Molecular evolution and population genetics of the *Nesospiza* buntings

by

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ABSTRACT

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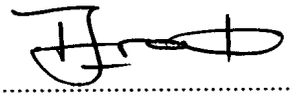
Nesospiza is a genus of buntings restricted to the Tristan da Cunha Islands in the central South Atlantic Ocean. They have undergone an adaptive radiation at the islands and currently two species are recognised based on morphology: the small-billed Tristan bunting (*N. acunhae*), which is a dietary generalist and the large-billed Wilkins' bunting (*N. wilkinsi*), a dietary specialist. Both species occur on Nightingale Island with no apparent hybridisation between them. On the neighbouring Inaccessible Island there appears to have been a breakdown of the species barrier and the two species hybridise extensively. Also two altitudinally segregated colour morphs of *N. a. acunhae* occur on Inaccessible Island. The morphological differentiation of *Nesospiza* is not reflected in either the mitochondrial DNA or the microsatellite data. Rather the data suggest that there are two island lineages and that the sympatric populations on each island are more closely related to each other than to their allopatric (presumed conspecific) island neighbours. The molecular data support sympatric speciation with parallel evolution in *Nesospiza,* possibly as a result of divergent selection, acting on the sympatric populations on each island, which could have resulted from a change in feeding ecology. Furthermore the molecular data differentiate between the two sympatric colour morphs of *N. a. acunhae*, which appear to be speciating as a result of assortative mating.

DECLARATION

I declare that the work presented in this thesis is my own, unaided work, except as acknowledged in the text. It is submitted as the requirement for the degree Master of Science at the University of Pretoria, Pretoria, South Africa. It has not been submitted for any degree or examination at this or any other university.



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Signed on this 1st day of March 2004

DEDICATION

For my parents

Thanks for your unconditional love, support and understanding

ACKNOWLEDGEMENTS

The duration of my masters was like taking a long road trip in an old car. At the start of the trip the car was in relatively good working condition and rearing to go. The itinerary had been meticulously planned and it seemed as if this was going to be a pleasant journey. Life however, always seems to throw one a curve ball. After all the careful planning there were some roads that had been closed or ended up being dead ends and thus a lot of backtracking was done and a few major detours were also taken. The secondary roads were often unchartered and full of potholes. The car thus took a major beating and on one occasion the wheel fell off and on another the engine seized. There were always however friendly mechanics, petrol attendants and fellow motorists who helped repair the car when it broke and set it on its journey again.

I would like to thank the chief mechanics (my supervisors) Paulette Bloomer and Peter Ryan for their invaluable advice, support and expertise. I am thankful for the opportunities that both of you have afforded me and sincerely appreciate your comments on the earlier drafts of my thesis.

To friendly passers by who came out of nowhere and where willing to assist, I am eternally grateful: Kenneth Petren, Michael Sorenson and Kristina Sefc for supplying the *Geospiza* and *Vidua* microsatellite loci. To Leo Joseph thanks for the *Melanodera* foot scrapings. Also to Kevin Burns for slotting my cytochrome b sequences in with your phylogeny to give me an idea of what was going on.

To my fellow motorists (MEEP members) who where taking similar trips with different destinations thanks for the moral support and insightful suggestions whenever I experienced major setbacks or obstacles in my research. I would specifically like to single out the following people: Wayne Delport who always seemed to be able to keep me positive whenever I was ready to concede defeat and for all of his assistance could probably qualify for mechanic status, Ute Kryger and James Sakwa for your assistance with the microsatellite analyses, Isa-Rita Russo for affording me the opportunity to get out into the field every now and again and for the use of your computer whenever mine decided to have an aneurysm. Also to Ernst Swartz, Heidi Roos, Lisel Solms, and all the other MEEP members thanks for your friendship and your willingness to discuss the dynamics of my project with me.

Money to put petrol in the car came from the National Research Foundation, University of Pretoria and National Geographic Society. Without this support the goals of this project would never have been realised.

To all the backseat drivers (my friends) from school, varsity and Boston BBQ you can all finally stop asking me when I am going to reach my final destination! Thanks for all the encouragement and support. To my girlfriend, Suretha thanks for being so understanding with your time, for supporting me and keeping me motivated throughout the last three years. My brother Clive thanks for always believing in me and encouraging me to finish.

My parents (the petrol attendants), you always made sure that there was more than enough petrol in the car and always ensured that it was clean and had a place to park at night. I cannot thank you enough. Thanks for your unwavering love and support and I am grateful that you put up with me for so long. Without your monetary assistance, the roof over my head and your motivation I don't think I could have ever finished this work or this journey.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

Ever since Charles Darwin developed his theory of evolution by natural selection based on his studies of natural populations of organisms (Darwin 1859), islands have become model environments for the study of evolution. Oceanic islands are ideal environments in which to study evolution because they are distinct geographical units with defined oceanic boundaries. For most terrestrial organisms the ocean is a formidable dispersal barrier, limiting gene flow between islands. As a result, island faunas and floras are relatively depauperate; often with vacant niches which promote evolutionary innovations among the organisms occurring on them (Emerson 2002). The small and isolated populations on islands have the potential for rapid evolutionary change and have all of the available niches on the island at their disposal, due to lower rates of interspecific competition. Natural populations on remote oceanic islands thus provide an excellent opportunity to study the processes of natural selection, speciation and microevolution (Grant 1979).

Inferences from environmental, geographical and ecological factors provide valuable insights into evolutionary processes (Grant 1993, Grant and Grant 1995) and have played a vital role in the development of biogeographical and evolutionary theories. Most studies that provide adaptive explanations for observed patterns have tended to focus on correlations between ecological and morphological variation between island populations. More recently however, the focus has shifted towards the analysis of genealogical relationships between species, using molecular markers, to better understand the forces driving the evolutionary process and to reconstruct the historical dispersal events that explain the current distribution of closely related island taxa (Avise *et al.* 1987, Avise 1992, Freeland and Boag 1999, Marshall and Baker 1999, Petren *et al.* 1999, Malhotra and Thorpe 2000, Ciofi *et al.* 2002, Clegg *et al.* 2002a, Holland and Hadfield 2002).

The *Nesospiza* buntings of the Tristan da Cunha islands provide a classic example of an adaptive radiation at an oceanic archipelago (Abbott 1978). The ecology and morphology of the buntings have been extensively studied (Ryan 1992, Ryan *et al.* 1994, Ryan 2001, Ryan and Moloney 2002) and the purpose of the present study was to elucidate the genetic relationships between these birds with a view to reconstructing past events which have led to the present diversity and distribution.

1.2 Taxonomic position

Nesospiza is a genus restricted to the Tristan da Cunha group of islands, which is assumed to be most closely related to *Rowettia*, a monospecific genus occurring on Gough Island some 320 km south west of the Tristan da Cunha group (Abbott 1978). Sibley and Monroe (1990) re-arranged the

subfamily Emberizinae and placed *Nesospiza* and *Rowettia* in the subfamily Thraupinae, which includes the tropical American finches, grass-quits, seed-eaters and all of the tanagers and honeycreepers. It is a wholly central and South American group with island forms occurring on the Galápagos and in the Caribbean. The closest extant relatives of *Rowettia* and *Nesospiza* are believed to be the Patagonian genus *Melanodera*, based on plumage similarities between *Rowettia* and *Melanodera* (Rand 1955).

It has been hypothesised that the ancestors of *Nesospiza* arrived on the islands by prevailing winds from South America and established a breeding population (Lack 1947). In much the same way as Darwin's finches (Grant 1986) and the Hawaiian honeycreepers (Raikow 1976, Olson and James 1982), *Nesospiza* buntings have undergone an adaptive radiation at Tristan da Cunha. The radiation has not been as extensive however, due to small island size, low habitat diversity and the small number of islands. The adaptive radiation at Tristan da Cunha has resulted in two morphologically well defined species: the small, abundant, small-billed Tristan bunting (*Nesospiza acunhae*) which is a habitat generalist and the large, scarce, thick-billed Wilkins' or Grosbeak bunting (*N. wilkinsi*), a habitat specialist, feeding mainly on the seeds of the *Phylica* trees which occur on the islands (Collar and Stuart 1985). Due to the apparent simplicity of the radiation it has been widely quoted as a simple two species adaptive radiation at an oceanic island group (Lack 1947, Abbott 1978, Williamson 1981).

Both species of bunting co-occur on Inaccessible and Nightingale Islands with two distinct subspecies described for each species (Lowe 1923, Hagen 1952). Standard avian evolutionary theory (Lack 1947, Grant and Grant 1997) would suggest that the two species of bunting speciated in allopatry on Nightingale and Inaccessible Islands, with the ocean acting as an isolating barrier. Subsequent to the speciation event the new species colonised the island on which they did not occur (Ryan 1992). A population of Tristan buntings (*N. acunhae*) occurred on Tristan Island but became extinct at some point during the 19th century as a result of habitat alteration and the introduction of terrestrial predators such as rats and mice (Collar and Stuart 1985, Stattersfield *et al.* 1998).

1.3 The Tristan da Cunha Islands

The Tristan da Cunha group of islands lie in the South Atlantic Ocean some 2800 km from South Africa and about 3200 km from the closest point in South America (Figure 1.1A). The group consists of three oceanic islands; Tristan, Nightingale and Inaccessible, which lie fairly close together near 37°18'S, 12°41'W (Figure 1.1B). The islands lie east of the mid-Atlantic Ridge, and near the western end of the Walvis lateral ridge. They are the remains of the summits of three

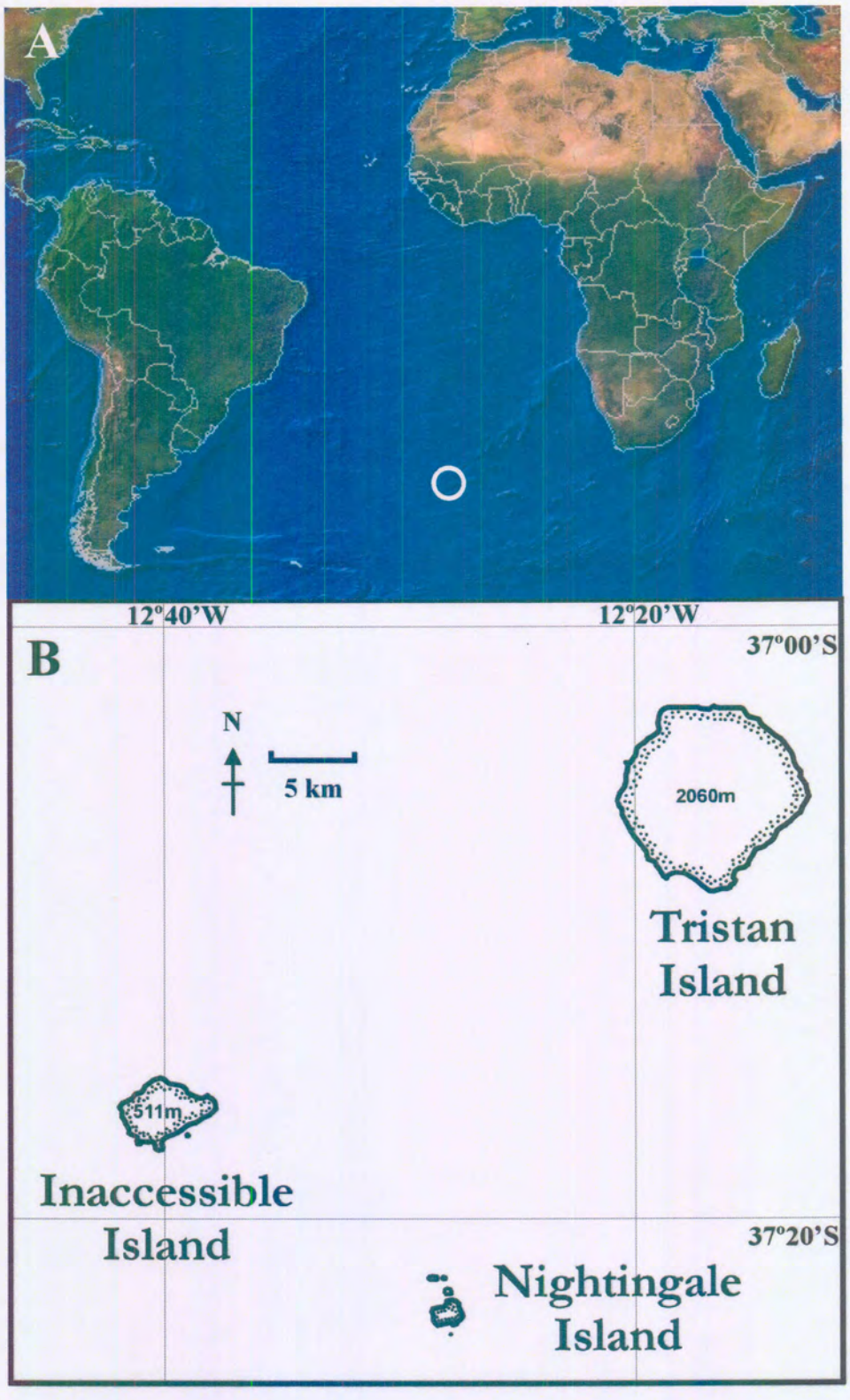


Figure 1.1: (A) Location of the Tristan da Cunha archipelago and Gough Island. The circle in the figure indicates the location of the Tristan da Cunha group near the site where the mid-Atlantic ridge and Walvis lateral ridge intersect (Map courtesy of National Geographic MapMachine). **(B)** The Tristan da Cunha group, Inaccessible Island and Nightingale Island support bunting fauna. Gough Island lies approximately 320 km SE of Tristan Island.

volcanoes and have never been connected above sea level or to a continental landmass (Baker *et al.* 1964). Maximum island ages estimated by potassium-argon dating are 200 000 years for Tristan, three million years for Inaccessible and 18 million years for Nightingale (Baker *et al.* 1964, Gass 1967, McDougall and Ollier 1982). Island size is inversely related to age with Tristan at 96 km^2, Inaccessible at 13 km^2 and Nightingale at 4 km^2. The climate of the Tristan islands is cool temperate oceanic, with fairly constant rainfall all year round and an annual mean of 1676 mm at the coast of Tristan and mean annual air temperature of 14.7°C, with relatively little seasonal variation (Wace and Holdgate 1976, Höflich 1984).

