expected to be higher than D only when mutation rate is low, when populations are recently separated or when the effects of drift (either gradual or due to recent bottlenecks) are most pronounced (Leng and Zhang 2011). We found that G_{ST} was only higher than D in pairwise comparisons involving the Selvagens archipelago, and only at LV loci. This is consistent with our previous detection of a bottleneck in the Selvagens (see chapter 4), and suggests the differences in G_{ST} and D can be used to make inferences about the evolutionary history of populations.

Novel coalescent and Bayesian approaches can estimate genetic structure, migration rates, recombination rates, effective population sizes and test hypotheses of colonisation history, while taking mutation rate into account (Pritchard et al. 2000; Beerli and Felsenstein 2001; McVean et al. 2002; Cornuet et al. 2008). Some have argued that with the development of these approaches, summary statistics such as F_{ST}, G_{ST} and D are increasingly of limited value (Pearse and Crandall 2004). However, these new approaches still make (often unrealistic) assumptions, such as mutation-drift equilibrium, and due to their complexity can be misleading when not properly understood and/or when assumptions are violated (Faubet et al. 2007; Kuhner 2009; Karl et al. 2012). In contrast it is much easier to quantify how simple summary statistics behave in different ecological and evolutionary scenarios, and interpret them accordingly (Holsinger and Weir 2009). G_{ST} and D are therefore (in our opinion rightly) going to continue be used in population genetic studies. However, it is crucial that these measures are interpreted in light of the variability of the markers being used, and the many mutational, ecological and evolutionary forces that influence them.

5.6 References

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Chapter 6: Gene conversion rapidly generates major histocompatibility complex variation in island bird populations

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Beach at Porto Santo

6.1 Abstract

Population bottlenecks can restrict variation at functional genes, reducing the ability of populations to adapt to new and changing environments. Understanding how populations generate adaptive genetic variation following bottlenecks is therefore central to evolutionary biology. Genes of the major histocompatibility complex (MHC) are ideal models for studying adaptive genetic variation due to their central role in pathogen recognition. While *de novo* MHC sequence variation is generated by point mutation, gene conversion can generate new haplotypes by transferring sections of DNA within and across duplicated MHC loci. However, the extent to which gene conversion generates new MHC haplotypes in wild populations is poorly understood. We developed a 454 sequencing protocol to screen MHC class I exon 3 variation across all 13 island populations of Berthelot's pipit. We reveal that just 11–15 MHC haplotypes were retained when the Berthelot's pipit dispersed across its island range in the North Atlantic *ca*. 75 000 years ago. Since then, at least 26 new haplotypes have been generated *in situ* across populations. We show that most of these haplotypes were generated by gene conversion across divergent lineages, and that the rate of gene conversion exceeded that of point mutation by an order of magnitude. Gene conversion resulted in significantly more changes at nucleotide sites directly involved with pathogen recognition, indicating selection for functional variants. We suggest that the creation of new variants by gene conversion is the predominant mechanism generating MHC variation in genetically depauperate populations, thus allowing them to respond to pathogenic challenges.

6.2 Introduction

Reductions in population size can result in loss of diversity at functional genes, compromising the ability of populations to adapt (Mayr 1965; Dlugosch & Parker 2008). While populations with low levels of genetic variation may be able to persist in stable environments, loss of adaptive genetic diversity is likely to be especially important in scenarios where changes in habitat, climate or pathogens occur (Lande & Shannon 1996). Following a population bottleneck, novel genetic diversity may be introduced by migration or mutation. In isolated populations where migration rates are low, mutation may be the only mechanism for generating new variation. Understanding how mutation generates adaptive genetic diversity in isolated populations is therefore central to evolution, and given the role of such diversity in the persistence of populations, is key from a long-term conservation perspective.

Vertebrate major histocompatibility complex (MHC) genes are arguably the best studied examples of how selection operates at the genetic level. The MHC genes code for cell surface glycoproteins that present pathogenic peptides to cells of the immune system, and so play a direct role in pathogen defence (Klein 1986). These genes display extraordinary levels of polymorphism, often with hundreds of alleles at individual loci (Garrigan & Hedrick 2003). Patterns of MHC variation are directly associated with pathogen resistance (Doherty & Zinkernagel 1975; Ilmonen et al. 2007; Oliver et al. 2009) and survival (Brouwer et al. 2010; Worley et al. 2010), suggesting that pathogen-mediated natural selection is the predominant force driving MHC diversity (reviewed in Jeffery & Bangham 2000; see also chapter 2). MHC-based mate choice (Penn 2002; Richardson et al. 2005) and selection against deleterious mutations at MHC-linked regions (e.g. van Oosterhout 2009) can operate alongside pathogen-mediated selection to maintain MHC variation, and a large body of research has been devoted to establishing the relative roles of these processes (Apanius et al. 1997; Piertney & Oliver 2005).

Demographic processes can also play an important role in regulating genetic diversity at the MHC (Alcaide 2010). In small, isolated populations, the effect of genetic drift can override the effects of selection at MHC loci, leading to depletion in diversity (e.g. Hedrick et al. 2001; Miller & Lambert 2004; Seddon & Ellegren 2004). Such reductions in MHC

variation may compromise the ability of populations to adapt to new and rapidly evolving pathogens, although establishing direct links between reduced MHC variation, and population viability has proved difficult (Edwards & Potts 1996; Radwan et al. 2010). Even less well understood is how, and at what rate, populations are able to recover from the reduced MHC variation caused by reductions in population size over evolutionary timescales. These are questions of fundamental importance for conservation biologists, and of intrinsic interest to molecular biologists, but are difficult to address, and so have received comparatively little attention.

New MHC sequence variation is generated by point mutation, with positive selection resulting in an excess of amino acid changing substitutions in the peptide-binding region (PBR)—the part of the molecule directly involved with pathogen recognition (Hughes & Nei 1988). Gene conversion-like processes (sometimes referred to as 'micro-recombination'; Geliebter & Nathenson 1988; hereafter referred to as 'gene conversion') can also generate new haplotypes, by shuffling existing MHC variation within or across duplicated loci (Ohta 1991). Gene conversion can occur between alleles within loci, or across duplicated loci, and is therefore an important feature of multigene families such as the MHC. Gene conversion may be particularly important for regenerating MHC haplotype variation in bottlenecked populations, as the rate at which it can do so may greatly exceed that of point mutation (Parham & Ohta 1996). The occurrence, and to some extent, the mechanics of gene conversion, have been documented (Baltimore 1981; Schulze et al. 1983; Reusch & Langefors 2005; Chen et al. 2007), including in the avian MHC (Wittzell et al. 1999; Burri et al. 2010). However, the ability of gene conversion to generate MHC variation relative to point mutation is still highly controversial, primarily due to a lack of empirical data from natural populations (Martinsohn et al. 1999; Klein et al. 2007). Moreover, statistical methods for detecting gene conversion lack power (Mansai & Innan 2010), and it can be difficult to separate gene conversion from convergent accumulation of point mutations due to selection (Kriener et al. 2000; Sato et al. 2011).

Here, we use the Berthelot's pipit as a model to study how MHC genes evolve in the early stages of differentiation. We first test whether MHC diversity in Berthelot's pipit has been

reduced to a similar extent as the neutral diversity as a result of the founder event. We then test whether novel MHC haplotype variation has been generated in situ post-colonization. Finally, we quantify the relative roles of point mutation and gene conversion in generating new MHC haplotypes in the pipit populations.

6.3 Methods

6.3.1 Sampling

Representative samples (*c.* 30 individuals) were obtained from all 13 Berthelot's pipit populations (see section 3.3 for full details). For this chapter, only samples from 2006 were used.

6.3.2 Molecular procedures

Genomic DNA was extracted from blood using a salt extraction method (Richardson et al. 2001), and diluted to 10–50 ng/µL. Degenerate fusion primers based on those designed by Westerdahl et al. (2004; DG2, TTGCGCTCYAGCTCYTTCTGCT; GENDG, TCCCCACAGGTCTCCACAC), incorporating the Roche 454 FLX adapter sequences were used to amplify 240 bp (out of 270 bp) of the MHC class I exon 3 locus of each individual. Exon 3 contains sites which code for the PBR of the class I molecule, and variation at these sites is likely to be directly associated with immune defence (Hughes & Yeager 1998).

Initial amplification and cloning was performed on five individuals from Tenerife and one from Madeira, using the amplification and cloning protocols outlined in Westerdahl et al. (1999). Twelve alleles were found, all of which were confirmed by subsequent 454 sequencing (Table 6.1). Between four and seven alleles were detected per individual, indicating that a minimum of four loci were present (Table 6.1). This is concordant with the number of class I loci found in other passerine birds (Richardson & Westerdahl 2003; Promerováet al. 2009).

We used population-level tagged primers and 454 sequencing to screen population-level MHC variation. Thirteen variants of the MHC primers were designed, each with a 7-bp

population-specific tag at the 5' end. To reduce the risk of sequencing errors, tags were preceded with a 'CCD' (D = G, A or T) motif, stretches of the same nucleotide longer than two were avoided, and all tags differed by at least 2 bp (Valentini et al. 2009). With primers and tags included, the amplicon was longer than the average read length of the 454 sequencer (the total target amplicon length including all primers and tags was 322 bp). Both forward and reverse primers were tagged, so that, amplicons could be sequenced using bi-directional beads and the exon could be re-assembled using the read overlap. Each individual within a population was amplified in a separate PCR using the same population tagged primer. The PCRs were performed in 15 µL reactions, containing 7.5 µL 2× Thermoprime PCR mastermix (ABgene), 2 µL each primer, 1.5 µL H2O and 2 µL template DNA, under the following conditions: initial denaturation at 96 °C for 3 min, followed by 27 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 60 s, with a final extension stage of 72 °C for 10 min. The reduced number of cycles and long extension time were used to reduce the formation of chimeric sequences (Lenz & Becker 2008). The PCR products were purified using MinElute PCR Purification Kits (Qiagen). Concentrations were assessed using 1.2% agarose gels and a NanoDrop ND-1000 spectrophotometer.

Table 6.1 Sequences obtained from amplifying and cloning duplicated MHC class I exon 3 loci from individual Berthelot's pipits from Tenerife (TF) and Madeira (M). All sequences were confirmed by subsequent 454 sequencing (see main text).

TF1	TF2	TF3	TF4	TF5	M1
ANBE 10	ANBE 10	ANBE 28	ANBE 10	ANBE 10	ANBE 10
ANBE 2	ANBE 9	ANBE 8	ANBE 8	ANBE 8	ANBE 9
ANBE 8	ANBE 8	ANBE 24	ANBE 6	ANBE 4	ANBE 8
ANBE 24	ANBE 6	ANBE 2	ANBE 2	ANBE 2	ANBE 23
	ANBE 19				ANBE 2
	ANBE 2				
	ANBE 4				

Amplicons from all individuals from a given population were equalized and pooled. The mixed amplicons from each separate population were then equalized and pooled into one overall sample containing all populations and individuals. The final sample was sequenced using bi-directional sequencing on half a 454 FLX plate.