1.4 Vegetation and habitat

Plant (Roux *et al.* 1992) and animal (Wace and Holdgate 1976, Ryan *et al.* 1990, Glass and Ryan 2003) communities on the islands are fairly depauperate, as is the case with most oceanic islands, with only a few vegetation types, which are altitudinally segregated. Nightingale Island is covered in a single vegetation type consisting of dense *Spartina arundinacea* tussock grass with small groves of *Phylica arborea* around the Ponds and in some of the inland gullies (Figure 1.2A). Inaccessible Island supports three main types of vegetation. *Spartina arundinacea* tussock covers the rocky ground on the western and northeastern coasts and all of the coastal cliffs between sea level and 200 to 500 m altitude. This habitat type is interspersed with a few *Phylica arborea* trees, forming a similar habitat to that found at Nightingale Island. The two other main vegetation types occur on the higher lying plateau of Inaccessible Island (Figure 1.2B), the low-lying eastern part of the plateau supports a *Phylica* woodland and the higher lying western plateau contains mostly *Blechnum palmiforme* wet heath (Roux *et al.* 1992).

Tristan Island is the only permanently inhabited island in the group and has undergone significant habitat modification over the last 200 years as a result of human activity (Wace and Holdgate 1976, Richardson 1984). The uninhabited islands, Inaccessible and Nightingale are still fairly pristine, since there has been limited interference by man and relatively few species have been introduced. There are currently no introduced mammals and the few plant and invertebrate introductions appear to have had little influence on the ecosystems of these two islands (Wace and Holdgate 1976, Glass and Ryan 2003).

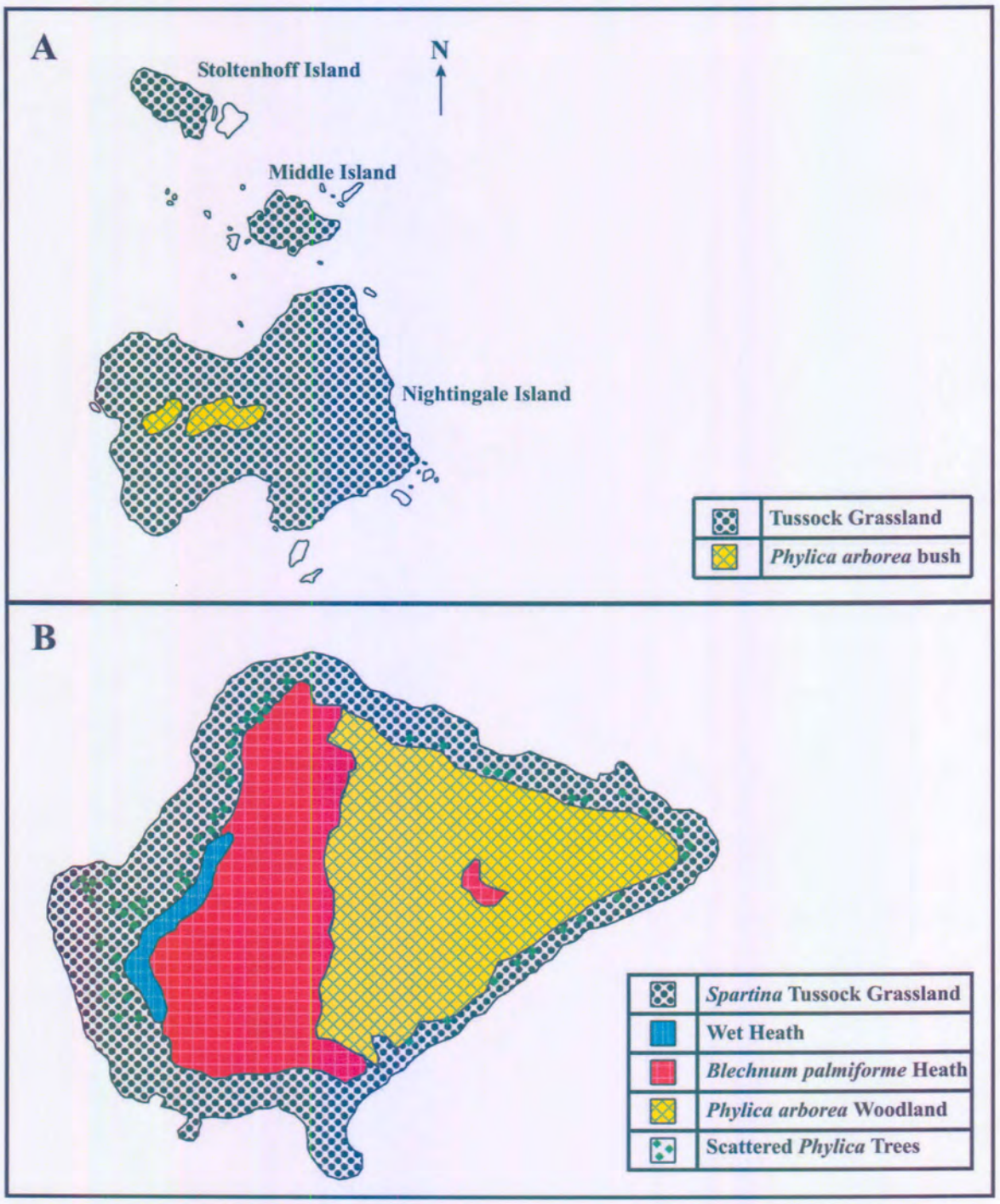


Figure 1.2: Distribution of the main vegetation types at **(A)** Nightingale Island and **(B)** Inaccessible Island, Tristan da Cunha (modified from Roux *et al.* 1992).

1.5 Nesospiza *revisited*

Inter-island movement of *Nesospiza* is believed to be infrequent due the threat posed by Subantarctic Skuas (*Catharacta antarctica*), which regularly feed on buntings (Ryan 1992). A bunting in flight between two islands would make an easy target for the skuas, thus most birds attempting to travel the 22 km between the two islands are unsuccessful (Fraser 1984, Ryan and Moloney 1991). A single Tristan bunting collected during the sampling for this study at Nightingale Island was 'larger' than all of the 'regular' Nightingale Tristan buntings indicating that it could possibly have been a migrant from Inaccessible Island (P.G. Ryan pers. comm.).

Both species of bunting occur on the two uninhabited islands (Table 1.1). On Nightingale Island they co-occur with overlapping distributions and do not appear to hybridise. The small size and simple habitat structure of Nightingale Island appears to have led to only a single bunting assemblage there. The larger size and more diverse vegetation of Inaccessible Island supports more ecological niches than Nightingale Island, and the situation is thus far more complex.

Table 1.1: Sub-specific taxonomy of *Nesospiza* buntings.

	Wilkins' Bunting	Tristan Bunting
Nightingale Island	*N. w. wilkinsi*	*N. a. questi*
Inaccessible Island	*N. w. dunnei*	"Upland" *N. a. acunhae**
		"Lowland" *N. a. acunhae**
Tristan Island	No known records	*N. a. acunhae* (Extinct)

* The populations on Inaccessible Island are allied to the type specimen from Tristan Island; there is however insufficient material surviving from this population to confirm that they are necessarily the same subspecies (Rand 1955).

During the Denstone Expedition to Inaccessible Island in 1982-1983 (Swales *et al.* 1985) two altitudinally and habitat segregated morphs of the Tristan bunting *N. a. acunhae* were identified (Fraser and Briggs 1992). The upland forms, occurring on the summit plateau, are brighter than those found on the coastal cliffs and at sea level and have larger relative body sizes and smaller bills, than the lowland morph (Ryan 1992). Variation in body size between morphs is consistent with the observed tendency of body sizes to increase with an increase in altitude (Moreau 1957, James 1970, Gill 1973), whereas differences in bill size mirror differences in seed sizes in their respective habitats (Ryan 1992). Age-related plumage differences were ruled out as a possible cause for the differences since the nestlings also showed large amounts of variation, the most notable being that

lowland birds have pink chicks and upland forms have yellowish chicks. The breeding ranges of the two morphs are parapatric with each found in different habitat types, namely the *Spartina* tussock grassland on the costal lowlands and the *Blechnum palmiforme* wet heath on the plateau. The colour differences between the two morphs of Tristan bunting have been found to be of dietary origin (Ryan *et al.* 1994) and appear to be promoting evolutionary differentiation of the birds as a result of assortative mating based on plumage colouration (Ryan 1992, Ryan *et al.* 1994).

The coastal cliffs of Inaccessible Island support a mosaic of *Spartina* tussock and *Phylica arborea,* forming a habitat type similar to that on Nightingale Island. Due to this similarity in habitat type it is not surprising that both *N. w. dunnei* and lowland *N. a. acunhae* coexist on the coastal cliffs of Inaccessible, with no apparent hybridisation between them. Small *Spartina* grass seeds and large *Phylica* fruits dominate the diets of the buntings in this habitat type (Figure 1.3A). The distribution of taxa thus mirrors seed availability and density with the small-billed *N. a. acunhae* feeding mainly on *Spartina* and the large-billed *N. w. dunnei* using their massive bills to feed on the *Phylica* fruits (Ryan 1992).

On the western plateau of Inaccessible Island the absence of *Phylica* trees accounts for the absence of *N. w. dunnei* on this part of the island. The habitat in this area is dominated by *Blechnum palmiforme* and several sedge grasses resulting in only small seeds being available for the buntings to feed on (Figure 1.3B). Due to this only upland *N. a. acunhae* occur on this part of the island. The smaller size of the sedge seeds relative to the *Spartina* seeds on the western plateau accounts for the smaller bill sizes of the larger-bodied upland *N. a. acunhae* morphs (Ryan 1992, Ryan *et al.* 1994).

The eastern plateau of Inaccessible Island supports a *Phylica arborea* woodland where there appears to be a breakdown in the species barrier between *N. w. dunnei* and upland *N. a. acunhae* as on this part of the island they frequently interbreed and produce fertile hybrids (Ryan 1992, Ryan *et al.* 1994). In Darwin's finches hybridisation has been shown to occur occasionally between species with no apparent loss of fitness (Grant and Grant 1992, 1993). It has further been found that hybridisation among bird species may lead to the formation of new species due to an increase in morphological variation, resulting in a few individuals who can specialise their diets and explore new ecological niches (Grant and Grant 1994).

Frequent hybridisation between bird species over large parts of their distributions is relatively rare and hybridisation events are usually associated with linear contact zones between species or subspecies (Moore 1977, Barton and Hewitt 1985). There have however been a few

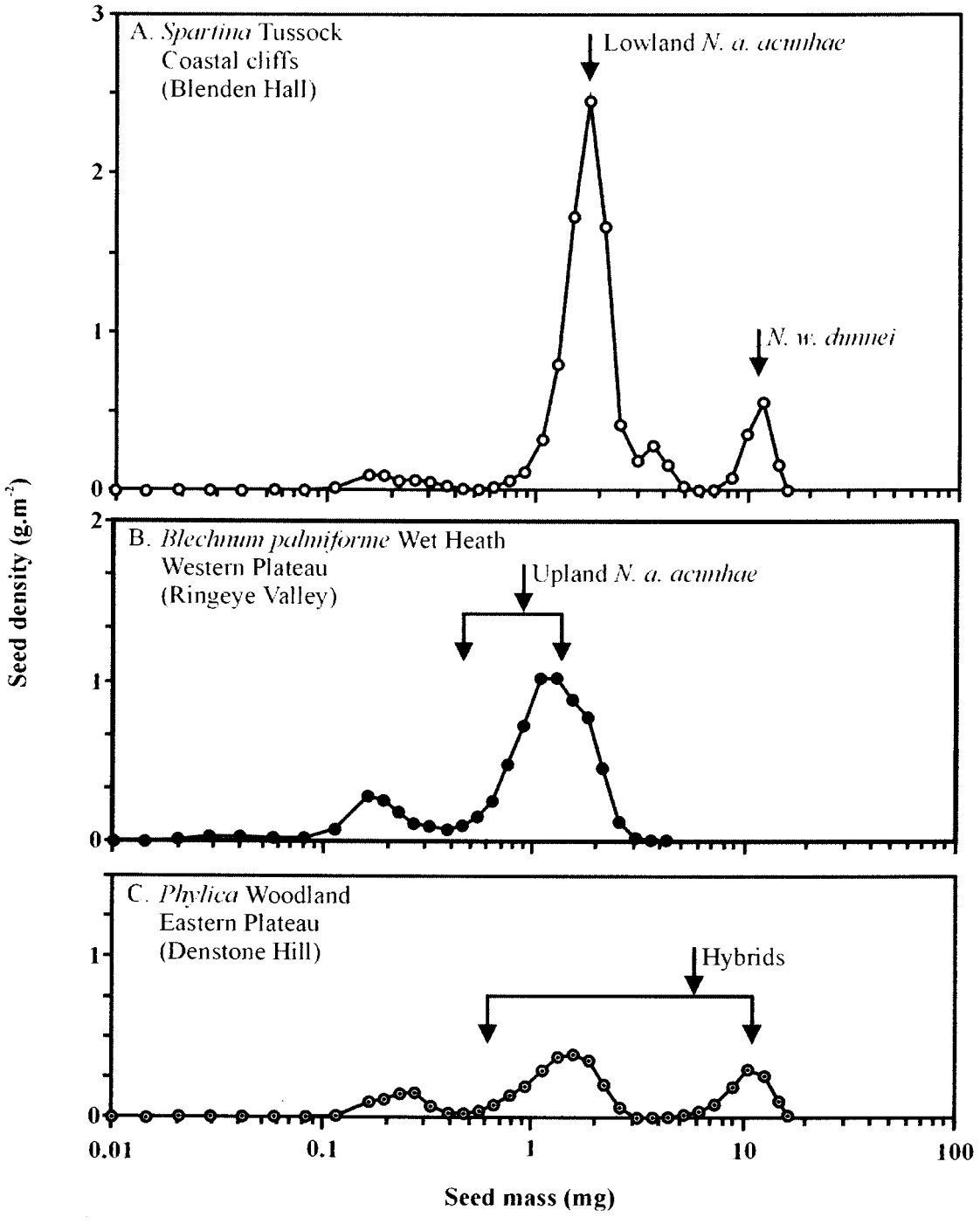


Figure 1.3: Seed profiles of the three main vegetation types at Inaccessible Island, depicting densities of seeds of different sizes (excluding species not recorded in bunting diets). Arrows indicate median seed sizes in the diet of *Nesospiza* taxa found in each habitat. Median values were calculated in terms of numerical and mass contribution to the diet; where these differed (B and C), both are shown, together with the arithmetic mean of the two values. In both cases where the two do not coincide numerical median<mass median (modified from Ryan 1992).