6.3.3 Identification of MHC haplotypes

Forward and reverse reads corresponding to each of the 13 population-specific tags were extracted using an in-house Perl script, and grouped into the populations. The reads from each population were assembled into contigs with the Roche 454 Newbler assembler version 2.3, using highly stringent settings (99% minimum overlap identity and 200 bp minimum overlap length). Most short and low frequency reads, which may represent PCR and sequencing artefacts (Babik et al. 2009; Galan et al. 2010), would not group into contigs using this procedure. Nonetheless, some may, and so contigs shorter than 220 bp and low frequency contigs (see below) were excluded. If each individual in a population (30 individuals) had eight different haplotypes (based on the estimate of four duplicated loci), the frequency of a single haplotype copy in the population would be ~0.4%. To minimize the possibility of missing very rare haplotypes, the identified haplotypes from each population were mapped back to the discarded contigs from all other populations, with the aim of identifying haplotypes that had been verified in some populations, but discarded in others.

6.3.4 Founder population size and drift

We developed a simple simulation to establish the minimum number of founder individuals required to introduce the observed number of MHC haplotypes, using a macro developed in Minitab version 12.1 (Minitab Inc.; available from the author on request). The rationale behind this simulation was that if the number of founder individuals required to introduce the total observed number of haplotypes is much larger than is plausible based on the mitochondrial diversity, then it is likely that some MHC variation has evolved *in situ*, post-colonization.

The simulation re-sampled eight haplotypes per individual (based on four duplicated loci) from the contemporary gene pool 1000 times, increasing the number of individuals until the observed number of MHC haplotypes occurred in 1%, 50% and 99% of simulations. The simulations assumed equal sex ratio and no genetic drift after the founder event. Although the second assumption is unrealistic, it will result in an underestimation of the number of founding individuals required. As our aim was to simulate the minimum

number of founding individuals required to introduce all MHC variation, it can be considered conservative.

The effect of genetic drift was examined by comparing population size with allelic richness at both the MHC and at the five microsatellite loci amplified by Illera et al. (2007) using linear regression.

6.3.5 Sequence analyses

To visualize the time frame over which MHC loci have been diverging across populations, it is necessary to obtain an estimate of how long the populations have been separated. To do so, time to most recent common ancestor (TMRCA) of the pipit mitochondrial cytochrome *b* sequences from Illera et al. (2007) was estimated using beast version 1.6.1 (Drummond & Rambaut 2007). Two independent MCMC analyses of 10,000,000 steps, each with a burn-in of 1,000,000 steps, were performed. We used the HKY model of nucleotide substitution (Hasegawa et al. 1985) and a rate of 0.01 substitutions per site per million years, corresponding to a divergence rate of 2% per million years. Convergence of the chains to a stationary distribution was assessed with Tracer version 1.4. Mutation rates at passerine mitochondrial genes can vary, and 2% per million years is likely to be an underestimate (Nabholz et al. 2009). The true date of dispersal may therefore be more recent than our conservative estimate of TMRCA.

At the MHC, nucleotide sites known to code for the PBR in humans (Brown et al. 1993) were used to estimate PBR and non-PBR sites in the pipit sequences. We calculated d_N and d_S separately for PBR and non-PBR regions (Nei & Gojobori 1986) in MEGA (Tamura et al. 2007). Differences between d_N and d_S, as well as differences across PBR and non-PBR regions, were assessed using Mann–Whitney U-tests.

We tested for individual gene conversion events using GENECONV (Sawyer 1999), and recombination using the RDP (Martin & Rybicki 2000), MaxChi (Smith 1992) and methods implemented in the RDP3 package. However, GENECONV, the most commonly used statistical method to detect gene conversion, has been shown to suffer from low power (Mansai & Innan 2010), and other methods are not designed to detect the transfer of

small sequence fragments. We also calculated rate of recombination (ρ) relative to point mutation (θ) across the global alignment using LDHat (McVean et al. 2002). This program uses a coalescent-based approach, and therefore while estimates of $\rho/\theta > 1$ are likely to be indicative of recombination, absolute estimates from data across populations need to be interpreted with caution.

We developed a macro to quantify the level of shared polymorphism across unrelated haplotypes (hereafter 'convergence'), using Minitab version 12.1. Each haplotype (the 'derived') is first paired with its closest matching haplotype (the 'ancestor') using a 3-bp sliding window, and the percentage sequence similarity between the two is calculated. The macro then searches for a 'donor' haplotype within the genepool that can explain 100% of sequence similarity at the regions where the ancestor and derived haplotypes differ. A 'construct' haplotype is then created by recombining the derived haplotype with the donor at the region of mismatch. The percentage sequence similarity between the construct haplotype and the ancestor haplotype is then calculated to give the level of sequence similarity after allowing 'convergence' with one donor. The average improvement in sequence similarity was compared to a data set simulated to evolve neutrally via point mutation only (see below). Differences between average levels of sequence divergence in the observed and simulated datasets were tested using Wilcoxon tests. For these, each of the haplotypes (41 empirical and 41 x 5 = 205 simulated) was a data point. The value represents the percentage similarity between that haplotype and its ancestor after simulating convergence. A significant increase in sequence similarity is predicted to arise from convergent evolution via either point mutation or by gene conversion. We therefore stress that the above method quantifies the level of convergence in haplotypes, rather than gene conversion per se. We assessed the likelihood of the convergent evolution occurring via point mutation by looking for independent point mutations at synonymous sites (see section 6.4, below).

Simulated sequences were generated in the program Seq-Gen v. 1.3.2 (http://tree.bio.ed.ac.uk/software/seqgen). This program simulates sequences along a given tree topology according to a user-specified model of sequence evolution. We generated a neighbour joining tree using the 41 pipit MHC class I exon 3 haplotypes. This tree was used to simulate nucleotide divergence assuming the HKY model of substitution.

We generated five simulated sets, each containing 41 240 bp sequences. As these sequences conform to the same tree as the real MHC sequences, levels of divergence prior to running the gene conversion macro were identical.

6.4 Results

6.4.1 454 sequencing and identification of MHC haplotypes

The 454 run yielded 268,267 reads, 257,708 (96%) of which exactly matched one of the 13 population-level tags. Thus, the rate of error was relatively low. The number of reads obtained per population ranged from 7,763 to 33,102. The Newbler assembler grouped these sequences into between 12 and 91 contigs per population, using 227,056 (88%) of the reads. After removing short contigs, and those with <0.4% of the reads (this corresponded to 38 reads in the population with the lowest read number), between 9 and 14 haplotypes per population were identified (Table 6.2) making use of 208,199 (81%) of the reads. None of the remaining haplotypes contained indels or stop codons. Some haplotypes occurred at high frequencies across most populations, whereas others were rare, and only present in one, or a subset of populations, making a total of 41 unique haplotypes (Table 6.3).

A phylogenetic network of the MHC haplotypes revealed eleven divergent lineages, each containing between one and twelve closely related haplotypes (Fig. 6.1). Lineages typically consist of one geographically widespread haplotype and a few rarer haplotypes, unique to just one or two populations (Table 6.2). Not all populations contain all 11 lineages (range 6–10), but with the exception of the more complicated L1 and L3 lineages, all populations possess only one haplotype from each of the lineages they do contain (Table 6.2). We tested whether MHC haplotypes within lineages were mutually exclusive in populations using a binomial mass function. For each haplotype lineage *i*, we calculated the binomial probability that the ancestral haplotype was absent in the populations where a derived haplotype was present. The probability that the ancestral haplotype was present in a population (A_i) was calculated as the number of populations in which the ancestral haplotype was observed (k) divided by the total number of populations ($A_i = k/13$). We then tallied the number of populations where the derived haplotype was present (n) and where ancestral and derived haplotypes were both present (m), and

calculated the binomial probability of finding *m* or fewer populations with the ancestral haplotype:

$$P(x_i \leq m) = \sum_{i=1}^{m} \binom{n}{m} A_i{}^m (1 - A_i)^{n-m}$$

These 11 p-values, one for each lineage, were then analysed using Fisher's combined probability test with 22 degrees of freedom. We found that haplotypes within lineages are mutually exclusive across populations (Fisher's combined probability test: $\chi 2 = 53.8$, d.f. = 20, $P = <0.001$, Table 6.2).

6.4.2 Colonization time, founder population size and drift

Analyses of the pipit mitochondrial DNA sequences using beast yielded a mean age estimate for the MRCA of extant mtDNA haplotype diversity of 75,000 years (HPD 20,000–170,000), which we take as a conservative estimate of when the Berthelot's pipit dispersed to form its current range. This assumes a single founding mtDNA lineage from which all extant lineages are derived, and it should be pointed out that if colonization had involved more than one of the four mtDNA haplotypes from which the single extant haplotype diversity is derived, or if our mutation rates are too low, colonization time would be closer to the present.

Table 6.2 Haplotype and lineage diversity at MHC class I genes across Berthelot's pipit populations. Columns L1-11 represent haplotype lineages, the numbers inside cells are the labels of individual MHC haplotypes. The colour of cells corresponds to whether, within each population, we found only the most widespread haplotype within a lineage (blue), a rare haplotype found in just a few populations (red), or cases where multiple haplotypes of the same lineage were present (yellow). Also included are the probabilities from binomial mass functions that haplotypes within lineages are mutually exclusive across populations (see text for details).

	Island	# MHC haplotypes	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
Madeira	**Deserta Grande**	12	10	9	8,6,21	2	1	7	26	4	3	5	
	Madeira	11	10	9	8,21	2	1	7	26	33	3	23	
	Porto Santo	12	10	35	8,6,21	2	1	40	32	36	3	23	
Selvagens	**Selvagem Grande**	11	10	30	8,6	2	27	37		4	3	11	38
Canary Islands	**La Graciosa**	9		9	6,42,25		1	7		4	3		16
	Lanzarote	10	10		8,24	2	1	7		4	31	11	16
	Fuerteventura	11	10,13	9	8,12,14	2	1	7			3	11	
	Gran Canaria	14	10,13,28		8,6,15,17	2	1	7		4	3	11	16
	El Teide	10	10		8,12	2	39	19		4	3	11	16
	Tenerife	13	10,28	9	8,6,24	2	41	19		4	3	11	16
	La Gomera	13	10,13		8,6,24,22	2	1	7		4	3	11	16
	La Palma	9	10,13		8,22,34	2	1	7			3		
	El Hierro	12	10,18,28	30	8,6	2	1	7		4	3		29
		Probability	1	0.2330	1	1	0.0123	0.0090	0.8462	0.0947	0.0769	0.0123	0.2899

Table 6.3 – Frequencies of MHC class I exon 3 haplotypes across 13 island populations of Berthelot's pipit, grouped by lineage.

Lineage	Haplotype	DG	FV	GOM	GRAC	GC	HIER	LZ	PAL	M	PS	SG	TEIDE	TF	Number pops
L1	**ANBE10**	0.402	0.272	0.225		0.178	0.297	0.375	0.315	0.407	0.441	0.271	0.282	0.285	12
	ANBE13		0.026	0.025		0.013			0.021						4
	ANBE28						0.012							0.011	2
	ANBE18					0.020	0.009								2
L2	**ANBE9**	0.078	0.030		0.478					0.050				0.053	5
	ANBE30						0.024					0.043			2
	ANBE35										0.005				1
L3	**ANBE8**	0.179	0.216	0.206		0.197	0.186	0.205	0.223	0.243	0.193	0.174	0.260	0.191	12
	ANBE6	0.025		0.029	0.079	0.012	0.007				0.046	0.072		0.017	8
	ANBE21	0.014								0.032	0.035				3
	ANBE42				0.059										1
	ANBE24			0.009				0.031						0.036	3
	ANBE14		0.069												1
	ANBE22			0.050					0.014						2
	ANBE15					0.026									1
	ANBE25				0.026										1
	ANBE34								0.035						1
	ANBE12		0.029										0.015		2
	ANBE17					0.009									1
L4	**ANBE2**	0.013	0.159	0.117		0.215	0.172	0.114	0.110	0.029	0.030	0.143	0.148	0.126	12
L5	**ANBE1**	0.069	0.033	0.021	0.045	0.007	0.016	0.030	0.076	0.053	0.062				10
	ANBE41													0.037	1
	ANBE39												0.029		1
	ANBE27											0.007			1
L6	**ANBE7**	0.083	0.080	0.118	0.196	0.122	0.076	0.076	0.141	0.098					9
	ANBE19												0.094	0.100	2
	ANBE37											0.073			1

Table 6.3 (cont.)

Lineage	Haplotype	DG	FV	GOM	GRAC	GC	HIER	LZ	PAL	M	PS	SG	TEIDE	TF	Number pops
L7	**ANBE26**	0.009								0.009					2
	ANBE32										0.021				1
L8	**ANBE4**	0.028		0.117	0.077	0.120	0.148	0.088				0.153	0.080	0.076	9
	ANBE36										0.027				1
	ANBE33									0.042					1
L9	**ANBE3**	0.033	0.032	0.031	0.028	0.040	0.036		0.034	0.015	0.021	0.046	0.048	0.034	12
	ANBE31							0.034							1
L10	**ANBE11**		0.053	0.021		0.038		0.029				0.008	0.025	0.020	7
	ANBE23									0.054	0.050				2
	ANBE5	0.065													1
L11	**ANBE16**			0.03	0.013	0.02		0.02					0.019	0.01	6
	ANBE38											0.01			1
	ANBE29						0.02								1

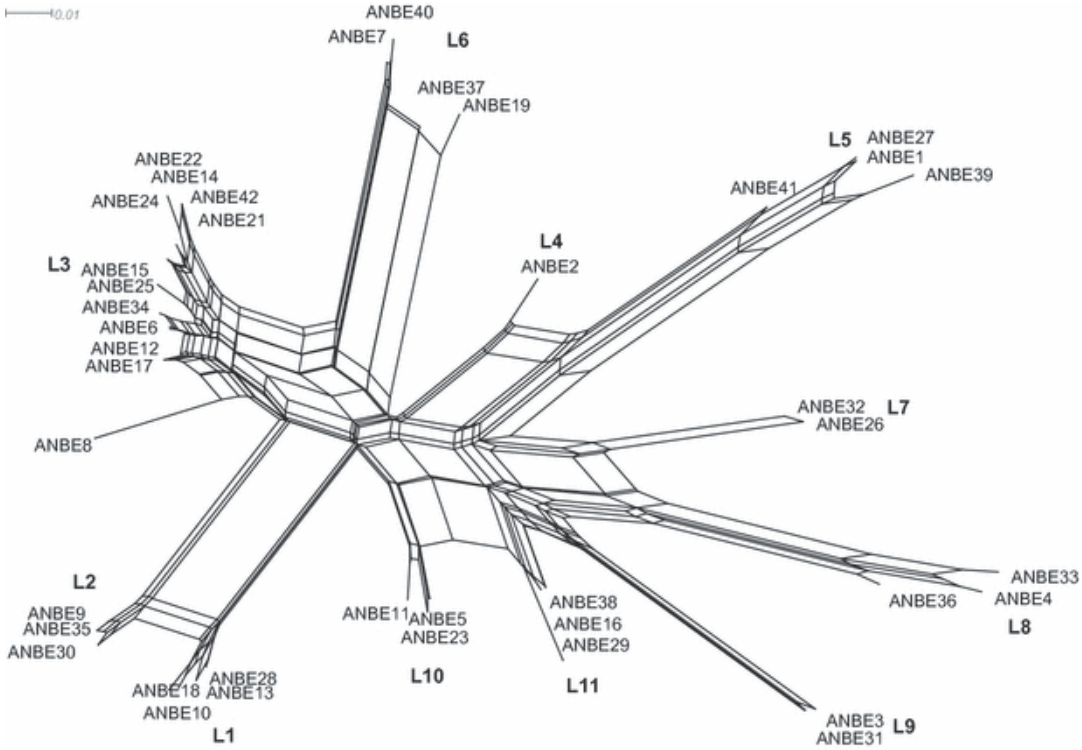

Figure 6.1 Neighbour-net constructed from 41 Berthelot's pipit MHC class I exon 3 haplotypes (from 371 individuals) using Jukes–Cantor distances. Labels L1-L11 represent putative lineages. The length along the lines between two sequences is proportional to their genetic distance, and intersections are formed when the relationship between sequences is ambiguous (for example, due to recombination, recurrent mutations or gene conversion).

Simulations revealed that a minimum founder population size of 320 (outbred individuals) is required to introduce the observed number of 41 MHC haplotypes. A founder size of ca. 500 birds will introduce a median of 41 MHC haplotypes, and more than 2000 birds are required to explain the presence of 41 haplotypes with 99% confidence (Fig. 6.2). Given that no drift was allowed after the founder event, these simulations are conservative (i.e. they are underestimating the 'true' founder population size if all extant MHC variation has to be introduced during founding). As a single ancestral mitochondrial haplotype was involved in colonization (Illera et al. 2007), such a large number of colonizing individuals seems highly unlikely, given the levels of mitochondrial haplotype diversity seen in other outbred bird populations (Grapputo et al. 1998; Questiau et al. 1998; Kimura et al. 2002). Furthermore, if all 41 haplotypes were present in the founding population, and the extant haplotype distribution was caused by stochastic patterns of loss across populations due to

drift, there should be a correlation between population size and number of MHC haplotypes. We found strong evidence for drift at neutral loci, indicated by a positive relationship between microsatellite variation and population size (R^2 = 0.87, d.f. = 11, P < 0.001; Fig. 6.3); however, this was not the case for the MHC, which showed a strikingly different pattern (R^2 = 0.001, d.f. = 11, P = 0.89; Fig. 6.3). Consequently, we suggest that the geographically widespread haplotypes of each lineage are ancestral, that novel haplotype variation has been generated in situ within each population, and that new haplotypes normally replaced the haplotype from which they were derived. Depending on the number of ancestral haplotypes in the complicated L1 and L3 lineages, there are between 11 and 15 founder MHC haplotypes, with between 26 and 30 haplotypes having evolved in situ after the initial founding event. In subsequent analyses, we assume 12 ancestral alleles—one from each lineage, and the two most widespread haplotypes from lineage L3 (ANBE6 and ANBE8). Fewer than 20 individuals are required to introduce 11–15 haplotypes to each island with 99% confidence (Fig. 6.2). This founder population size appears to be more consistent with the low mtDNA variation of the species.

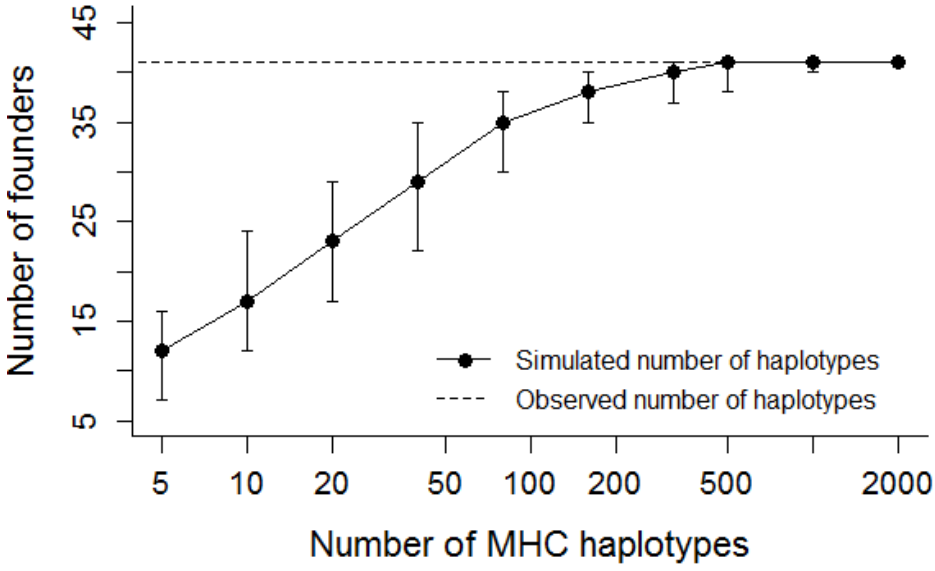

Figure 6.2 Mean (± 99% CI) number of MHC haplotypes in the Berthelot's pipit metapopulation as a function of the number of founders. The estimates are based on a simulation of 4 MHC genes (8 haplotypes) per individual drawn from the contemporary gene pool with the haplotype frequencies based on those observed across the entire range of pipits (see Table 6.3). The simulations make the following conservative assumptions: equal sex ratio, no sperm storage and no genetic drift after the founder event.

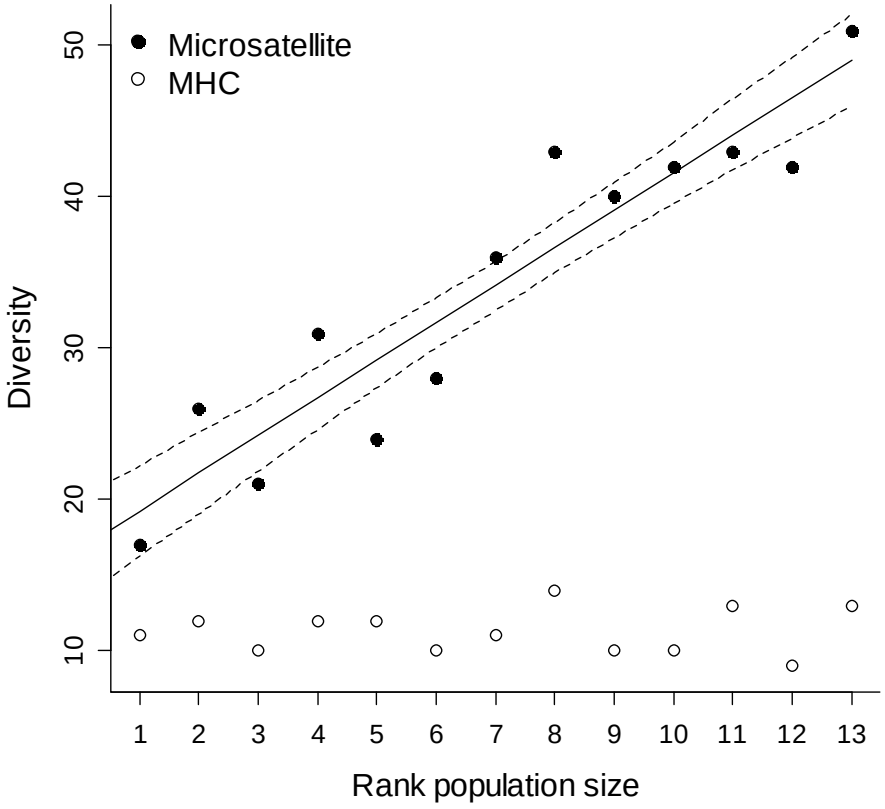

Figure 6.3 Variation at major histocompatibility complex (MHC) and microsatellite loci in relation to rank population size (Illera 2007) 13 Berthelot's pipit populations (371 individuals). For the MHC, this is the number of haplotypes found in each population. For microsatellites, it is the total number of haplotypes detected across five loci (Illera et al. 2007). Dotted lines represent 95% confidence intervals.