documented instances of bird species hybridising without restraint over large parts of their range (Sibley 1954, Mayr and Short 1970). The frequency of hybridisation at Inaccessible Island and the fact that it is largely restricted to one habitat type differs greatly from the relatively rare hybridisation amongst Darwin's finches (Grant 1993).

The plumage of the *Nesospiza* hybrids closely resembles that of the upland Tristan buntings (upland *N. a. acunhae*). Hybridisation may be adaptive on the eastern plateau, where seed abundance is far lower than in the other habitat types; overall seed density on this part of the island is lower than on the coastal cliffs and western plateau and the peaks for seed availability are less well defined than in the other habitat types (Figure 1.3C). Coupled with the large range of hybrid bill sizes, this may allow the hybrids to utilise the full range of seed sizes in this habitat type, which would otherwise not be able to support sympatric populations of large and small-billed buntings (Ryan 1992). Hybridisation may therefore be adaptive and thus favourable in terms of maximum utilisation of the available resources in this habitat type. The reproductive success of the *Nesospiza* hybrids is equal to or greater than that of the other taxa (Ryan 1992); this observation is in agreement with Moore's (1977) hybrid superiority hypothesis.

The situation at Inaccessible Island can thus be summarised as follows: upland and lowland morphs of *N. a. acunhae*, which can be distinguished from each other by plumage colouration and body size, occur and breed assortatively. The two morphs are known to hybridise, but this is infrequent (Ryan 1992). Wilkins' buntings are found on all parts of the island that support *Phylica* trees, and are scarce on the treeless western plateau. On the eastern plateau of Inaccessible Island *N. w. dunnei* hybridise extensively with the upland forms of *N. a. acunhae*. The hybrid offspring are fully fertile and previously did not appear to be breeding outside of the hybrid zone (Ryan 1992). Hybridisation thus appears to be the best adaptive solution for maximum utilisation of available resources. A recent field survey (1999–2000) found that hybrids were found to be breeding more extensively outside of the hybrid zone (P.G. Ryan pers. comm).

Morphological variation between the different *Nesospiza* populations with regards to size and shape is evident from morphological character measurements. Wilkins' buntings are considerably larger than Tristan buntings for all characters (Figure 1.4, see Ryan 1992). Multivariate analysis clearly shows the extent of morphological differences between the different populations (Figure 1.4). The size differences between the different populations are largest at Nightingale Island where the largest subspecies of *N. wilkinsi* and the smallest subspecies of

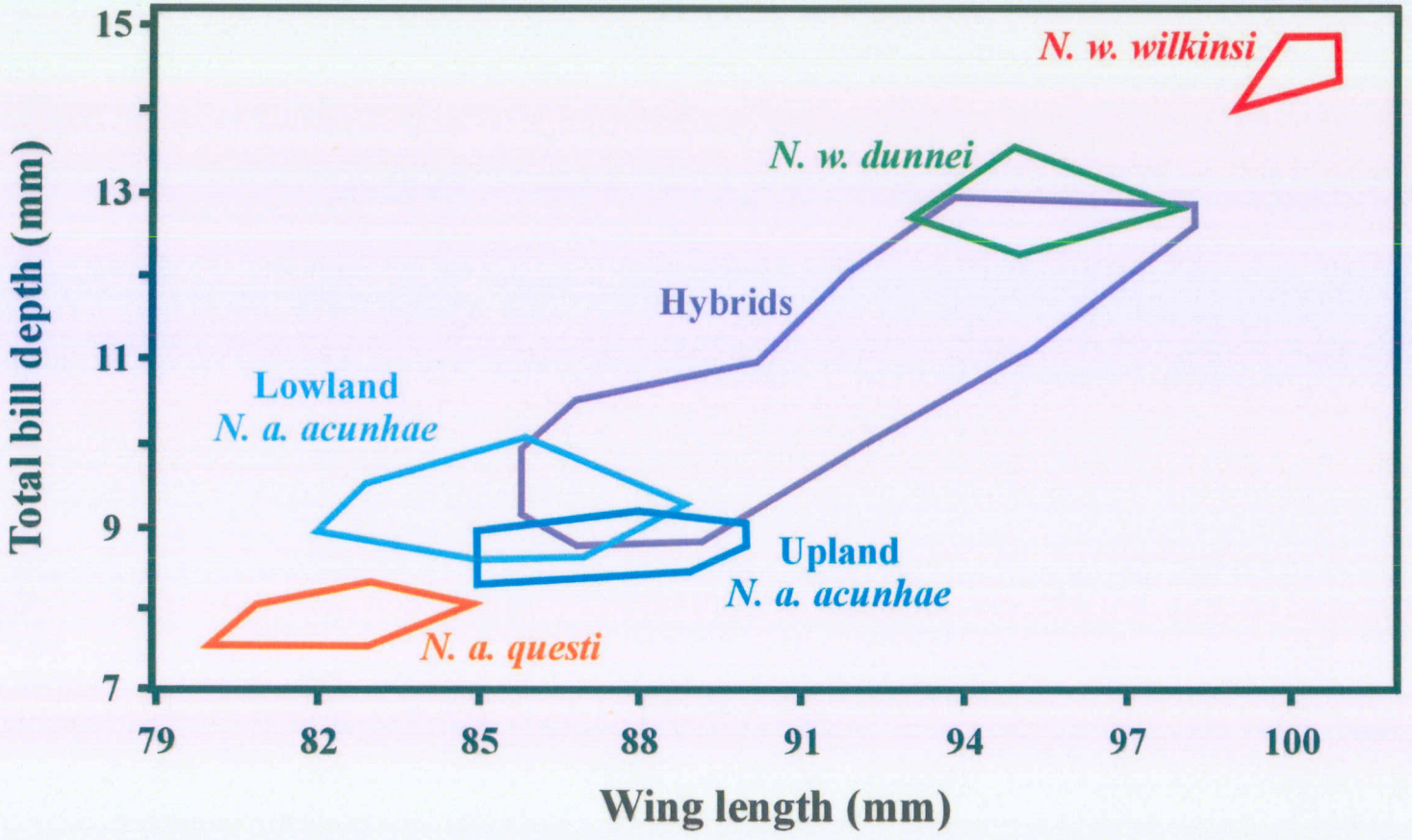


Figure 1.4: Biplot of total bill depth as a function of wing length among male *Nesospiza*. Polygons incorporate all measured individuals in each taxon. The purple polygon represents the hybrids between *N. w. dunnei* and upland *N. a. acunhae* (modified from Ryan 1992).

N. acunhae occur. At Inaccessible Island interspecific size differences are less noticeable but still remain extensive. The two *N. a. acunhae* colour morphs show considerable overlap in characters but are however still significantly differentiated in terms of size. Upland birds are characterised by larger overall body sizes and smaller bills than the lowland birds, which show smaller body sizes along with larger bill sizes. Interspecific hybrids exhibit character sizes covering the entire range of character space between upland *N. a. acunhae* and *N. w. dunnei* (Ryan 1992).

1.6 The significance of bill size differences

The occurrence of sympatric large and small-billed bird species is by no means unique to Tristan da Cunha (or *Nesospiza*); rather it has been shown to be the outcome of natural selection in several bird species. Natural selection, the driving force behind evolution, is the process that leads to the adaptation of organisms to their specific environments. If environments differ, natural selection by means of adaptation may lead to large-scale genetic divergence between subpopulations (provided there is little or no introgression) with the result that the organisms may speciate. If on the other hand environments are similar; then natural selection by adaptation may result in the prevention of genetic divergence between subpopulations (Hartl 1998). Speciation occurs due to selection of morphological characters that allow populations to utilise different niches and thus speciate. In several granivorous passerines bill size differences appear to be strongly selected for and are correlated with differences in feeding ecology: larger bills allow species to exploit larger and harder seeds (Grant 1986, Smith 1990, Grapputo *et al.* 1998, Matessi *et al.* 2002). Changes in bill depth and width usually implies a modification of function (Gould 1966) and in the case of *Nesospiza* it is consistent with the dietary differences between the Tristan and Wilkins' buntings.

Inter- and intraspecific variation in bill size in the Darwin's finches (*Geospiza*) has been studied intensively (Grant 1986). Interspecific variation in bill size is greater than intraspecific variation, but hybridisation and oscillating selection causes bill depth to vary intraspecifically in relation to changing climatic conditions. Directional selection favours birds with larger bills in drier years when they can utilize larger and harder seeds, than their smaller billed counterparts. However, in wet years an abundance of small seeds favours smaller billed birds. The African finch (*Pyrenestes ostrynus*) (Smith 1987, 1990) and reed bunting (*Emberiza schoeniclus*) (Grapputo *et al.* 1998) also display variations in bill size, with sympatric forms that utilise different seed types. In these cases it appears that disruptive selection has resulted in the evolution of two bill morphs, reducing intraspecific competition for food by allowing the different morphs to utilise different niches (Smith 1990; Matessi *et al.* 2002).

1.7 Gough Island and Rowettia

Gough Island lies about 320 km south-south-east of the Tristan da Cunha archipelago at 40°18'S, 9°56'W. Its annual rainfall is far higher than the Tristan da Cunha group with a mean of 3154 mm and a mean annual temperature of 11.3°C (Wace and Holdgate 1976, Jones *et al.* 2003). Gough Island has a single endemic bunting species *Rowettia goughensis*. *Rowettia* is a monospecific genus, which is presumed to have shared a common ancestor with *Nesospiza* at some point in the past. Likelihood of movement would suggest that the two genera resulted from separate colonisation events rather than as a result of island hopping (Ryan 1992). *Rowettia* is also the only passerine on Gough Island and is a generalist in the true sense of the word (Collar and Stuart 1985).

1.8 Speciation and the concept of species

Biologists have long sought ways to compartmentalise natural biological diversity by means of the systematic classification of organisms. However, due to the complex nature of natural systems, this is not always possible because species continuously evolve and adapt to their environments. The processes by which *Nesospiza* speciated are of interest for the present study, however these processes can only be understood within the context of a workable species definition. Species concepts thus form an integral part in the understanding of speciation and without a conceptualisation or definition of what a species is, it is almost impossible to describe the processes that lead to the creation of new species. Table 1.2 gives the definitions for the species concepts most commonly used by biologists.

In the General Lineage Concept of Species (de Querioz 1998) it is argued that all modern species concepts are variations on the same general theme although there are some fundamental differences between the different concepts. Isolation and cohesion are two important facets of the Biological Species Concept (BSC) and the original definitions of Mayr (1963) and Dobzhansky (1940, 1970) both refer to these two requirements. Templeton (1989) however went one-step further and defined species as inclusive groups recognising genetic exchangeability as well as ecological exchangeability as important requirements for defining species. The Cohesion Concept (Templeton 1989) comprises of several testable hypotheses dealing with morphological and molecular discrimination between groups as well as gene flow between populations and hybridisation.

The BSC (Mayr 1963, Dobzhansky 1970), Recognition (Paterson 1985) and Cohesion Concepts (Templeton 1989) are all strongly motivated by the process of speciation, and specifically by the mechanisms that result in isolation and cohesion and allow patterns of variation to be predicted. In these models, processes generate patterns (Templeton 1989). The Phylogenetic Species

Table 1.2: Various species concepts and definitions (modified from Avise 1993, Harrison 1998).

1. Biological Species Concept "Groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr 1963) "Systems of populations, the gene exchange between these systems is limited or prevented in nature by a reproductive isolating mechanism or by a combination of such mechanisms" (Dobzhansky 1970)
2. Cohesion Species Concept "The most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms" (Templeton 1989)
3. Recognition Concept "The most inclusive population of individual bi-parental organisms which share a common fertilisation system " (Paterson 1985)
3. Phylogenetic Species Concept A monophyletic group composed of "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent" (Cracraft 1983)
4. Genealogical Species Concept "Exclusive groups of organisms, where an exclusive group is one whose members are all more closely related to each other than to any organisms outside the group" (Baum and Shaw 1995)

Concept on the other hand uses a character-based approach and thus places more emphasis on observed patterns of variation (Cracraft 1989). However, in the end all species concepts eventually rely on inference of processes from observed patterns of variation (Harrison 1998).