6.4.3 Sequence analyses

Putative derived MHC haplotypes differed from their ancestral forms by distinctive nucleotide clusters that were present in ancestral haplotypes from other lineages (Fig. 6.4). The effect of these recurring motifs was apparent in the phylogenetic network (Fig. 6.1), which reveals widespread conflicting splits, indicating ambiguous relationships among the MHC haplotypes. The RDP, GENECONV and MaxChi methods of recombination detection found 3, 11 and 19 recombination events respectively, strongly suggesting that micro-recombination or gene conversion has occurred at the pipit MHC. However, these methods failed to detect instances of gene conversion that were very clear when inspected by eye (the event shown in Fig. 6.5, for example, was not detected). Considering all sequences together, the estimated population recombination rate (ρ) was 26.94 (5% and 95% bounds were 18.34 and 38.22 respectively), compared to a mutation

rate (θ) of 15.89, giving a recombination–mutation ratio of 1.695. This indicates that recombination is more prevalent than point mutation.

Our macro developed to detect convergence among haplotypes revealed that MHC haplotypes were highly similar to their closest relative, differing on average by just over 1% (Table 6.4). In contrast, putative donor haplotypes were on average 10% different to the derived haplotypes across the sequence as a whole, but usually matched perfectly in the donor regions. The size of donor regions varied, with minimum insert size ranging from 2 to 35 bp (Table 6.4). There was an increase in sequence similarity between haplotypes and their closest relatives after allowing a single donor haplotype, and after allowing two donors, 100% of the observed nucleotide polymorphisms could be explained in all but two putative derived haplotypes, which both differed from their closest relative by a single point mutation (Table 6.4). Sequence variation in the putative ancestral haplotypes could be less well explained by donor haplotypes (Table 6.4). Moreover, putative ancestral haplotypes were identified by the macro as donor sequences in 26 of 27 cases. Thus, these analyses strongly support our identification of ancestral and derived haplotypes. The average level of convergence in the observed MHC sequences was much greater than that at sequences simulated to evolve neutrally via point mutation (Wilcoxon tests, $P < 0.001$; Fig. 6.6). In other words, to explain the shared polymorphisms observed across lineages, either gene conversion or convergent evolution by point mutation must be operating. Assuming 12 ancestral and 29 derived sequences, a combination of 27 gene conversion events and two point mutations could potentially explain all the MHC sequence variation in the pipit populations.

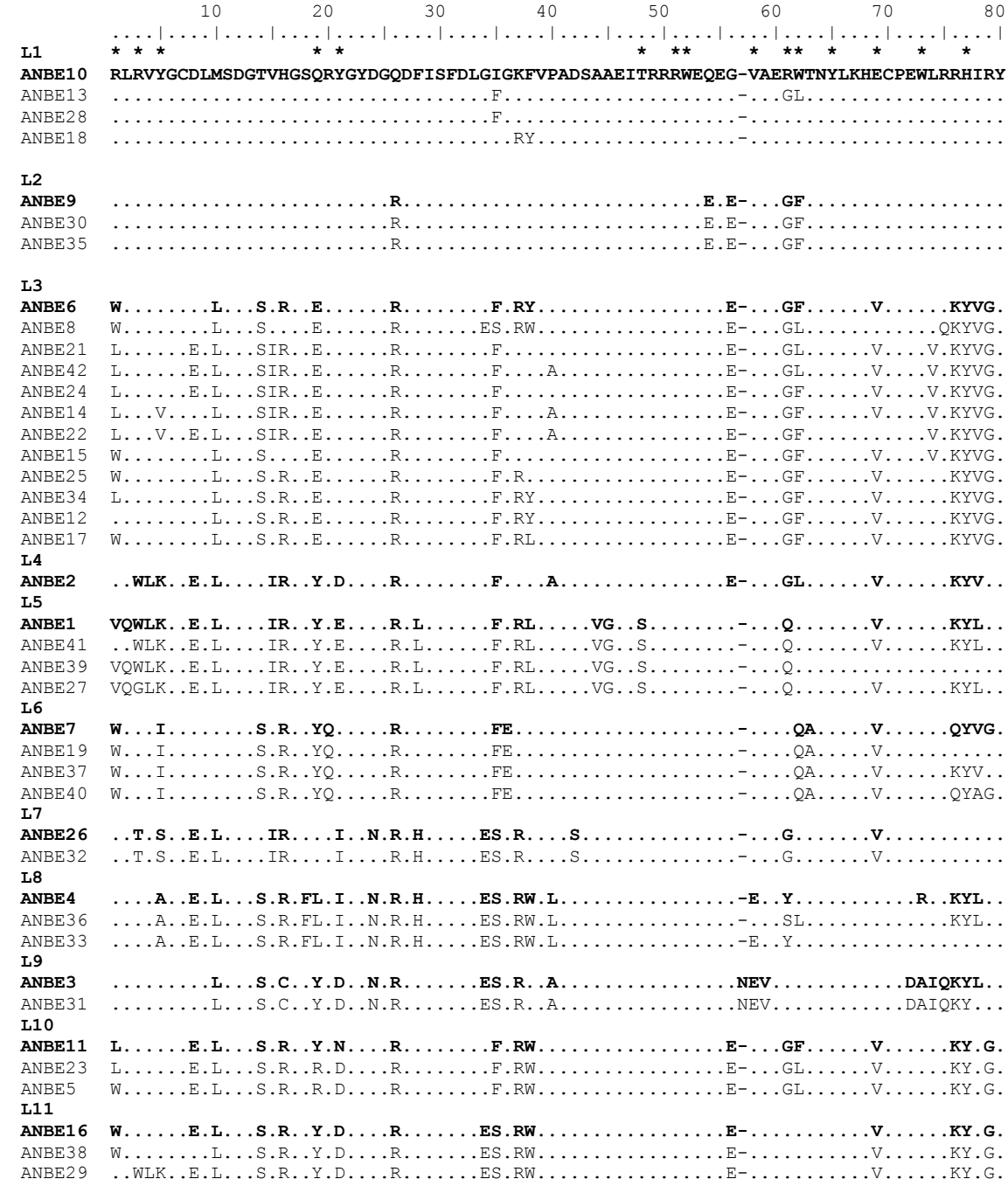

Figure 6.4 Amino acid alignment of 41 Berthelot's pipit major histocompatibility complex (MHC) class I exon 3 haplotypes, grouped by lineage according to a neighbour-net (Fig. 6.1). Putative ancestral haplotypes are at the top of each lineage, and highlighted in bold. Sites marked with a * indicate those that encode for the human peptide-binding region.

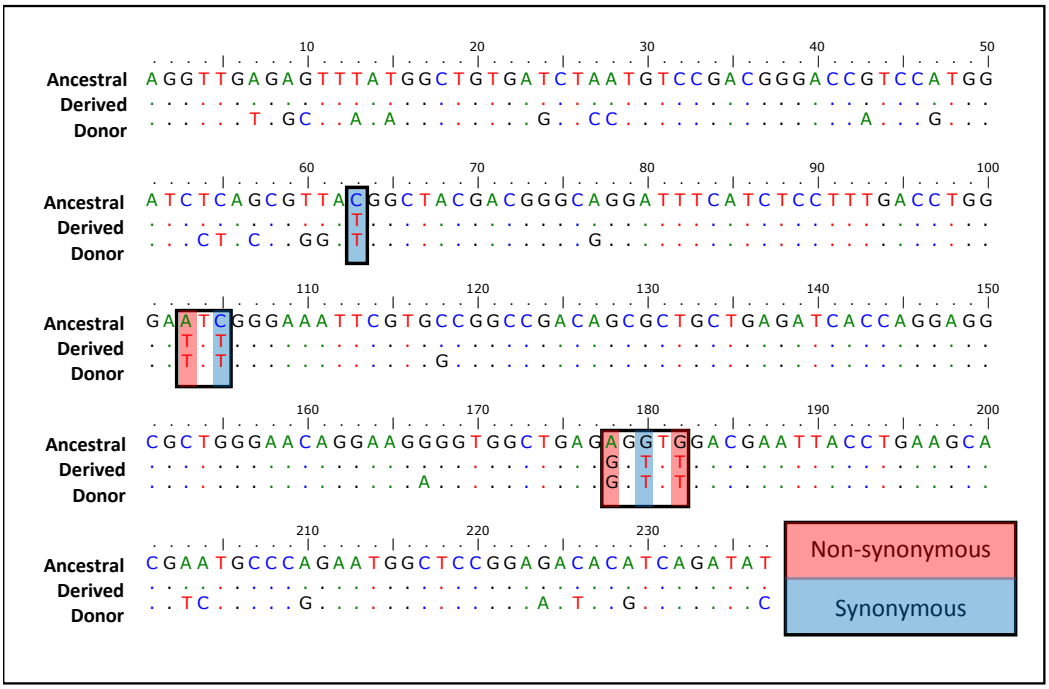

Figure 6.5 Nucleotide alignment showing an example of gene conversion in three Berthelot's pipit MHC sequences. This example consists of a widespread ancestral haplotype (ANBE10), a closely related haplotype derived from this (derived, ANBE13), and another distantly related ancestral haplotype (donor, ANBE2). Areas in which the ancestral and derived haplotypes differ are outlined with shaded boxes, and non-synonymous and synonymous substitutions are highlighted in red and blue respectively. All differences between the ancestral and derived sequences can be explained by variation present in the donor, strongly suggesting that gene conversion has occurred.

To assess the likelihood that convergent accumulation of point mutations, rather than gene conversion, explained our observed patterns, patterns of variation at (mostly neutral) third codon positions were examined. If all within-lineage variation could be explained by point mutation, we would expect to see, by chance, some evidence of independent neutral mutations within lineages. We did not see this: of 80 third codon sites, 50 were conserved across all haplotypes, 14 sites were variable across (but not within) lineages and 15 sites exhibited within-lineage variation. Importantly, all within-lineage variation could be explained by polymorphism found across lineages, with no evidence of independent mutations within lineages. The probability of 15 neutral mutations occurring at the same nucleotide positions in different lineages by chance given the number of variable sites is extremely low ($\chi 2 = 29.17$, d.f. = 1, $P < 0.001$). Considering all 240 sites, only two unique within-lineage mutations occurred, which were also identified by the macro and appear to represent point mutations.

Table 6.4 Output from a sliding window analysis created to detect gene conversion in Berthelot's pipit MHC haplotypes (see text for details). Donor haplotypes identified by the macro that we had previously identified as ancestral are highlighted in bold.

Haplotype	Similarity to CMH (%)	1st donor	Similarity to 1st donor (%)	Minimum insert size 1st donor (bp)	Similarity to 1st construct sequence (%)	2nd donor	Similarity to 2nd donor (%)	Insert size 2nd donor (bp)	Similarity to 2nd construct sequence (%)
Putative ancestral									
ANBE10	99.16	**ANBE2**	87.25	6	100		87.25		100
ANBE11	97.90	**ANBE16**	96.36	7	99.16	**ANBE9**	89.78	3	99.58
ANBE16	99.58	**ANBE11**	96.36	3	100		96.36		100
ANBE9	99.58	**ANBE10**	96.64	3	100		96.64		100
ANBE6	99.86	**ANBE16**	91.18	1	100		91.18		100
ANBE8	95.80	ANBE35	87.68	17	98.46	ANBE13	90.90	5	99.16
ANBE2	93.28	ANBE42	92.44	35	98.74	**ANBE1**	91.18	8	99.86
ANBE1	99.58	ANBE39	97.34	3	100		97.34		100
ANBE7	99.58	**ANBE6**	90.76	3	100		90.76		100
ANBE26	99.58	**ANBE3**	83.61	3	100		83.61		100
ANBE4	97.76	**ANBE1**	81.93	12	99.58		81.93		99.58
ANBE3	99.58	**ANBE1**	81.51	3	100		81.51		100
Putative derived									
ANBE13	98.32	**ANBE8**	90.90	10	100		90.90		100
ANBE18	99.16	**ANBE6**	90.48	6	100		90.48		100
ANBE28	99.16	**ANBE2**	88.10	5	100		88.10		100
ANBE23	99.72	**ANBE11**	97.90	2	100		97.90		100
ANBE5	99.72	**ANBE16**	96.22	2	100		96.22		100
ANBE29	97.76	**ANBE2**	93.28	12	100		93.28		100
ANBE38	99.58	**ANBE3**	90.20	3	100		90.20		100
ANBE30	99.58	**ANBE6**	91.74	3	100		91.74		100

Table 6.4 (cont.)

Haplotype	Similarity to CMH (%)	1st donor	Similarity to 1st donor (%)	Minimum insert size 1st donor (bp)	Similarity to 1st construct sequence (%)	2nd donor	Similarity to 2nd donor (%)	Insert size 2nd donor (bp)	Similarity to 2nd construct sequence (%)
ANBE35	99.58	**ANBE10**	96.64	3	100		96.64		100
ANBE12	99.86	**ANBE10**	89.78	1	100		89.78		100
ANBE14	98.74	**ANBE6**	96.78	7	100		96.78		100
ANBE15	98.46	**ANBE8**	94.12	8	99.58	**ANBE38**	90.34	3	100
ANBE17	99.16	**ANBE1**	85.29	6	100		85.29		100
ANBE21	99.58	**ANBE8**	93.42	3	100		93.42		100
ANBE22	98.74	ANBE39	82.49	5	99.72	**ANBE2**	90.76	2	100
ANBE24	99.58	**ANBE11**	92.86	3	100		92.86		100
ANBE25	99.58	**ANBE10**	90.06	3	100		90.06		100
ANBE34	99.72	**ANBE11**	94.12	2	100		94.12		100
ANBE42	99.58	**ANBE2**	92.44	3	100		92.44		100
ANBE27	99.58				99.58				99.58
ANBE39	97.34	**ANBE10**	86.13	16	100		86.13		100
ANBE41	98.32	**ANBE2**	92.86	5	100		92.86		100
ANBE19	98.60	**ANBE10**	90.20	9	100		90.20		100
ANBE37	98.60	**ANBE2**	89.78	9	100		89.78		100
ANBE40	99.58				99.58				99.58
ANBE32	99.58	**ANBE10**	88.52	3	100		88.52		100
ANBE33	97.76	**ANBE10**	84.03	15	100		84.03		100
ANBE36	97.06	**ANBE8**	87.68	17	99.58	**ANBE16**	90.20	2	100
ANBE31	99.58	**ANBE10**	84.31	3	100		84.31		100
Mean (all)	**98.85**		**90.33**	**6**	**99.85**		**90.39**	**4**	**99.93**
Mean (derived)	**99.04**		**90.07**	**6**	**99.93**		**90.30**	**2**	**99.97**

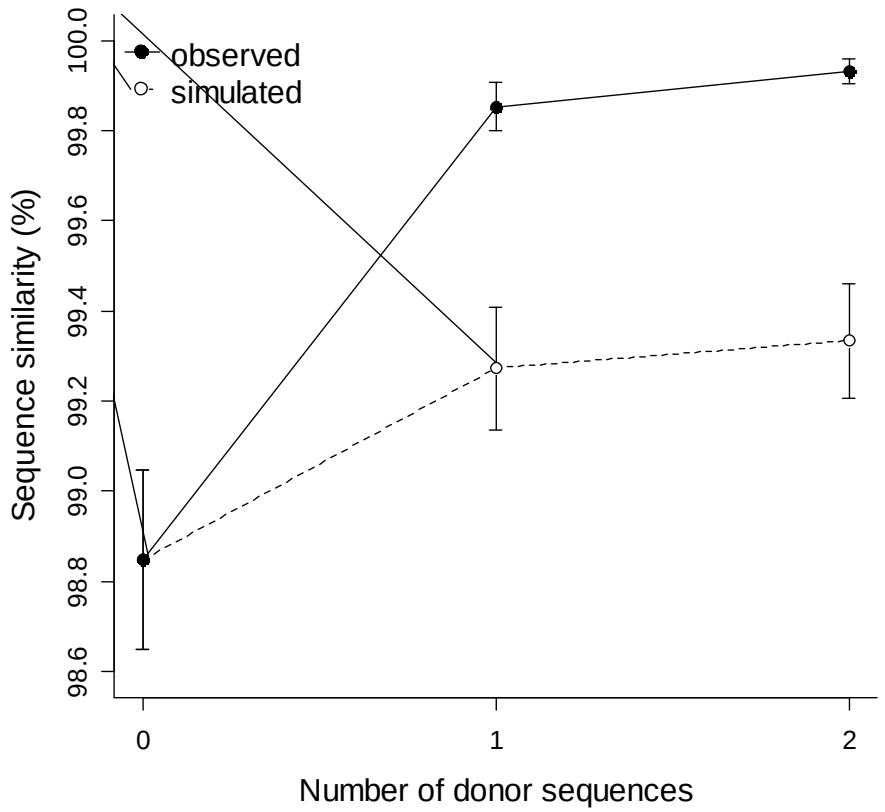

Figure 6.6 Convergence and mean (± s.e.) sequence similarity between MHC haplotypes. Each sequence was matched to its closest relative, and percentage similarity was calculated using a sliding window analysis. This value was then re-calculated allowing for the transfer of sections of DNA from one or two donor haplotypes from within the dataset. The analysis was performed on Berthelot's pipit MHC class I exon 3 haplotypes (observed), and sequences that were simulated to evolve via point mutation (see text for details).

Gene conversion resulted in more changes at putative PBR sites than at non-PBR sites ($\chi 2 = 10.89$, d.f. = 1, $P = 9.6 \times 10^{-4}$; Fig. 6.7). This is reflected in rates of non-synonymous (d_N) and synonymous (d_S) substitutions across the pipit sequences. Both d_N and d_S were significantly higher at PBR compared to non-PBR sites (Fig. 6.7), but d_N was not significantly higher than d_S in the PBR. However, d_S was significantly higher than d_N at non-PBR sites (Fig. 6.7) indicating purifying selection in these areas.

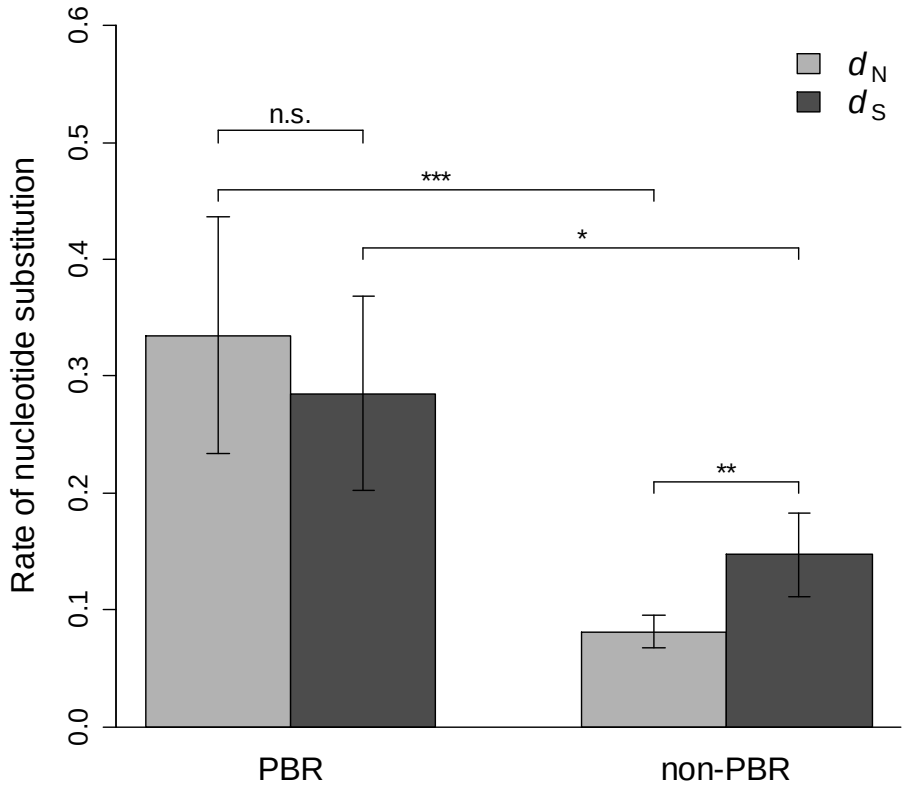

Figure 6.7 Rates of non-synonymous (d_N) and synonymous (d_S) substitutions in peptide-binding (PBR) and non-PBR regions in 41 Berthelot's pipit major histocompatibility complex (MHC) class I exon 3 sequences. Significance levels were evaluated with Mann–Whitney U-tests (*P < 0.05, **P < 0.01, ***P < 0.001). Error bars represent 95% confidence intervals.

6.5 Discussion

Here, we show that, in the Berthelot's pipit, only a limited number of divergent MHC haplotypes persisted when this species dispersed across its range ca. 75 000 years ago. Importantly, we were able to characterize the generation of MHC diversity subsequent to the founding event. We found that gene conversion rapidly generated functional MHC haplotype diversity across populations, with conversion events outstripping point mutations by as much as an order of magnitude.

The argument could be made that the recombinant haplotypes we observe are chimeras—artefacts of the PCR process known to occur when amplifying multiple loci. We believe that this is highly unlikely. First, we used a PCR protocol designed to minimize the formation of chimeras (Lenz & Becker 2008). Second, chimeras are expected to be rare in

comparison to true haplotypes (Babik et al. 2009), whereas we find the same recombinants occurring at high frequencies both within a population, and most importantly, across multiple independently amplified populations. To invoke an explanation of chimeras for our data would require independent non-random generation of the same chimeric sequence in independent PCR reactions, from independent populations. Third, by virtue of the dynamics of their formation, chimeras should co-occur with the parental sequences, from which they formed in a PCR reaction (Lenz & Becker 2008). In contrast, we find that recombinant and parental haplotypes do not typically co-occur within populations (Table 6.2). Fourth, there would be no reason to expect chimeras to form preferentially at the PBR, as we have found. Finally, we would generally expect chimeras to resemble single breakpoint recombination events within our short fragment, and many (but not all) of the recombination events we observe would require multiple breakpoints (Fig. 6.4). We can thus exclude any explanation of chimeric origin for our recombinant sequences.

Using population-level tagged primers and 454 sequencing allowed us to MHC genotype a large number of individuals rapidly and efficiently. With this approach, however, individual-level variation could not be resolved. While initial cloning indicated that four duplicated class I loci are present in Berthelot's pipit—a similar number to other studies on passerines (Richardson & Westerdahl 2003; Promeováet al. 2009)—we cannot exclude the possibility that more loci are present. However, given the number of haplotypes we detected per population (between 9 and 14), it is unlikely that we have drastically underestimated the number of loci present.