The General Lineage Concept of Species (de Querioz 1998) shows that when species concepts are viewed as descriptions of species there is not much agreement between the different concepts. When species concepts are broken down into species criteria however, there is a large amount of concordance between the different concepts. Each criterion of a specific concept provides different information with regards to the separation of lineages or it describes a different stage in the divergence of lineages (de Querioz 1998).

The fundamental problem with species concepts and definitions occurs because speciation takes place over a continuum, thus not all species concepts will necessarily be applicable at any specific point in the evolution of a species. In support of this, Harrison (1998) proposed that species have a life history, which depends on geography, demography, natural selection and cohesion. Depending on when a study is undertaken during this 'life history' the species studied will conform to different requirements of the different species concepts.

Speciation may be initiated when populations are isolated (allopatric speciation) or perhaps more rarely, when they remain in contact (sympatric speciation). Allopatric speciation, especially in birds (Grant and Grant 1997), is believed by many to be the only workable mechanism of speciation (Mayr 1963). Allopatric divergence assumes that new species arise from geographically isolated populations of the same ancestral species (Mayr 1963). Sympatric speciation (where new species arise without being geographically isolated) has often been dismissed as not being plausible, although recently there has been an ever-increasing body of empirical and theoretical work supporting the likelihood of sympatric speciation in vertebrates (Schliewen *et al.* 1994, Johannesson *et al.* 1995, Dieckmann and Doebeli 1999, Schliewen *et al.* 2001, Via 2001) including birds (Sorenson *et al.* 2003), albeit under rather specific conditions. There is however no empirical support for sympatric speciation occurring among island birds, with no known radiations occurring among birds at small, single islands (Grant 1986). In some bird species (e.g. island silvereyes *Zosterops lateralis*) successive colonisation events have been shown to lead to multiple species at some islands (Clegg *et al.* 2002b).

1.9 Molecular markers

In this study I used two genetic markers to clarify the genetic relationships among *Nesospiza* subpopulations: mitochondrial DNA (mtDNA) cytochrome b sequences and size variation at nuclear DNA microsatellite loci. These markers have contrasting modes of inheritance and mutation and are under different selection pressures. Thus each marker system has a different evolutionary history to the other, allowing a more holistic interpretation of the genetic relationships.

In animals mtDNA is a double-stranded, extra-nuclear, closed circular, independently replicating molecule of approximately 16-20 kb in length (Avise 1986). The mtDNA molecule is made up of 37 genes, classified into 13 protein coding messenger RNAs, 22 transfer RNAs, two genes encoding ribosomal RNAs and a hyper-variable, non-coding control region (Boore 1999). Mitochondrial DNA is the molecule of choice in phylogenetic studies for elucidating evolutionary relationships between closely related species and between populations of the same species (Avise *et al.* 1987, Moritz *et al.* 1987, Harrison 1989). Mitochondrial DNA is maternally inherited (Lansman *et al.* 1983) and the molecule does not undergo recombination (Clayton 1982, Hayashi *et al.* 1985). It has higher rates of evolution (5-10 times faster) than single copy nuclear DNA (Brown *et al.* 1979, Barton and Jones 1983) and within the mtDNA genome itself the genes (and control region) have variable rates of evolution (Brown *et al.* 1993). These characteristics make it an accurate tracer of the maternal genealogy of a species (Moritz *et al.* 1987). However, with increasing phylogenetic depth, mtDNA becomes less useful due to its rapid rate of evolution.

The fact that the mtDNA molecule is strictly maternally inherited results in individuals being effectively haploid for their mtDNA. The haploid nature and mode of inheritance of mtDNA result in a smaller effective population size than for diploid nuclear alleles (at single copy loci), resulting in mtDNA haplotypes becoming fixed at four times the rate of nuclear alleles (Barton and Jones 1983). Low-levels of paternal leakage of mitochondria have however been documented in studies involving long-term back crossing experiments (Lansman *et al.* 1983, Gyllenstein *et al.* 1991) a situation which could be mirrored in nature by severe bottlenecking with subsequent inbreeding.

The presence of copies of mitochondrial genes in the nuclear genome (numts) presents a particularly challenging situation especially with regard to avian studies (Sorenson and Fleischer 1996, Sorenson and Quinn 1998). One of the most frequently sampled biomaterials in bird studies is blood due to the ease with which it can be obtained but its use is a double-edged sword. Bird red blood cells are nucleated, resulting in copious amounts of nuclear DNA being present with a relatively poor mtDNA content (Quinn 1992). Thus the probability of amplifying and sequencing

nuclear copies of the mitochondrial genes is greatly increased when working with bird blood (Sorenson and Fleischer 1996, Sorenson and Quinn 1998). This problem may however be resolved by purifying mtDNA from tissue, if it is available, or by amplification of the entire mitochondrial genome (Cheng *et al.* 1994, Sato *et al.* 2001).

The maternal inheritance of mtDNA may have limitations in phylogeographic studies where the males of a species disperse and females do not. It is thus wise to include bi-parentally inherited nuclear markers in genetic studies to ensure that the entire phylogeographic history is represented, reflecting both the paternal and maternal lineages. The most commonly used nuclear markers in population studies are DNA microsatellites. These genetic markers consist of short tandemly repeated sequence motifs of one to six base pairs (Hamada *et al.* 1982, Tautz *et al.* 1986). The hypervariable nature of tandem repeat loci is due to their high mutation rates (10^{-5} to 10^{-3}) that result in either an increase or decrease in the number of repeats and thus a change in the size of an allele (Wright 1994). The high mutation rate together with the corresponding high levels of polymorphism of loci in natural populations has made microsatellites a very important marker in the study of population genetics (Jarne and Lagoda 1996, Stefanini and Feldman 2000).

The mechanism creating microsatellite polymorphism is hypothesised to be 'strand slippage' during DNA replication (Levinson and Gutman 1987, Schlotterer and Tautz 1992). Strand slippage occurs when the DNA polymerase enzyme slips past a repeat unit within a repeat sequence during replication and then resumes normal base pairing resulting in the addition or deletion of (usually) a single repeat unit (Garza *et al.* 1995). Several theoretical mutational models are used to describe allele length variation at microsatellite loci. The infinite-alleles model (IAM) as proposed by Kimura and Crow (1964) assumes that each mutation forms a new allele since the possible number of alleles at a locus is very large; most classical distance measures are based upon this model of mutation. Most mutations at microsatellite loci are, however, stepwise in nature resulting in single additions or deletions of repeat units and thus most distances designed for the study of microsatellite variation assume Ohta and Kimura's (1973) stepwise mutation model (SMM).

Microsatellites are extremely useful for addressing questions at a large variety of scales and have been used extensively in addressing questions relating to comparisons among closely related species (Roy *et al.* 1994, Petren *et al.* 1998), determination of genetic structure of populations (Estoup *et al.* 1995, Grapputo *et al.* 1998, Haavie *et al.* 2000), kinship and parentage (Queller *et al.* 1993, McDonald and Potts 1994, Primmer *et al.* 1995), sexing (Longmire *et al.* 1993) and forensics (Frequeau and Fourney 1993). Due to the properties of microsatellites they are generally favoured

over other markers such as minisatellites, RAPDs, RFLPs, AFLPs and allozymes. The bi-parental Mendelian mode of inheritance, high levels of polymorphism, ease of use and scoring and the fact that microsatellites are usually selectively neutral and co-dominant are all properties which make them attractive markers for use in evolutionary studies (Bruford and Wayne 1993, Schlotterer and Pemberton 1994, Bruford *et al.* 1996).

1.10 Study Objectives

The aim of this study was to determine the phylogeographic and evolutionary history of the genus *Nesospiza*, with a view to determine the systematic relationships within the group. I also attempted to elucidate the relationship between *Nesospiza* and *Rowettia*. Specifically, I tested the following hypotheses:

Hypothesis 1: There are two species of *Nesospiza* bunting present at Tristan da Cunha, namely the small-billed *Nesospiza acunhae* and the large-billed *Nesospiza wilkinsi*, which are further distinguished by differences in plumage colouration, morphology and vocalisations.

Hypothesis 2: Each of the two *Nesospiza* species is further divided into two subspecies dependant on their island of origin. These subspecies are monophyletic and support the classical theory of a single, allopatric origin of large and small-billed forms in birds.

Hypothesis 3: Due to morphological differences between the upland and lowland forms of *N. a. acunhae* at Inaccessible Island, natural selection will continue to lead to adaptive divergence between these two populations, ultimately resulting in the formation of two new species.

Hypothesis 4: Hybridisation between upland *N. a. acunhae* and *N. w. dunnei* at Inaccessible Island is occurring on a small scale and is not affecting the genetic integrity of these two species.

Following on from these hypotheses, I asked the following research questions:

Question 1: What is the level of genetic variation within and among the *Nesospiza* subspecies based on mtDNA?

Question 2: What is the level of genetic variation within and among the *Nesospiza* subspecies based on DNA microsatellites?

Question 3: Are the genetic data supportive of the current species and subspecies taxonomy?

Question 4: What is the level of genetic structure and partitioning within the genus *Nesospiza*?

Question 5: What conclusions can be drawn with regards to the current and historical evolutionary processes, which have led to the current diversity of *Nesospiza* taxa at Tristan da Cunha?

The results of the genetic analyses performed were used to elucidate the taxonomic relationships within *Nesospiza*, make inferences on the evolutionary history of the genus and provide insights into the current observed morphological groups.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Sample collection

Three hundred and sixty buntings were caught in hand nets or mist-nets by P.G. Ryan and C.L. Moloney during the September 1999 to February 2000 field season to Tristan da Cunha. Approximately 10-20ì l of blood was taken from the brachial vein of each bird and stored in EDTA. Samples were collected at random from the three islands in the group, which support buntings: Inaccessible Island (37°18'S, 12°41'W), Nightingale Island (37°26'S, 12°29'W) and Gough Island (40°18'S, 9°56'W). At Inaccessible Island, birds were caught at three main locations: Blenden Hall on the west coast, Ringeye Valley on the western plateau and Denstone Hill on the eastern plateau (Figure 2.1). Sympatric populations of Wilkins' buntings (*N. w. dunnei*) and lowland Tristan buntings (*N. a. acunhae*) were collected at Blenden Hall, an area of *Spartina arundinacea* tussock grassland with several copses of *Phylica* trees near sea-level. Ringeye Valley on the western plateau of Inaccessible at an altitude of 450 m has a combination of *Blechnum palmiforme* and wet heath vegetation that mainly supports upland Tristan buntings (*N. a. acunhae*). Interspecific hybrids were collected from the ecotone between the *Phylica* woodland and *Blechnum palmiforme* heath at Denstone Hill on the eastern plateau (elevation 250 m). Hybridising pairs were classified as pairs containing one Wilkins' bunting and one Tristan bunting or pairs that contained at least one hybrid. Birds were classified as hybrids if they had bill sizes falling outside the 95% confidence intervals of 'pure' Tristan and Wilkins' buntings (Ryan 1992). At Nightingale Island, blood was collected from Tristan buntings (*N. a. questi*) and Wilkins buntings (*N. w. wilkinsi*) and on Gough Island blood was obtained from the resident Gough bunting, *Rowettia goughensis*.

2.2 DNA extraction

Total genomic DNA was extracted using standard phenol:chloroform methods (Sambrook *et al.* 1989). Approximately 50ì l of whole blood was mixed with 450ì l of extraction buffer (0.05M Tris-HCl, 0.5M Na_2· EDTA, 1M NaCl, 10% SDS). After the addition of 0.5mg Proteinase K (Roche Diagnostics) samples were digested overnight at 55 °C, followed by a 1 hour digestion with 0.1mg RNAse A (Roche Diagnostics) at 37 °C. Samples were then extracted twice with phenol and once with a 24:1 solution of chloroform:isoamyl alcohol. Precipitation of the DNA was performed overnight at –20 °C after the addition of 0.1 volumes of 3M sodium acetate and 2.5 volumes of 96% ethanol. The DNA was pelleted in a microcentrifuge at 13,000 rpm for 30 minutes. DNA pellets were washed twice with 70% ethanol and left to air dry. The DNA was finally resuspended in 50µl Sabax water (Adcock Ingram).