A second challenge that our approach presented was that our amplicon exceeded the available read length, meaning that we had to assemble forward and reverse reads into contigs. With this approach, there is a possibility that chimeric sequences could arise if overlapping sequences are incorrectly assembled. However, it is important to remember that the observed differences within lineages can be explained by variation across lineages (i.e. 'donor' sequences in Table 6.4). This difference between "ancestral" and "derived" sequence (which is also the similarity between the "derived" and "donor" sequence) we suggest arises by gene conversion across lineages. In order for this same

result to arise from incorrect assembly would require sequences from across lineages to be assembled together into contigs. We are confident that our bioinformatics procedures eliminated the possibility of this happening. In order for a contig to assemble, the forward and reverse reads must have 200bp overlap, and 99% sequence identity within that overlap. Yet the haplotype lineages observed are much more divergent than this (10% on average across the sequence). Indeed, no two sequences from different lineages meet the levels of similarity required from our assembly settings. The longer read-lengths now available with 454 sequencing are likely to eliminate the need for this bioinformatics phase for amplicons up to ~300 bp (see for example Galan et al. 2010), although our paired-end approach offers the opportunity for longer amplicons (up to ~700 bp) to be genotyped.

As derived haplotypes appear to have replaced their ancestors within the pipit populations, gene conversion has not increased haplotype diversity within each independent population. This same pattern has been found in humans (Parham & Ohta 1996), suggesting that it may be a widespread phenomenon in MHC evolution. The replacement of ancestral MHC haplotypes by new haplotypes within populations may be because of direct positive selection of the derived haplotype, or because derived haplotypes share the same 'sheltered load' of deleterious mutations at MHC-linked regions as their ancestors (van Oosterhout 2009). Importantly, gene conversion generated different MHC haplotypes in eight of the 13 populations. Consequently, these results show that while haplotype diversity did not increase within populations, gene conversion can rapidly increase MHC haplotype diversity at the metapopulation level.

Although there is a large body of evidence suggesting that gene conversion operates within the MHC, the evolutionary significance of this has remained both poorly understood and controversial (Martinsohn et al. 1999; Klein et al. 2007). This is largely due to the difficulty of detecting gene conversion in wild populations. In populations that have evolved independently for long periods of time, homoplasy arising from recurrent gene conversion events and/or point mutations will make the two difficult to distinguish. In Berthelot's pipit, the combination of multiple populations and limited migration has allowed us to separate ancestral and derived variation, and as a result, clearly observe

gene conversion. Moreover, the recent divergence among populations has provided a 'window of opportunity' to characterize how MHC genes evolve in the first stages of diversification, without the effects of homoplasy. Additional research is now required to determine how selection and/or drift operate to maintain new haplotypes within populations, and how migration may affect how patterns of MHC variation and newly evolved haplotypes are distributed across populations.

An additional factor underpinning the difficulty of quantifying gene conversion in natural populations is that detecting individual gene conversion events using statistical methods is highly problematic, although all three methods implemented in the present study yielded positive results. This is a particular problem when gene conversion does not result in large changes—for example, when small sequence tracts are transferred. We present a simple method for detecting the overall degree of convergence between sequences. Again, we stress that this method does not distinguish between gene conversion and convergence of point mutations; in our study, this was only achieved by looking at patterns of synonymous variation. The probability that point mutation can explain the high levels of convergence seen in the pipit MHC is exceedingly small ($P = 6.7 \times 10^{-8}$). We acknowledge, however, that we were not able to statistically identify all of the gene conversion events, and cannot completely rule out the possibility that some of the convergent substitutions observed were the result of point mutations.

No evidence was found for elevated d_N/d_S in the PBR, which is widely regarded as the classic sign of selection at MHC genes (Hughes & Nei 1988). Instead, we found that both d_N and d_S were higher in PBR compared to non-PBR regions. This pattern is to be expected under a scenario of gene conversion combined with positive selection (Ohta 1995), whereby conversion events that contain beneficial non-synonymous mutations at the PBR are positively selected, and synonymous mutations hitchhike across with them (Fig. 6.5). While a number of other population genetic studies have found elevated *d*S at PBR compared to non-PBR sites (e.g. Richardson & Westerdahl 2003; Ekblom et al. 2007), to our knowledge, none have considered the possibility that gene conversion may be the cause.

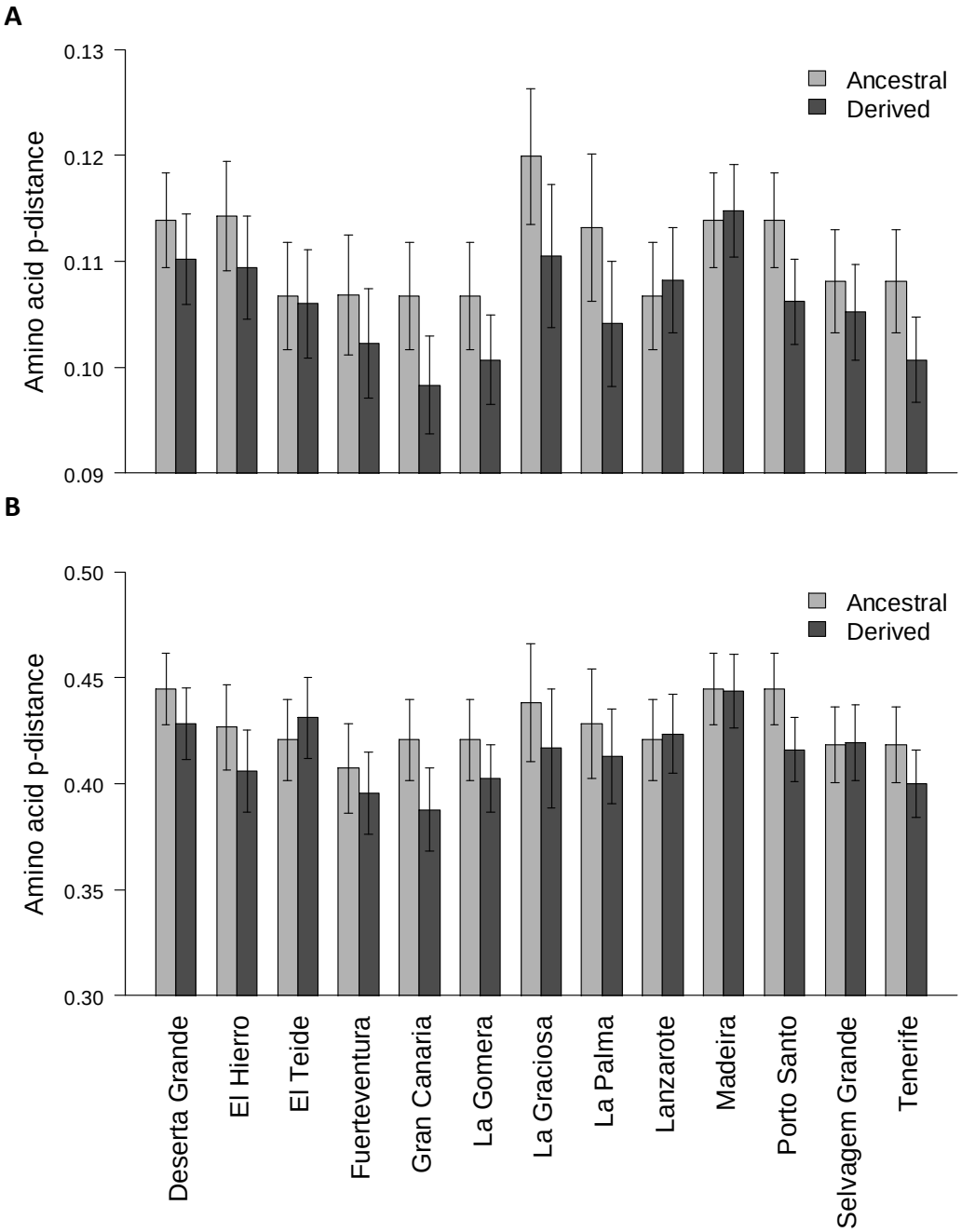

Figure 6.8 Mean amino acid p-distance (proportion of amino acid differences between pairs of sequences) between all pairwise combinations of Berthelot's pipit MHC haplotypes in populations for amino acids coded by the **a)** non-PBR and **b)** PBR codons. The pale bars show the p-distances of populations in which all derived haplotypes have been replaced by their ancestral form. This removes the effect of gene conversion. The dark bars show the p-distances based on all observed haplotypes. The rate of loss is lower for the PBR sites (3.48%) than for the non-PBR sites (4.50%), (paired t-test: t = 2.48, *P* = 0.024). Error bars represent standard error.

Gene conversion can occur across alleles or across loci when genes have been duplicated (Chen et al. 2007). Thus, gene conversion across loci has the potential to generate new haplotypes, even if the population is entirely homozygous at each of the paralogous MHC

loci. Nonetheless, gene conversion can only re-shuffle existing (intra- or inter-locus) polymorphisms to create new variants. Therefore, although it can rapidly generate variation at the haplotype level, variation at the sequence level is expected to be homogenized over time, especially when larger fragments are being transferred (Takuno et al. 2008). In populations of Berthelot's pipit, gene conversion did cause a reduction in amino acid variation, although to a lesser extent in the PBR than non-PBR regions, and not in all populations (Fig. 6.8). It therefore appears that in these populations, the beneficial effect of novel haplotype variation from gene conversion outweighed the effect of sequence variation becoming marginally homogenized. This is to be expected when sequences within a population are divergent—a common observation at the MHC following population bottlenecks (Hedrick et al. 2000, 2002; Richardson & Westerdahl 2003; van Oosterhout et al. 2006). In such scenarios, we suggest that gene conversion may be the primary mechanism generating new functional haplotypes.

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Chapter 7: General discussion

El Teide, Tenerife, viewed from La Gomera

7.1 General discussion

In this thesis, I set out to explore how neutral and adaptive genetic diversity are partitioned across recently separated populations of Berthelot's pipit. As is often the case with research projects of this size and nature, some aspects of the research were serendipitous, whereas other analyses and approaches I had planned at the start of the project turned out not to be feasible. In this final chapter, I discuss: i) what I believe to be the most salient overall messages from this work; ii) some research that I had planned but which turned out to be unfeasible, and; iii) some future directions for research into the Berthelot's pipit system.

7.1.1 Selection and drift in Berthelot's pipit

In Berthelot's pipit I found that demography, and consequently genetic drift, was an important factor shaping patterns of genetic diversity within and across populations at both neutral and adaptive loci. In an earlier study on Berthelot's pipit, Illera *et al.* (2007) suggested that the low mitochondrial and microsatellite variation observed were indicative of a recent dispersal across the islands. However, the lack of evidence for recent bottlenecks that this study found, along with indications of isolation-by-distance, suggested that the populations had reached mutation-drift equilibrium (Illera *et al.* 2007). By revisiting the same data set with an increased number of markers in chapters 4 and 5, I have confirmed that there are low levels of neutral genetic diversity across all three archipelagos, but in contrast to the previous work, found that recent bottlenecks have occurred in two of them. The patterns of divergence across populations observed by Illera *et al.* (2007) and in this study, including the isolation-by-distance effect, were in fact an artefact of this. The low levels of intra-island MHC diversity observed in chapter 6 are also consistent with a recent bottleneck, though I was unable to explicitly compare patterns of variation at MHC and neutral loci (see below). Thus, Berthelot's pipit is an example of a recent colonisation, characterised by a series of bottlenecks resulting from founder events.