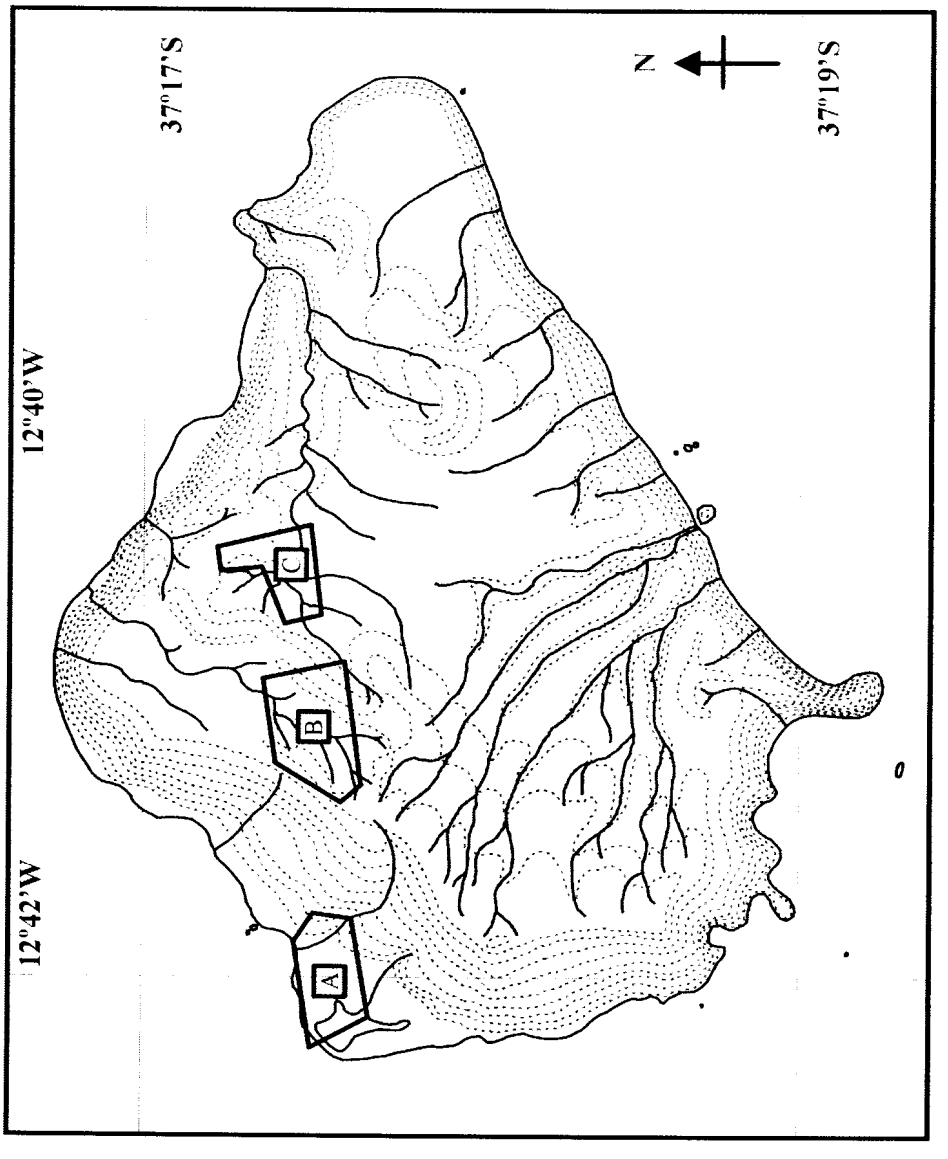


Figure 2.1: The three main sampling areas on Inaccessible Island: **(A)** Blenden Hall, **(B)** Ringeye Valley and **(C)** Denstone Hill. Contour interval is 60m (modified from Ryan 1992, Ryan *et al.*1994).

2.3 PCR amplification and sequencing of mtDNA

The universal PCR primers L14841 5'-CCAACATCTCAGCATGATGAAA-3' (Kocher *et al.* 1989) and H15499 5'-GGTTGTTTGAGCCTGATTC-3' (Avise *et al.* 1994) were used to amplify a 658bp fragment of the mitochondrial cytochrome b gene from 35 individuals, representing five individuals from each of the seven morphologically described bunting groups: *N. w. wilkinsi,* and *N. a. questi* from Nightingale Island; upland *N. a. acunhae*, lowland *N. a. acunhae*, *N. w. dunnei* and hybrids from Inaccessible Island and *R. goughensis* from Gough Island. The polymerase chain reaction (PCR; Saiki *et al.* 1988) was performed in a total volume of 50µl, containing ~100ng DNA, 1.5mM $MgCl_2$, 12.5 picomol of each primer, 0.2mM of each of the four deoxyribonucleotides and 1.5U of Supertherm DNA polymerase (Southern Cross Biotechnology). PCR was performed using the following conditions: 2 min initial denaturation at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 30 sec and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min on a GeneAmp 9700 PCR System (PE Applied Biosystems). Elecrophoresis was performed using 1% agarose gels (Laboratory Specialist Services) and 8µl of each PCR product to determine whether amplification had been successful. Negative controls were included during PCR and elecrophoresed to ensure that no cross contamination of samples or stock solutions occurred during setting up of the PCR reactions. Fragments, which amplified successfully, were purified using the High Pure PCR Product Purification kit (Boehringer Mannheim) and resuspended in 30µl Sabax water. Electrophoresis was repeated using 5µl of the purified PCR products to ensure that the purification process had been successful and that the products had not been lost during the purification process.

The risk of amplifying nuclear copies of mitochondrial genes (numts) when using the PCR method in phylogenetic studies is always present (Quinn 1992, Sorenson and Fleischer 1996, Sorenson and Quinn 1998). Long PCR was therefore performed on five samples that displayed double peaks at some sites in their sequences so as to control for numts and thus ensure that the sequences obtained were not of nuclear origin. Long PCR reactions were performed using primers RR32 5'-TATCTCTGACGTTGAGTAGCTCGGTTCTCGTGAG-3' and RR33 5'-CTCCTTGCTCTTCACA GATACAAGTGGTCGGTTG-3' (Sato *et al.* 2001). The principle behind performing long PCR is to amplify the complete mitochondrial genome using a pair of PCR primers and then to perform a nested PCR on the amplified product. This reduces the risk of amplifying nuclear copies of the mitochondrial genes, because it is unlikely that the entire mtDNA genome will be incorporated into the nuclear genome during an integration event (Cheng *et al.* 1994). Long PCR reactions were performed using an amplification profile consisting of: initial denaturation at 94 °C for 5 min followed by 10 cycles of 10 sec at 94 °C, 30 sec at 64 °C and 25 min at 68 °C, and then 20 cycles of

10 sec at 94 °C, 30 sec at 64 °C and 25 min plus a 20 sec elongation for each successive cycle at 68 °C, followed by a final extension at 68 °C for 10 min. Reactions were performed in 50µl volumes, using the Expand High Fidelity PCR System (Roche Diagnostics), containing: 20mM Tris-HCl pH 7.5, 100mM KCl, 1.5mM $MgCl_2$, 2.5µM of each primer, 50µM of each dNTP, and 2.6U Expand High Fidelity System enzyme mix. Long PCR products were electrophoresed on agarose gels and the PCR products were excised with sterile scalpel blades and purified. Nested PCR was performed using 1µl of the long PCR product as a template with primers L14841 and H15499 and the conditions described above for the amplification of the 658 bp cytochrome b fragment.

Each PCR purification product was sequenced bi-directionally in a reaction consisting of ~100ng purified DNA, 3.2pmol of either primer H15499 or L14841 in a total reaction volume of 10µl with the Big Dye Ready Reaction Kit (PE Applied Biosystems) on a GeneAmp 9700 thermocycler. Cycle sequencing products were precipitated using the sodium-acetate precipitation procedure and were run on an ABI 3100 automated DNA sequencer (PE Applied Biosystems) to determine the sequence for each individual.

2.4 Sequence analysis

Sequences were imported into Sequence Navigator (PE Applied Biosystems) and the heavy and light strands from each individual were aligned and proof read and a 510 bp fragment of the PCR product was used further. All sequences were aligned in CLUSTALX version 1.81 (Thompson *et al.* 1997). The aligned sequences were further analysed using the Phylogenetic Analysis Using Parsimony (PAUP) program version 4.0 (Swofford 2002). A neighbour-joining (Saitou and Nei 1987) tree was constructed using the absolute number of differences with bootstrap replicates (Felsenstein 1985) performed to establish the robustness of the nodes. The tree was rooted with Uniform Finch *Haplospiza unicolor* (GENBANK AF290156) and Sharp-beaked Ground Finch *Geospiza difficilis* (GENBANK AF108787), both members of the Thraupinae (Sibley and Monroe 1990).

2.5 Sequence divergence

There is no fossil data available for the Thraupini with which to calibrate the molecular clock, however standard avian molecular evolutionary theory suggests a sequence divergence of approximately 2% per million years for the mtDNA genome, as in most vertebrates (Shields and Wilson 1987, Burns 1997, Sato *et al.* 2001). Sequence divergence in the Hawaiian honeycreepers has however been shown to be approximately 1.6% per million years when determined using the origin of the different islands in the Hawaiian archipelago to calibrate the molecular clock (Fleischer

et al. 1998). Both sequence divergences were thus used to give an upper and lower limit on the approximate time of divergence between the different taxa.

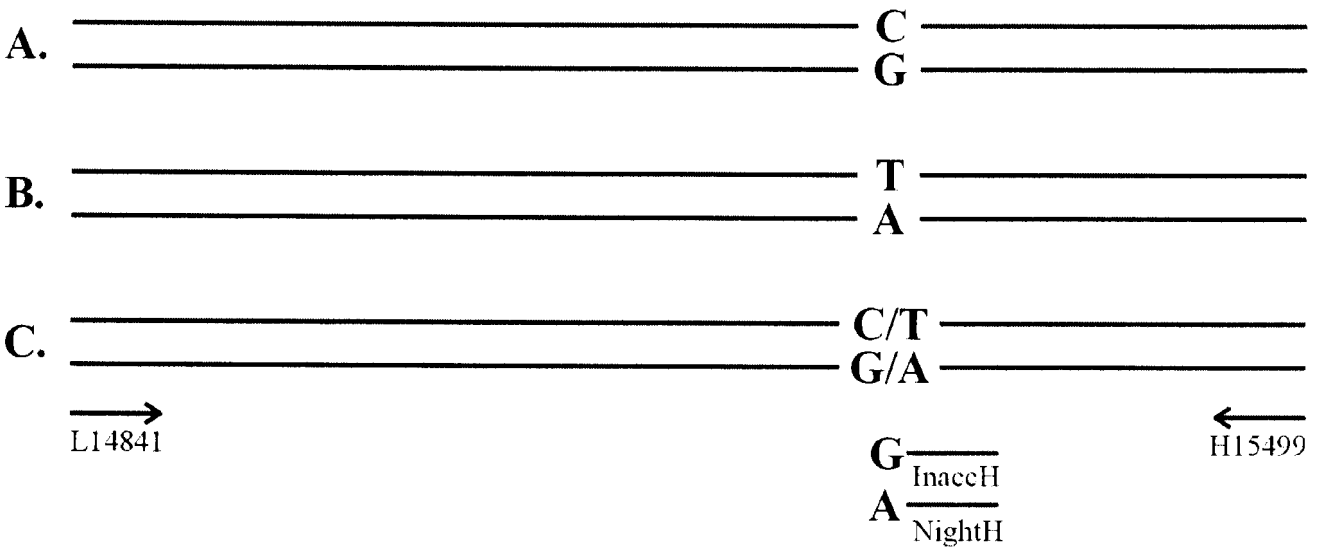
2.6 Diagnostic determination of haplotypes

Given the limited sequence divergence among individuals, especially within *Nesospiza,* a diagnostic PCR test was designed to determine the haplotype of each individual bird. Two PCR primers were designed each with a selective base at the 3' end allowing amplification of only one of the two *Nesospiza* haplotypes (Figure 2.2). For the diagnostic test either InaccH 5'-GCCCCTCAGAA TGATATTTGG-3' or NightH 5'-GCCCCTCAGAATGATATT TGA-3' were used to replace primer H15499 in the PCR reaction. With these diagnostic primers it was possible to determine the island from which an individual originated. The primers were however unable to elucidate the island of origin of individuals which had nuclear copies of the cytochrome b gene.

2.7 Microsatellite data analysis

Seven microsatellite loci developed for the Medium Ground Finch *Geospiza fortis* (Petren 1998) and 12 loci from the Village Indigobird *Vidua chalybeata* (Sefc *et al.* 2001) were tested on several *Nesospiza* samples. The PCR was performed in a total volume of 10µl, containing *ca* 30ng of genomic DNA, 20mM Tris-HCl pH 7.5, 100mM KCl, 1.5mM $MgCl_2$, 0.5µM of each primer, 0.125mM of each dNTP and 0.2U Expand High Fidelity PCR System enzyme mix (Roche Diagnostics). Optimal PCR temperatures were determined for each locus using a Mastercycler gradient (Eppendorf) thermocycler and the following conditions: 4 min denaturation at 94 °C followed by 35 cycles of 40 sec at 94 °C, 40 sec at 61 °C ± 10 °C, 40 sec at 72 °C, and a final extension at 72 °C for 4 min. PCR products were electrophoresed on 1% agarose gels to determine if amplification had been successful and to ascertain the optimal amplification temperature for each locus. Table 2.1 shows the optimal annealing temperatures for each of the 19 loci tested.

PCR was repeated across a test panel of 15 individuals consisting of three representatives of each of the *Nesospiza* subspecies (*N. a acunhae, N. a. questi, N. w. dunnei* and *N. w. wilkinsi*) and three hybrids to determine whether the loci were polymorphic and would thus be informative with regards to the evolutionary history and genetic relationships of *Nesospiza*. PCR products were sized on an ABI 377 sequencer by comparison to the internal TAMRA GS 500 (PE Applied Biosystems) size standard using GENESCAN (PE Applied Biosystems) software. Alleles from homozygous individuals were sequenced to ensure that loci amplified were in fact microsatellites. Markers were rejected on the basis of non-amplification, monomorphism or inconsistent size.