In addition to drift, natural selection appears to have played an important role during the recent evolutionary history of Berthelot's pipit. By analysing spatio-temporal patterns of

pathogen prevalence (chapter 3) I have shown that differences in pathogen fauna across populations were consistent, and constrained by basic biogeographic features. This, combined with the known virulence of avian malaria and pox (van Riper et al. 1986; Jarvi et al. 2008), and an observed effect on pipit body condition, suggests different levels of pathogen-mediated selection operate across the different island populations (chapter 3). I also found evidence of selection in the microsatellite outlier analyses in chapter 4, where I showed that one EST-linked marker had much higher structure across populations than expected by chance. Finally, I found indirect evidence of selection at the MHC, where gene conversion appeared to disproportionately target amino acid sites involved in pathogen recognition, suggesting that alleles with functional, phenotypic differences are positively selected, and thus recruited into the population (chapter 6).

Many studies have looked for associations between patterns of MHC diversity and pathogenic infection, which can help differentiate between mechanisms of pathogen-mediated selection (e.g. Paterson et al. 1998; Westerdahl et al. 2005; Bonneaud et al. 2006; see also chapter 2). In this thesis I screened both population-level pathogen load and MHC variation, but have not explicitly analysed them together. There are various reasons for not doing so: one key reason was to do with the method I used to screen population-level MHC variation, using 454 sequencing and population-level tagged primers. This method was rapid, efficient and in many ways very effective, but is not without its problems. A lack of individual MHC profiles severely hinders the ability and power one has to make associations between MHC variability and pathogen regime. Furthermore, as discussed in chapter 2, relationships between MHC variability and pathogen regime are often highly complex: sometimes there is selection for specific alleles, sometimes for heterozygosity, and sometimes a combination of both (see also Smith et al. 2010; Worley et al. 2010). Such associations will almost certainly be obscured by population-level pooling. Recently, other research groups have utilised the increased read-length available from 454 sequencing and developed approaches to efficiently genotype MHC genes at the individual level (Galan et al. 2010). However, when we nlevel typing would have been prohibitively costly (for this PhD project) and inefficient due to the shorter read-lengths available at that time (see section 6.5). In the future, individual-

level MHC screening across the pipit populations should provide interesting insight into the nature of selection at the MHC.

Unlike the MHC, I did have individual-level microsatellite data, and it would have been possible to test for correlations between multi-locus microsatellite heterozygosity and susceptibility to pathogen infection (*cf.* heterozygosity-fitness correlations; see Chapman et al. 2009 for a review). Associations between microsatellite heterozygosity and pathogen infection may be expected if i) if heterozygosity is a proxy for inbreeding, and inbred individuals are more susceptible to disease; or ii) individual microsatellite loci are linked to genes involved with disease resistance (Coltman et al. 1999). However, numerous problems exist with this approach. First, it has been shown that multi-locus heterozygosity is a poor proxy for inbreeding unless a very large number of loci (>100s) are used (Balloux et al. 2004; Slate et al. 2004). The 22 loci I used differed widely in levels of heterozygosity (Chapters 4 and 5), suggesting that using them to estimate "genome-wide" heterozygosity would be very weak indeed. Second, there is no reason to expect that we would choose, by chance, microsatellites linked to disease resistance genes. Third, pathogen load varied markedly across the pipit populations (chapter 3), and as a result relationship between heterozygosity and pathogen infection will be confounded by population-specific effects, and it is unlikely that I have sufficient sample sizes to detect subtle, within-population effects.

Another commonly employed analysis in studies of selection at the MHC is to compare patterns of population structure at MHC and microsatellite markers (e.g. Ekblom et al. 2007; Alcaide et al. 2008; Cammen et al. 2011; see also chapter 2). However, a major problem with many of these studies is that they cannot amplify individual MHC loci, which presents a real problem when calculating population structure. In my study, in addition to being unable to amplify individual MHC loci, I estimated allele frequencies from pooled population-level samples based on read depth from 454 sequencing. While I am confident that the 454 approach provided an accurate estimate of the presence/absence of MHC alleles across the pipit populations, it is unclear how accurate estimates of allele frequencies obtained using this method would be. Even slight error in

estimating allele frequencies will introduce further error into estimates of population structure. Finally, even if it were possible to calculate individual heterozygosity and population structure at duplicated MHC genes with high accuracy, comparing MHC and microsatellites is highly problematic due to the different mutation rates and levels of variability at the two marker types (see chapter 2). Indeed, in this thesis (chapters 4 & 5) I have shown that marker variability has enormous effects on estimates of population structure, even across sets of markers of the same type. These problems, when considered in concert, led me to decide not to conduct any MHC analyses that were heavily dependent on accurate estimates of allele frequencies or heterozygosity.

7.1.2 General conclusions and future directions

Selecting the appropriate model system for evolutionary and ecological studies is of utmost importance (Bernasconi et al. 2009; Clutton-Brock and Sheldon 2010). This is a message that I have tried to emphasise throughout this thesis – a study which adds to the large and growing body of evolutionary research using island systems as models for population and species level research (Whittaker 1998; Emerson 2002). It is unlikely that the main results of this thesis would have been so clear were it not for the replicated and simple nature of the study system. In chapter 3, the island setup enabled me to use populations as replicates, and demonstrate clear, consistent biogeographic patterns of pathogenic infection. In chapters 4 and 5, the lack of migration between the island populations enabled me to observe clear patterns of population structure and signatures of bottlenecks, and thus to disentangle the population history of the pipit. Then in chapter 6, the replicated populations and lack of migration were essential for identifying ancestral and derived MHC sequences, without which it would have not been possible to observe so clearly how gene conversion generates new MHC alleles.

The other overall theme that I think is apparent throughout this research is the importance of interpreting population genetic and ecological analyses in light of their assumptions, and of fully considering alternative explanations for any given finding. When I first began to read the literature on pathogen-mediated selection at the MHC it became clear that there was a great deal of confusion about how to differentiate between

mechanisms of selection, and that many studies had not fully considered alternative explanations for their findings. Chapter 2 of this thesis is an attempt to clarify some of this confusion. Chapters 4 and 5 originated in similar circumstances: when I revisited the previous work by Illera *et al.* (2007) on population history in Berthelot's pipit with an increased number of markers, my results differed. This led me to explore in more detail the effects of marker variability on different population genetic analyses, and find that it can severely affect outcomes. The confusion that can occur when interpreting population genetic data and analyses was also a central theme to producing chapter 6. When I first looked for evidence of recombination and gene conversion in the pipit MHC sequences, I found that some published algorithms detected extensive evidence for gene conversion within our dataset, whereas others did not. It was this that led us to develop the visual method presented in chapter 6.

The research presented in this thesis goes some way to establishing the Berthelot's pipit as a model system for population and ecological genetics and leaves many exciting questions to be addressed. For example, temporal analyses of genetic diversity were beyond the scope of the project, though I was able to analyse temporal patterns of pathogenic infection (which was central to establishing whether the populations faced different selective pressures). Very few study systems have good multi-population, multi-year sampling regimes, and thus further temporal sampling of the pipit populations could provide novel insight into the micro-evolutionary processes that operate across wild populations. Examining spatio-temporal patterns of variation across the pipit populations would be particularly interesting if done from both "top-down" and "bottom-up" perspectives. Using newly developed genomic techniques, such as restriction-site associated DNA (RAD) sequencing, it is now possible to generate genome-scale sets of markers in any non-model species (Baird et al. 2008; Etter et al. 2011). Moreover, as costs of high-throughput sequencing decrease, it is now becoming possible to run large marker panels on a population-scale. At the same time, many new candidate genes of functional importance are being identified, and markers developed for a wide range of organisms (Piertney and Webster 2010). The pipit system, in which founder events, genetic drift and natural selection have all played a role promoting adaptation and differentiation across

populations, provides an interesting opportunity to utilise these approaches, and explore how different evolutionary forces operate across the genome.

7.3 References

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Appendix 1

Allele frequencies at 22 microsatellite loci across island populations of Berthelot's pipit. SG: Selvagem Grande, DG: Deserta Grande, M: Madeira, PS: Porto Santo, EH: El Hierro, PAL: La Palma, GOM: La Gomera, TEID: El Teide, TF: Tenerife, GC: Gran Canaria, FV: Fuerteventura, LZ: Lanzarote, GRAC: La Graciosa.

Locus	SG	DG	M	PS	EH	PAL	GOM	TEID	TF	GC	FV	LZ	GRAC
TG03-002	-	0.103	0.18	0.192	0.15	0.056	0.117	0.069	0.1	-	0.067	0.05	-
	1	0.293	0.46	0.308	0.75	0.759	0.767	0.845	0.767	0.793	0.85	0.85	0.935
	-	0.017	0.02	0.154	0.083	0.13	0.1	0.086	0.133	0.172	0.083	0.1	0.065
	-	0.431	0.26	0.346	0.017	0.056	0.017	-	-	0.017	-	-	-
	-	-	0.08	-	-	-	-	-	-	0.017	-	-	-
	-	0.155	-	-	-	-	-	-	-	-	-	-	-
TG13-009	1	0.828	0.688	0.673	0.914	0.962	0.897	0.926	0.967	0.821	0.95	0.87	0.826
	-	0.172	0.313	0.327	0.086	0.038	0.103	0.074	0.033	0.179	0.05	0.13	0.174
TG01-047	0.893	0.883	0.96	0.981	0.8	0.648	0.786	0.607	0.75	0.607	0.683	0.615	0.891
	0.107	0.117	0.04	0.019	0.2	0.352	0.214	0.393	0.25	0.393	0.317	0.385	0.109
TG02-088	1	1	1	1	1	0.981	0.958	1	0.981	1	0.933	0.962	0.933
	-	-	-	-	-	0.019	0.042	-	0.019	-	0.067	0.038	0.067
TG03-098	-	-	-	-	0.35	0.13	0.411	0.089	0.214	0.259	0.259	0.154	0.13
	0.357	0.042	0.075	0.043	0.233	0.093	0.125	0.232	0.107	0.086	0.259	0.269	0.022
	0.643	0.958	0.925	0.957	0.417	0.778	0.464	0.679	0.679	0.655	0.483	0.577	0.848
TG04-004	-	-	0.021	-	0.1	0.143	0.31	0.172	0.1	0.172	0.155	0.183	0.196
	1	0.517	0.646	0.788	0.75	0.804	0.655	0.759	0.867	0.724	0.776	0.7	0.717
	-	0.483	0.333	0.212	0.15	0.018	0.034	0.069	0.033	0.069	0.052	0.05	0.065
	-	-	-	-	-	0.036	-	-	-	0.034	0.017	0.067	0.022
TG09-018	-	-	-	-	-	0.019	-	-	-	-	-	-	-
	-	0.172	0.065	-	0.017	0.115	0.069	0.052	0.1	0.034	0.107	0.052	0.109
	0.517	0.414	0.391	0.417	0.267	0.288	0.19	0.155	0.183	0.224	0.107	0.155	0.065
	0.328	0.138	0.239	0.146	0.267	0.077	0.224	0.207	0.167	0.138	0.214	0.276	0.217
	0.034	-	-	-	0.017	-	-	-	-	-	0.018	0.017	-
	0.121	0.224	0.261	0.25	0.283	0.154	0.121	0.172	0.317	0.31	0.143	0.207	0.022
	-	0.052	0.043	0.188	0.133	0.288	0.379	0.379	0.217	0.276	0.375	0.276	0.565
	-	-	-	-	-	0.019	0.017	0.034	0.017	-	-	0.017	0.022
	-	-	-	-	0.017	0.038	-	-	-	0.017	0.036	-	-