Primer Pair	Haplotype A	Haplotype B	Nuclear Copy C
L14841 & H15499	Y	Y	Y
L14841 & NightH	N	Y	Y
L14841 & InaccH	Y	N	Y

Figure 2.2: Representation of the diagnostic test to determine the individual haplotypes of *Nesospiza* buntings. **(A)** The haplotype of individuals from Inaccessible Island showing the single base difference found in these birds. **(B)** The single base difference found in most individuals from Nightingale Island. **(C)** Representation of haplotypes of individuals, which possess a nuclear copy of the mitochondrial cytochrome b gene and thus amplified with both sets of diagnostic primers. The amplification profile is shown in the table below the figure where Y represents amplification of a band and N indicates non-amplification; as can be seen individuals with a nuclear copy of the cytochrome b amplified with both diagnostic primer pairs.

Table 2.1 Description of *Vidua* and *Geospiza* microsatellites tested for use in this study, giving the annealing temperature used in *Nesospiza*, the number of alleles found and the expected heterozygosity (H_E).

Locus Name	Anealing temperature	No. of alleles	H_E	Reference
Gf01	54°C	1	0	Petren (1998)
Gf04	-	-	-	Petren (1998)
Gf05	54°C	1	0	Petren (1998)
Gf06	-	-	-	Petren (1998)
Gf10	57°C	1	0	Petren (1998)
Gf12	62°C	1	0	Petren (1998)
Gf14	54°C	1	0	Petren (1998)
INDIGO 7	64°C	1	0	Sefc *et al.* (2001)
INDIGO 8	60°C	2		Sefc *et al.* (2001)
INDIGO 15	60°C	4	0.506	Sefc *et al.* (2001)
INDIGO 27	-	-	-	Sefc *et al.* (2001)
INDIGO 28	-	-	-	Sefc *et al.* (2001)
INDIGO 29	-	-	-	Sefc *et al.* (2001)
INDIGO 30	60°C	3	0.308	Sefc *et al.* (2001)
INDIGO 31	62°C	3	0.479	Sefc *et al.* (2001)
INDIGO 37	-	-	-	Sefc *et al.* (2001)
INDIGO 38	60°C	1	0	Sefc *et al.* (2001)
INDIGO 40	53°C	2	0.347	Sefc *et al.* (2001)
INDIGO 41	60°C	9	0.79	Sefc *et al.* (2001)

2.8 Hardy-Weinberg equilibrium and genotypic disequilibrium

To test for deviations from Hardy-Weinberg equilibrium (HWE) exact tests were performed using GENEPOP version 3.3 (Raymond and Rousset 1995). The null hypothesis tests for the random union of gametes. The program performs a score test (U-test) using a Markov chain (with 2500 dememorization steps, 100 batches and 1000 iterations per batch) to determine the exact probability of departure from HWE using the algorithm of Guo and Thompson (1992). Significance levels were calculated globally for each population at all loci and also for each locus in all populations.

Genotypic disequilibrium between all locus pairs was tested using GENEPOP version 3.3 (Raymond and Rousset 1995). The null hypothesis assumes that genotypes at one locus are independent from genotypes at another locus and determines an exact P-value after performing pairwise comparisons using the Markov chain simulation. The program also performs a global test for genotypic linkage disequilibrium across all populations for all loci by means of a Fisher's exact test.

The critical significance level applied to all statistical tests was 0.05. The chance of falsely rejecting the null hypothesis (type I error) was reduced by applying Bonferroni corrections to all simultaneous statistical tests (Rice 1989). One major drawback of applying Bonferroni corrections is that the reduction in chance of making a type I error leads to an increase in the chance of making a type II error, where a false hypothesis is accepted (Rice 1989, Sokal and Rohlf 1994).

2.9 Allelic diversity

Genetic diversity within each population was calculated using POPGENE version 1.31 (Yeh *et al.* 1997). The program was used to determine allele frequencies, number of polymorphic loci, mean number of alleles per locus and observed and unbiased estimates of heterozygosity (H_O and H_E) (Nei 1978) at each locus; values for mean H_O and H_E for each population were also determined.

2.10 Population differentiation

The significance of population genetic differentiation was tested using Fisher's exact tests for population differentiation in GENEPOP version 3.3 (Raymond and Rousset 1995). The tests assess differences in allelic and genotypic frequencies among populations at each locus with the null hypothesis that there are no differences in the frequencies of alleles among the populations. The test was performed using a Markov chain (5000 dememorization steps, 1000 batches and 1000 iterations per batch). Sequential Bonferroni corrections were applied to correct for the number of tests (Rice 1989, Sokal and Rohlf 1994).

As a result of lack of consensus with regards to the nature of microsatellite evolution, two estimates of genetic differentiation were determined: F_{ST}, which assumes the infinite alleles model (IAM) and R_{ST} (Slatkin 1995), which assumes the stepwise mutation model (SMM). Pairwise population differentiation was calculated by means of Wright's fixation index, F_{ST} (Wright 1951, Weir and Cockerham 1984), using ARLEQUIN version 2.0 (Excoffier *et al.* 1992). Unbiased, multilocus estimates of R_{ST} were made using R_{ST}CALC (Goodman 1997). R_{ST} has been developed specifically for microsatellite data analysis because it has been assumed that the mutation process of microsatellite loci is characterised by single-step insertions or deletions of the core repeat motif (Slatkin 1995). Biased results may be produced if R_{ST} is calculated as indicated by Slatkin (1995), because it assumes populations of equal sample size and loci with equal variances. To circumvent this, the data set was globally standardized (Goodman 1997) before performing the calculation of R_{ST}. In this way alleles are expressed as standard deviations from the global mean rather than as repeat unit number or size. Statistical significance of F_{ST} and R_{ST} values was estimated using 1000 permutations for both sets of calculations.

The interindividual microsatellite genetic distance Dps (1 – proportion of shared alleles) (Bowcock *et al.* 1994) was estimated with MICROSAT (version 1.5d) and subjected to 100 bootstrap iterations. The distance matrices obtained were used to construct a UPGMA dendrogram (Sneath and Sokal 1973) using the program NEIGHBOUR in PHYLIP version 3.57c (Felsenstein 1993). In addition, the standard genetic distance of Nei, D_S (Nei 1972, 1978) was calculated for all pairwise comparisons of populations over the loci. From the matrix of genetic distances a UPGMA tree (Sneath and Sokal 1973) was constructed to graphically represent the relationships between the populations of *Nesospiza* buntings in POPULATIONS version 1.2.28 (Langella 1999). Significance of internal nodes of the tree topology was assessed using 100 bootstrap iterations. The program TREEVIEW (Version 1.6.6) was used to draw the trees (Page 1996).

The relationships between the *Nesospiza* bunting populations was further examined using principal components analysis (PCA) using the program PCAGEN version 1.2.1 (Goudet 1999). The relationship between individuals was determined by ordinating individual genotypes according to their allelic state at each of the loci. In this case a homozygote for an allele is scored as 1, a heterozygote as 0.5 and the absence of an allele is scored as 0. This method is performed for each allele resolved at each locus over all populations. Results of the PCA were plotted showing the first two principal components (PCs) using PCAGEN (Goudet 1999).

2.11 Population assignment

Several approaches have been developed recently which use microsatellite allele frequency and individual multilocus genotypic data to identify the population of origin of individuals (Paetkau *et al.* 1995, Rannala and Mountain 1997, Cornuet *et al.* 1999, Banks and Eichert 2000, Prichard *et al.* 2000). These so called assignment tests are also useful for detecting dispersal and immigration between populations (Rannala and Mountain 1997). Assignment tests were conducted using the exclusion-simulation approach to obtain a level of certainty (P-value) for each individual assignment (Cornuet *et al.* 1999) under the likelihood-based Bayesian method (Rannala and Mountain 1997) in the program GENECLASS (Cornuet *et al.* 1999).

The probability of an individual belonging to a population was calculated by simulating 10000 genotypes using the observed allele frequencies for each population, the observed frequency of each genotype was then determined for each population. The threshold P-value was set at 0.05, thus individuals with probabilities lower than 0.05 in a particular population would not be assigned to that population. In this study, it was also assumed that the population to which an individual was assigned with the highest posterior probability was the individuals' population of origin. Two assignment tests were performed; the first was performed assuming that all six populations, namely the hybrids, *N. w. dunnei*, upland and lowland *N. a. acunhae*, *N. w. wilkinsi* and *N. a. questi,* were good populations; and the second assignment test was performed assuming that the hybrids, *N. w. dunnei* and upland *N. a. acunhae* birds formed a single interbreeding population. Individuals with missing data in their genotypes were excluded from all assignment tests (thus n = 97).

2.12 Extra-pair paternity

Composite genotypes, based on the microsatellite loci were used to compare with the allele sizes of the putative parents to determine whether extra-pair fertilizations occur within *Nesospiza*. Blood samples were obtained from 43 chicks from 26 hybrid, upland *N. a. acunhae* and lowland *N. a. acunhae* nests at Inaccessible Island where both members of the breeding pair were known (and had been sampled). *Nesospiza* buntings are strongly territorial while breeding, with a single male and female defending each territory and sharing the responsibilities of raising the offspring(Fraser and Briggs 1992, Ryan 1992), making it easy to identify the 'parents' of each nest (Ryan 2001).

CHAPTER THREE

RESULTS

3.1 Mitochondrial DNA

The results obtained from sequencing the 510 bp cytochrome b fragment showed that *Rowettia* and *Nesospiza* are well-defined, monophyletic groups. Sequence divergence between *Rowettia* and *Nesospiza* was 4.7%, whereas divergence between the populations of buntings at Inaccessible and Nightingale islands was only 0.2% (a single, silent, third position transition) suggesting a far more recent divergence between the latter lineages. The divergence between *Nesospiza* and the Gough bunting (*Rowettia*) suggests that these two groups shared a common ancestor within the last 2.4 to 2.9 million years. Likewise the sequence divergence between the Nightingale and Inaccessible Island lineages suggests divergence between these two groups occurred far more recently (within the last few hundred thousand years). Confidence in this estimate is low, however, because the difference between the lineages is only a single base pair.

The neighbour-joining analysis of mtDNA haplotypes showed a clear split among *Nesospiza* individuals according to their island of origin and not their putative species as would be expected. All of the individuals from Inaccessible Island had haplotype A and all but one individual from Nightingale Island had haplotype B (Figure 3.1). The exception was an individual caught on Nightingale Island that in terms of morphology resembled *N. a. acunhae* and probably was an immigrant to Nightingale Island from Inaccessible Island. Low levels of haplotype diversity were observed in the *Rowettia* population, however the presence of more than one haplotype in this population suggests that *Rowettia* may have been at Gough Island longer than *Nesospiza* has been at the Tristan da Cunha group, thus allowing more time for evolution of different haplotypes.

From the neighbour-joining tree (Figure 3.1) and the diagnostic determination of haplotypes (Table 3.1) it was clear that there are two mitochondrial lineages at Tristan da Cunha. The lineages are split from each other based on the island of origin of the individuals. Incorrectly assigned haplotypes were detected at low frequencies (4-6%) at both islands, due to 'errors' in the *N. w. wilkinsi*, lowland *N. a. acunhae* and interspecific hybrid populations (Table 3.1). The low frequency of individuals with the incorrect haplotype may be attributed to infrequent migration of birds between the islands.

Nuclear copies of the mtDNA only appear to occur in the populations at Inaccessible Island (Table 3.1). The presence of nuclear copies may be due to the integration of a section of the mtDNA into the nuclear genome after the two island lineages had been established. Alternatively the absence of nuclear copies of the cytochrome b gene in the Nightingale island populations may be as a result of purging of the nuclear copies due to founder effects and selection during the bottleneck this population most likely went through upon its establishment.