TG01-124	1	0.271	0.676	0.525	1	1	0.983	1	1	0.982	0.933	1	1
	-	0.729	0.324	0.475	-	-	0.017	-	-	0.018	0.067	-	-
TG05-053	-	-	-	-	-	-	-	-	-	0.018	-	0.018	0.065
	-	-	-	-	0.017	0.04	0.183	0.056	0.067	0.018	0.083	0.036	0.022
	1	1	1	0.864	0.567	0.58	0.367	0.556	0.567	0.482	0.5	0.518	0.696
	-	-	-	0.136	0.417	0.38	0.45	0.389	0.35	0.482	0.417	0.429	0.217
	-	-	-	-	-	-	-	-	0.017	-	-	-	-
TG06-009	0.817	0.183	0.558	0.288	0.733	0.704	0.867	0.804	0.75	0.845	0.517	0.724	0.717
	0.183	0.55	0.442	0.712	0.267	0.278	0.117	0.196	0.25	0.155	0.467	0.276	0.283
	-	0.267	-	-	-	0.019	0.017	-	-	-	0.017	-	-
PCA7	1	1	1	1	0.967	0.804	0.917	0.948	0.9	0.966	0.95	0.85	0.826
	-	-	-	-	0.033	0.196	0.083	0.052	0.1	0.034	0.05	0.15	0.174
CAM13	0.768	0.56	0.5	0.643	0.9	0.907	0.948	0.857	0.85	0.875	0.933	0.926	0.87
	0.232	0.16	0.167	-	0.1	0.093	0.052	0.143	0.15	0.125	0.067	0.074	0.13
	-	0.28	0.333	0.357	-	-	-	-	-	-	-	-	-
PDO46	-	0.05	0.06	-	0.05	-	-	0.018	0.033	0.017	-	-	-
	0.31	0.95	0.9	1	0.783	0.714	0.717	0.857	0.733	0.776	0.75	0.852	0.739
	-	-	0.04	-	0.083	0.232	0.183	0.107	0.083	0.155	0.217	0.093	0.196
	-	-	-	-	-	-	-	-	0.017	-	-	-	-
	0.69	-	-	-	0.067	0.054	0.1	-	0.117	0.034	0.033	0.037	0.065
	-	-	-	-	0.017	-	-	0.018	0.017	0.017	-	0.019	-
CAM2	1	0.357	0.188	0.34	0.25	0.482	0.217	0.304	0.276	0.19	0.35	0.36	0.087
	-	-	-	-	-	-	0.017	0.018	0.069	-	0.017	-	0.043
	-	0.161	0.063	0.04	0.033	0.054	0.083	0.018	0.034	0.052	0.033	0.02	0.217
	-	-	-	-	-	-	0.033	-	-	-	-	-	-
	-	0.089	0.083	0.1	0.2	0.125	0.25	0.268	0.224	0.19	0.233	0.04	0.196
	-	-	-	-	0.05	-	-	-	-	-	-	-	-
	-	-	0.021	-	0.133	0.107	0.167	0.125	0.086	0.121	0.1	0.16	0.065
	-	-	-	-	0.033	0.054	0.067	0.054	0.034	0.052	0.017	0.06	0.065
	-	0.375	0.417	0.5	0.05	0.036	-	0.054	0.034	0.052	0.033	0.04	0.043
	-	-	-	-	-	0.036	0.05	0.018	0.086	0.103	-	-	-
	-	0.018	0.229	0.02	0.133	0.071	0.067	0.107	0.069	0.172	0.183	0.18	0.065
	-	-	-	-	0.067	-	-	0.018	0.052	0.034	-	0.06	-
	-	-	-	-	0.033	0.036	-	0.018	0.017	0.017	0.017	0.06	0.217
	-	-	-	-	0.017	-	0.05	-	0.017	0.017	0.017	0.02	-
CAM23	-	-	-	-	0.1	0.089	0.1	0.017	0.1	0.034	0.083	-	-
	-	-	-	-	-	0.054	-	-	-	-	-	-	-

	-	-	-	-	0.017	0.036	0.017	0.034	0.017	0.052	0.05	0.115	-
	-	-	-	-	-	-	-	-	-	0.052	-	0.038	0.087
	-	-	-	-	-	0.018	-	0.017	0.083	0.017	-	0.038	0.043
	0.414	-	-	-	0.067	0.089	0.3	0.31	0.267	0.224	0.217	0.154	0.348
	-	-	-	-	0.067	0.036	-	-	-	-	0.033	0.019	-
	0.517	0.259	0.458	0.6	0.633	0.464	0.333	0.293	0.333	0.414	0.3	0.365	0.217
	0.069	0.741	0.542	0.4	0.083	0.125	0.217	0.19	0.1	0.103	0.2	0.173	0.152
	-	-	-	-	-	0.054	0.033	0.103	0.05	0.034	0.067	0.038	0.043
	-	-	-	-	0.033	-	-	0.017	0.05	0.017	0.05	0.058	0.109
	-	-	-	-	-	0.036	-	0.017	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	0.017	-	-	-
	-	-	-	-	-	-	-	-	-	0.034	-	-	-
CAM4	0.638	-	0.02	-	0.267	0.25	0.183	0.268	0.2	0.207	0.183	0.2	0.13
	-	-	-	-	0.133	0.036	0.05	0.018	0.233	0.069	0.017	0.16	0.196
	0.31	0.862	0.56	0.54	0.45	0.571	0.583	0.5	0.4	0.603	0.633	0.48	0.565
	0.052	0.138	0.42	0.46	0.15	0.143	0.183	0.214	0.167	0.121	0.167	0.16	0.109
CAM8	0.036	0.396	0.717	0.568	0.317	0.444	0.65	0.259	0.417	0.393	0.467	0.313	0.391
	0.964	0.604	0.283	0.432	0.683	0.556	0.35	0.741	0.583	0.607	0.533	0.688	0.609
PDO47	-	-	-	-	0.083	0.018	-	-	0.017	0.017	0.052	-	-
	0.086	-	-	-	0.033	0.054	-	0.036	0.017	-	0.034	0.017	0.022
	0.5	-	-	-	0.05	0.25	0.121	0.089	0.083	0.052	0.017	0.117	0.043
	-	-	-	-	0.15	-	0.052	-	0.067	0.069	0.069	-	0.043
PDO47	-	-	-	0.058	-	-	-	-	-	-	-	-	-
	-	-	-	-	0.067	0.018	0.034	0.071	0.017	0.017	0.034	0.017	0.022
	-	0.431	0.375	0.192	0.1	0.107	0.19	0.036	0.05	0.052	0.155	0.033	0.217
	0.345	0.414	0.417	0.615	0.117	0.196	0.19	0.143	0.217	0.259	0.103	0.267	0.109
	-	0.052	-	-	0.083	0.071	0.155	0.107	0.1	0.103	0.052	0.183	0.174
	0.069	0.103	0.167	0.135	0.083	0.107	0.052	0.179	0.117	0.138	0.155	0.117	0.283
	-	-	-	-	0.033	-	0.034	0.036	0.017	0.034	0.017	0.017	-
	-	-	-	-	0.067	0.036	0.052	-	0.05	0.017	0.069	0.033	0.022
	-	-	0.042	-	0.05	0.054	0.069	0.107	0.1	0.086	0.103	0.067	0.065
	-	-	-	-	0.017	0.018	0.017	0.054	0.033	0.017	-	0.083	-
	-	-	-	-	0.033	0.018	-	0.036	0.067	0.069	0.052	0.033	-
	-	-	-	-	0.017	0.036	0.034	-	0.033	0.017	0.034	-	-
	-	-	-	-	0.017	0.018	-	0.089	0.017	0.052	0.052	0.017	-
	-	-	-	-	-	-	-	0.018	-	-	-	-	-
PPI2	-	-	-	-	-	0.037	-	-	0.017	-	0.093	-	-
	-	-	-	-	-	0.037	-	-	0.017	-	-	-	-
	-	-	-	-	0.083	0.019	0.069	0.052	0.083	0.052	0.037	0.093	0.022
	0.267	0.37	0.318	0.5	0.367	0.222	0.19	0.19	0.25	0.241	0.167	0.093	0.37
	-	0.037	0.091	0.045	0.033	0.148	0.086	0.069	0.067	0.086	0.056	0.111	0.065

	-	0.019	-	-	0.217	0.259	0.241	0.138	0.117	0.155	0.074	0.111	0.152
	0.2	0.037	0.159	0.091	-	0.037	0.052	0.034	0.017	0.017	0.037	0.019	0.022
	0.067	0.296	0.364	0.318	0.183	0.056	0.259	0.328	0.217	0.259	0.259	0.426	0.239
	-	-	0.045	0.045	0.083	0.13	0.017	0.052	0.067	0.069	0.093	0.148	-
	0.117	0.222	-	-	0.017	0.019	0.017	0.086	0.083	0.052	0.074	-	0.022
	0.35	-	-	-	0.017	0.019	0.034	-	-	-	-	-	0.043
	-	-	-	-	-	-	0.017	0.017	0.017	0.017	0.019	-	0.065
PPI2	-	0.019	0.023	-	-	-	-	-	0.033	0.017	0.019	-	-
	-	-	-	-	-	-	-	-	-	-	0.019	-	-
	-	-	-	-	-	-	-	0.017	-	-	-	-	-
	-	-	-	-	-	0.019	0.017	0.017	0.017	0.034	0.056	-	-
CAM18	-	-	-	-	-	-	-	-	0.033	-	0.056	-	0.043
	0.143	0.452	0.615	0.8	0.411	0.241	0.362	0.315	0.45	0.446	0.407	0.185	0.239
	0.857	0.548	0.385	0.167	0.375	0.463	0.483	0.537	0.333	0.429	0.444	0.519	0.543
	-	-	-	0.033	0.214	0.296	0.155	0.148	0.183	0.125	0.093	0.296	0.174
PDO5	-	0.224	0.104	0.091	0.15	0.231	0.283	0.111	0.1	0.25	0.2	0.148	0.065
	-	-	-	-	0.1	0.038	0.05	0.019	0.017	0.036	-	0.037	0.065
	-	-	-	-	-	-	-	-	-	-	-	0.019	-
	1	0.759	0.771	0.841	0.617	0.538	0.55	0.611	0.75	0.5	0.65	0.593	0.609
	-	0.017	0.125	0.068	0.1	0.077	0.05	0.037	0.017	0.089	0.083	0.019	0.065
	-	-	-	-	0.033	0.019	0.067	0.037	0.067	0.036	-	0.111	0.022
	-	-	-	-	-	0.096	-	0.148	0.05	0.071	0.067	0.074	0.174
	-	-	-	-	-	-	-	-	-	0.018	-	-	-
	-	-	-	-	-	-	-	0.037	-	-	-	-	-
HRU5	0.741	0.367	0.577	0.481	0.617	0.732	0.617	0.586	0.655	0.638	0.417	0.531	0.63
	0.259	0.633	0.423	0.519	0.383	0.268	0.383	0.414	0.345	0.362	0.583	0.469	0.37