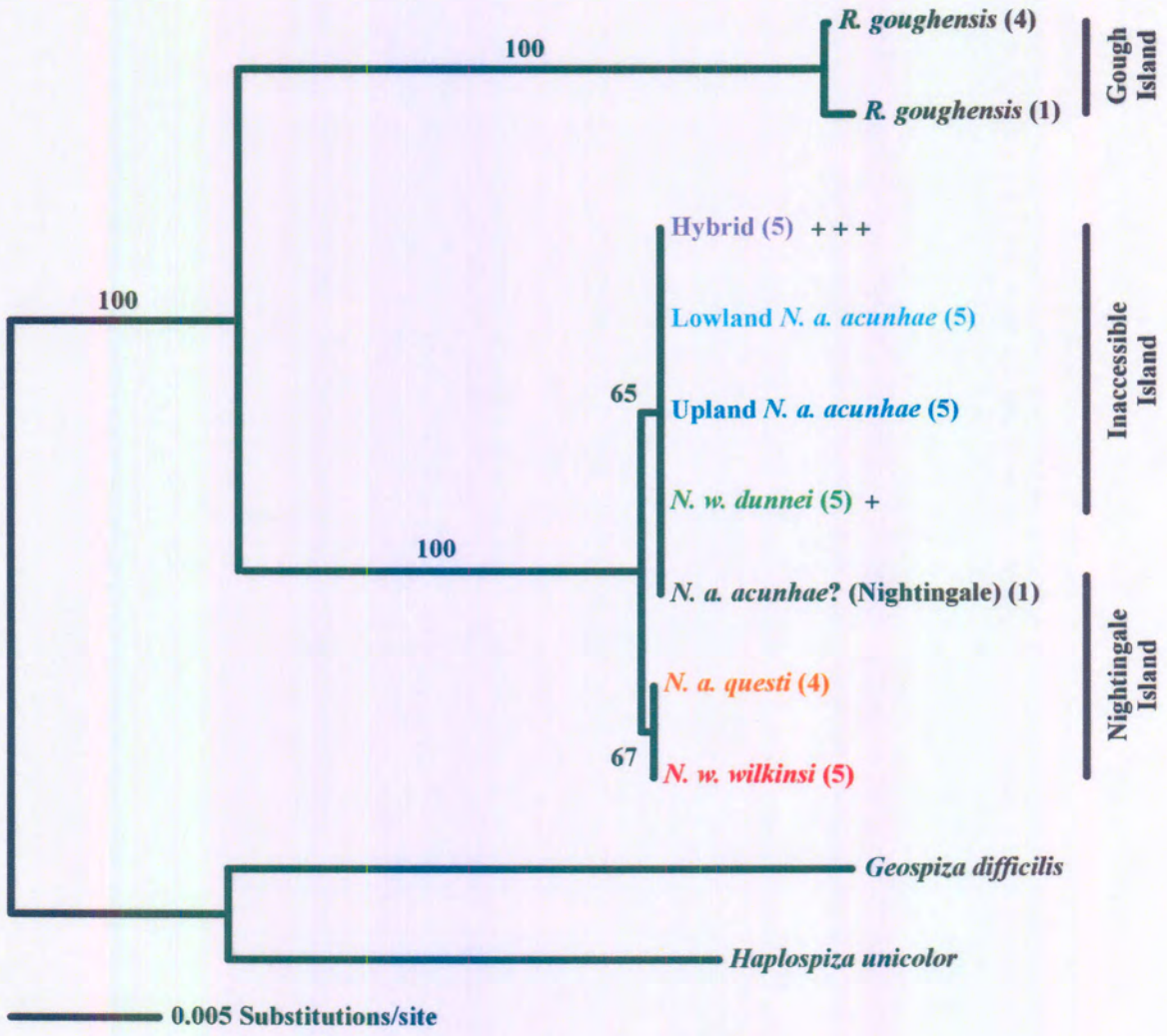


Figure 3.1: Neighbour-joining phylogram of *Nesospiza* bunting cytochrome b sequences. The tree was constructed using absolute number of differences and 1000 bootstrap replicates. Numbers following species names indicate the number of individuals, with that particular haplotype, sequenced per subspecies. Plus signs following a species name (+) indicate the identification of a nuclear copy of the mitochondrial gene in an individual's DNA. The individual labelled as: *N. a. acunhae*? (Nightingale) was an individual that morphologically resembled *N. a. acunhae* but was sampled on Nightingale Island. The tree was rooted using the Uniform Finch (*Haplospiza unicolor*) and Sharp-beaked Ground Finch (*Geospiza difficilis*) as outgroups.

Table 3.1 Distribution of mitochondrial cytochrome b haplotypes in *Nesospiza* buntings. Most of the birds from Inaccessible Island contained haplotype A and the birds from Nightingale Island had haplotype B, clearly showing that there are two mitochondrial lineages based on the island of origin of an individual. The numbers in the table indicate the number of individuals within a particular subspecies group possessing a particular haplotype. Nuclear copy indicates individuals that had positive amplification with both primer combinations and where a nuclear copy is thus present.

	Inaccessible Island				Nightingale Island	
			Upland	Lowland		
	Hybrids	*N. w. dunnei*	*N. a. acunhae*	*N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
Haplotype A	20	13	12	8	2	-
Haplotype B	1	-	-	1	13	16
Nuclear Copy	4	6	4	4	-	-

3.2 Microsatellites

Several microsatellite studies have shown that heterologous amplification is possible when primers developed in one species are used in a closely related species. Polymorphism declines and primers tend to work less effectively with increasing genetic distance, due to the presence of null alleles caused by point mutations in the primer binding sites (Primmer *et al.* 1996). The presence of null-alleles may impact severely on the conclusions made from microsatellite DNA data in population genetic studies. Nineteen *Vidua* and *Geospiza* microsatellite loci were tested to facilitate a broader understanding of the situation at Tristan da Cunha in terms of the genetic structure and history of the *Nesospiza* species, subspecies and populations. Of the 19 loci tested only six (INDIGO 8, INDIGO 15, INDIGO 30, INDIGO 31, INDIGO 40 and INDIGO 41) amplified consistently. Allele sizes were similar to those found in the studies for which they were developed and different alleles were consistent with the gain or loss of the integral repeat motif, for that particular locus. An interesting observation from the microsatellite optimisations was that all of the *Geospiza* loci, for which amplification was achieved, were monomorphic, whereas six of the eight successfully amplifying *Vidua* loci were polymorphic. This is somewhat contrary to the findings of Primmer *et al.* (1996) because *Geospiza* is in the same tribe as *Nesospiza*, whereas *Vidua* is in a different family (Sibley and Monroe 1990, Sorenson and Payne 2001).

The analysis of bunting genotypes using six microsatellite loci revealed a reasonable degree of polymorphism in all populations across the Tristan da Cunha archipelago. All loci showed a continuous distribution of alleles within the *Nesospiza* buntings and a disjunct distribution of alleles between *Nesospiza* and *Rowettia* buntings. In total 161 individuals were genotyped, with the number of alleles at each locus varying from two (INDIGO 40) to nine (INDIGO 41; Appendix 1). Due to the disjunct distribution of alleles between *Nesospiza* and *Rowettia*, the *Rowettia* samples were excluded from all subsequent population analyses. The *Rowettia* population was monomorphic for four of the six loci but had eight and five alleles respectively at the INDIGO 15 and INDIGO 31 loci (Appendix 1). Data obtained from bunting chicks with known parents who had been genotyped also were excluded from the population analyses to prevent skewing of allele frequencies due to resampling of the same alleles. This resulted in a final data set of 104 individuals.

3.3 Hardy-Weinberg equilibrium

After sequential Bonferroni correction, significant departure from Hardy-Weinberg equilibrium was found at the INDIGO 8 locus, indicating a global heterozygote deficit in all populations (Table 3.2). Measures of population differentiation assume Hardy-Weinberg equilibrium; therefore the INDIGO 8 locus was excluded from all further analyses. Heterozygote

deficiency can possibly be explained by the presence of so called 'null' alleles, which are alleles that are not amplified due to random mutations at the primer binding sites (Callen *et al.* 1993, Koorey *et al.* 1993, Paetkau and Strobeck 1995).

Significant departure from Hardy-Weinberg equilibrium (exact probability test $P < 0.05$) was observed in the lowland *N. a. acunhae* population, after adjustment for multiple tests (Table 3.3). Departure from HWE in the lowland *N. a. acunhae* population is due to a heterozygote deficit in this population, which may result from the presence of null alleles in this population or be due to random genetic drift as a result of small population size and fixation of alleles due to selective mating within this population.

3.4 Linkage disequilibrium

After correcting for heterozygote deficiency, no deviation was found in the test for statistical independence of genotypes (genotypic linkage disequilibrium). None of the 60 exact tests for pairwise linkage disequilibrium between the remaining five loci showed a significant probability ($P < 0.05$) that would result in the rejection of the hypothesis (Appendix 2A – 2F). However, 18 of the 60 pairwise comparisons were 'not possible', which occurs when one or both of the loci in the comparison are monomorphic in the populations from which this result was obtained. Monomorphic loci result in so called 'empty tables' and therefore pairwise comparisons cannot be performed. The Fisher's exact test performed for each locus pair over all populations corrects for this situation and produced no significant values (Table 3.4). It was therefore assumed that there was independent segregation of all genotypes at the five remaining loci and all were retained for subsequent analyses.

3.5 Allelic diversity

The five loci were all polymorphic, with 21 alleles observed in the 104 *Nesospiza* samples (Appendix 3A – 3E). Six private alleles were observed, four of which were found in the upland *N. a. acunhae* population; two at the INDIGO 15 locus (n=1) and single alleles at the INDIGO 30 (n=2) and INDIGO 31 (n=1) loci. The two remaining private alleles were observed at the INDIGO 41 locus in *N. w. wilkinsi* (n=1) and in the lowland morph of *N. a. acunhae* (n=4). The Wilkins' buntings from Nightingale Island (*N. w. wilkinsi*) showed low levels of polymorphism and were monomorphic for three loci (INDIGO 30, 31 and 40). This may be due to genetic drift as a result of small population size (Ryan and Siegfried 1994) resulting in fixation of the alleles at these loci or it may be due to the presence of null alleles. Single monomorphic loci were also observed in the lowland *N. a. acunhae* and *N. a. questi* buntings at INDIGO 15 and INDIGO 40, respectively.

Table 3.2: Global Hardy-Weinberg equilibrium (HWE) exact test results for each of the six microsatellite loci used in this study. Probabilities for departure from Hardy-Weinberg equilibrium are given in the first column and standard errors in the second; significant values after Bonferroni correction are shown in bold (n=104).

	P-Value	S.E.
INDIGO 8	**0.0009**	0.000
INDIGO 15	0.7215	0.005
INDIGO 30	0.8809	0.002
INDIGO 31	0.2970	0.004
INDIGO 40	0.3684	0.003
INDIGO 41	0.0998	0.008

Table 3.3: Global Hardy-Weinberg equilibrium (HWE) exact test results for each population of *Nesospiza* buntings over five loci. Probabilities for departure from Hardy-Weinberg equilibrium are given in the first column and standard errors in the second; significant values after Bonferroni correction are shown in bold.

	Sample size	P-Value	S.E.
Hybrids	25	0.5700	0.011
N. w. dunnei	19	0.4921	0.009
Upland *N. a. acunhae*	16	0.3596	0.106
Lowland *N. a. acunhae*	13	**0.0000**	0.000
N. w. wilkinsi	16	0.2244	0.005
N. a. questi	15	0.8473	0.005

Table 3.4: Matrix of probabilities of genotypic linkage disequilibrium for pairwise comparisons among five microsatellite loci in *Nesospiza* buntings. All values were not significant according to the Fisher's exact test.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41
INDIGO 15	-				
INDIGO 30	0.788	-			
INDIGO 31	0.264	0.633	-		
INDIGO 40	0.200	0.196	0.757	-	
INDIGO 41	0.490	0.990	0.651	0.179	-

Allele frequency distributions for each population at each locus are shown in Figure 3.2. Most loci showed the expected normal distribution of alleles. Allele frequencies, number of observed alleles and observed and expected heterozygosity (Nei 1978) are given for each population at each locus in Appendix 3A - 3E. The mean expected heterozygosity (H_E) ranged from 0.111 in the *N. w. wilkinsi* population to 0.490 in the upland morph of *N. a. acunhae* while observed heterozygosity (H_O) within each population ranged from 0.085 to 0.563 (Table 3.5). Within each locus observed and expected heterozygosity ranged from 0.240 to 0.691 with a mean value of 0.375 ± 0.183 for H_O and from 0.308 to 0.790 with a mean of 0.486 ± 0.190 for H_E. The mean number of alleles per locus (# alleles) varied from 1.6 in *N. w. wilkinsi* to 3.8 in upland *N. a. acunhae* (Table 3.5). Mean number of alleles (# alleles) could however be influenced by sample size (13 to 25 individuals per subspecies).

3.6 Population differentiation

The exact tests for population differentiation based on allele and genotypic frequencies (Table 3.6) showed that most populations are significantly differentiated from one another with the exception of three population pairs: the hybrids and *N. w. dunnei*; the hybrids and the upland *N. a. acunhae*; and the upland *N. a. acunhae* and *N. w. dunnei*. The fact that these population pairs are not differentiated from each other based on allele and genotypic frequencies resulted in the failure to reject the null hypothesis of identical allelic distribution for them and the rejection of it for all of the other population pairs. This is perhaps to be expected given that these populations hybridise extensively at Inaccessible Island. It is however intriguing that, although these populations are not genetically distinct, *N. w. dunnei* and upland *N. a. acunhae* remain morphologically distinct throughout most of their ranges. It was further interesting to note that the lowland *N. a. acunhae* population was detectably differentiated from upland *N. a. acunhae.* These populations are differentiated based on plumage colouration and to some extent on song and bill morphology (Ryan 1992). This observation supports the assumption that assortative mating is occurring between the two morphs of *N. a. acunhae* leading to differentiation and possibly incipient speciation of these morphs.

Population genetic differentiation using F_{ST} and R_{ST} gave similar results to the exact tests for population differentiation (Table 3.7). The upland *N. a. acunhae*, *N. w. dunnei* and hybrids from Inaccessible Island were not significantly differentiated from each other ($P>0.05$). All other populations were strongly differentiated ($P<0.001$), apart from the upland and lowland morphs of *N. a. acunhae* which were only weakly significant ($F_{ST}=0.069$ $P<0.05$). However, the upland and lowland morphs were not significantly differentiated based on R_{ST} ($R_{ST}=0.051$ $P>0.05$). The two

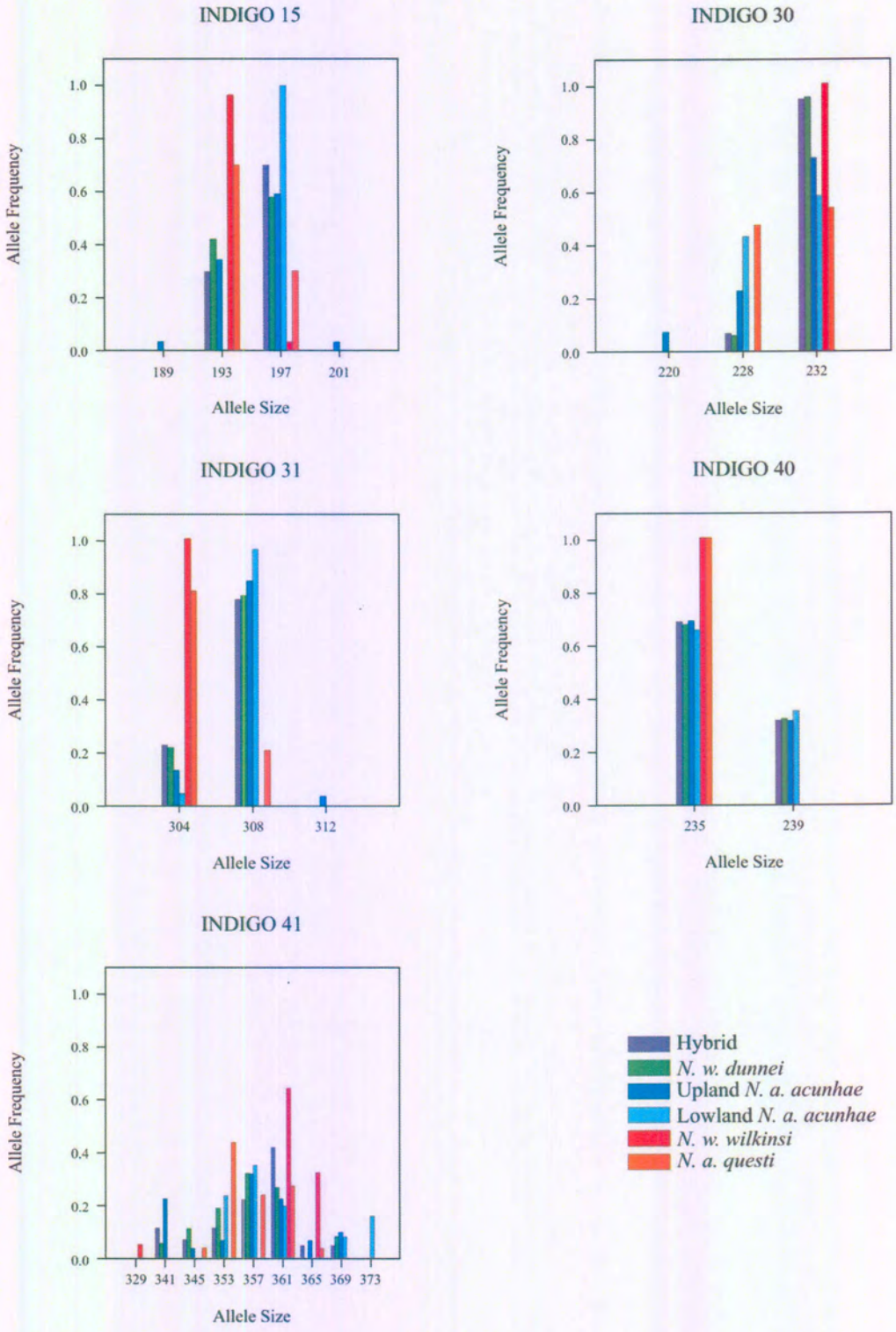


Figure 3.2: Frequency distribution of alleles at five microsatellite loci among six *Nesospiza* populations classified according to sub-specific classification and island of origin. Horizontal axes are representative of allele size classes and vertical axes are representative of the frequency of the specific allele. The locus represented is indicated above each graph and the colour-coded key indicates the species represented by each bar.

Table 3.5: Descriptive statistics of genetic diversity within populations of *Nesospiza* buntings showing sample sizes, total number of observed alleles, mean number of alleles per locus (# alleles), number of monomorphic loci, observed heterozygosity (H_O) and Nei's (1973) unbiased expected heterozygosity under HWE (H_E).

	Inaccessible Island				Nightingale Island	
	Hybrids	*N. w. dunnei*	Upland *N. a. acunhae*	Lowland *N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
Sample size	25	19	16	13	16	15
No of Alleles	15	14	19	12	8	11
# Alleles	3	2.8	3.8	2.4	1.6	2.4
Mono Loci	0	0	0	1	3	1
H_O	0.416	0.432	0.563	0.246	0.085	0.44
H_E	0.411	0.426	0.49	0.355	0.111	0.384

Table 3.6: Fishers' exact tests for allelic/genotypic differentiation among the six *Nesospiza* populations; P < 0.001 = ***, P < 0.01 = **, P < 0.05 = *, n.s. = non significant, n.i. = no information and o.g. = one genotype. HYB = Hybrids, IW = *N. w. dunnei*, ITU = Upland *N. a. acunhae*, ITL = Lowland *N. a. acunhae*, NW = *N. w. wilkinsi* and NT = *N. a. questi*. Overall results for each pairwise comparison are presented in the final column; values of observed and expected heterozygosity (H_O and H_E) for each locus are also presented.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41	Overall
HYB & IW	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.
HYB & ITU	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.
HYB & ITL	**/**	***/**	n.s./n.s.	n.s./n.s.	n.s/*	***/***
HYB & NT	**/*	***/***	***/***	**/**	n.s./n.s.	***/***
HYB & NW	***/***	n.s./n.s.	***/***	**/**	**/***	***/***
IW &ITU	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.
IW & ITL	***/***	**/**	n.s./n.s.	n.s./n.s.	n.s./n.s.	***/***
IW & NT	n.s./n.s.	***/***	***/***	**/**	n.s./n.s.	***/***
IW & NW	***/***	n.s./n.s.	***/***	**/**	***/***	***/***
ITU & ITL	**/***	n.s./n.s.	n.s./n.s.	n.s./n.s.	n.s./n.s.	**/***
ITU & NT	n.s./n.s.	n.s./n.s.	***/***	**/**	**/**	***/***
ITU & NW	***/***	**/*	***/***	**/**	***/***	***/***
ITL & NT	***/***	n.s./n.s.	***/***	**/*	n.s./n.s.	***/***
ITL & NW	***/***	***/***	***/***	**/*	***/***	***/***
NT & NW	*/n.s.	***/***	*/*	n.i./o.g.	***/***	***/***
H_O	0.375	0.279	0.240	0.291	0.691	
H_E	0.506	0.308	0.479	0.347	0.790	

Table 3.7: Matrix of pairwise comparisons of F_{ST} (below diagonal) and R_{ST} (above diagonal) values among and between island populations of *Nesospiza* bunting subspecies using five microsatellite loci. Significance levels are indicated by: * = P < 0.05, ** = P < 0.01, *** P < 0.001, values without asterixes indicate non-significant differentiation.

	Inaccessible Island				Nightingale Island	
	Hybrids	*N. w. dunnei*	Upland *N. a. acunhae*	Lowland *N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
Hybrids	-	-0.017	0.027	0.126^{***}	0.487^{***}	0.305^{***}
N. w. dunnei	-0.008	-	0.024	0.163^{***}	0.466^{***}	0.289^{***}
Upland *N. a. acunhae*	0.011	0.005	-	0.051	0.414^{***}	0.201^{***}
Lowland *N. a. acunhae*	0.117^{***}	0.128^{***}	0.069^{*}	-	0.647^{***}	0.431^{***}
N. w. wilkinsi	0.468^{***}	0.443^{***}	0.475^{***}	0.693^{***}	-	0.288^{***}
N. a. questi	0.284^{***}	0.244^{***}	0.241^{***}	0.378^{***}	0.263^{***}	-

species from Nightingale Island were significantly differentiated from each other and all of the birds on Inaccessible Island in all statistical tests ($P<0.001$). It is interesting to note that estimates of both F_{ST} and R_{ST} are high between island populations but much lower within island populations, suggesting infrequent movement between islands and closer genetic affinities between populations from the same island.

The UPGMA tree based on inter-individual Dps (1 – proportion of shared alleles) microsatellite distances (Bowcock *et al.* 1994) in Figure 3.3, showed that most of the birds are split into two major clusters, linked to their island of origin. There are however several outliers which group with the incorrect island cluster, which could be indicative of low levels of movement between the two islands or result from low allelic diversity at the microsatellite loci resulting in similar genotypes being observed in the different populations. The tree clearly showed that the genetic relationships are not consistent with the current morphological classification of these birds. In addition, a UPGMA tree constructed using Nei's standard genetic distance, D_S (Nei 1972, 1978) resolved the same major partition between subspecies (populations) from the different islands (Figure 3.4), showing that the island of origin, and not morphology, was the determining factor in the phylogenetic relationships between the bunting subspecies. This separation between the two islands was distinct and robust and was supported by high bootstrap support (91%). This demonstrated that *N. a. questi* and *N. w. wilkinsi* from Nightingale Island are genetically more closely related to each other than *N. w. wilkinsi* and *N. w. dunnei* (the so-called Wilkins' buntings). Likewise, the Tristan buntings, *N. a. questi* and *N. a. acunhae,* are more closely related to their *N. wilkinsi* counterparts from their respective islands of origin that to each other as would be expected based on morphology and their sub-specific classification.

The PCA plot of the individual genotypes clearly showed the separation of *N. w. wilkinsi* and *N. a. questi* from the other subspecies, indicating that these two groups are relatively distinct from the other populations (Figure 3.5). *Nesospiza w. dunnei*, *N. a. questi* and hybrids had overlapping point distributions, which is expected due to hybridisation. Lowland *N. a. acunhae* tended to group together with some degree of overlap with the other birds from Inaccessible Island. Consistent with the proportion of shared alleles UPGMA tree (Figure 3.3) there is an apparent split between the two islands in terms of the individual allelic states in the PCA plots.

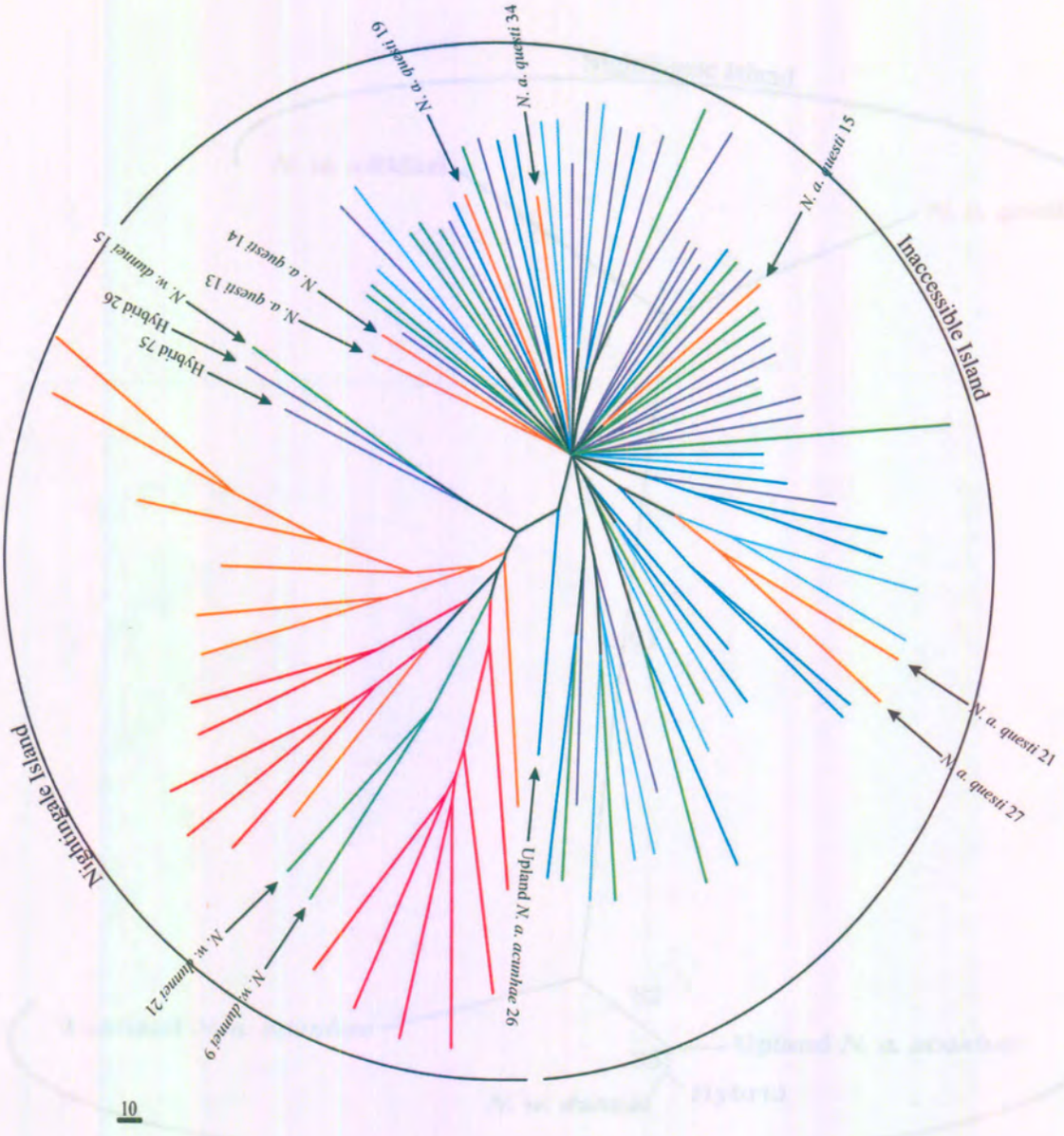


Figure 3.3: Unrooted UPGMA tree of individual *Nesospiza* buntings from Inaccessible and Nightingale Islands constructed using pairwise Dps distances (Bowcock *et al* 1994). Each branch represents an individual. Red and orange branches represent *N. w. wilkinsi* and *N. a. questi* individuals, respectively, from Nightingale Island. Branches representing birds from Inaccessible island are: Green *N. w. dunnei*, light blue Lowland *N. a. acunhae*, dark blue Upland *N. a. acunhae* and purple branches represent hybrids. Birds clustered mainly according to their island of origin except for a few outliers that appear to be misclassified; these individuals are indicated by arrows and sample labels.

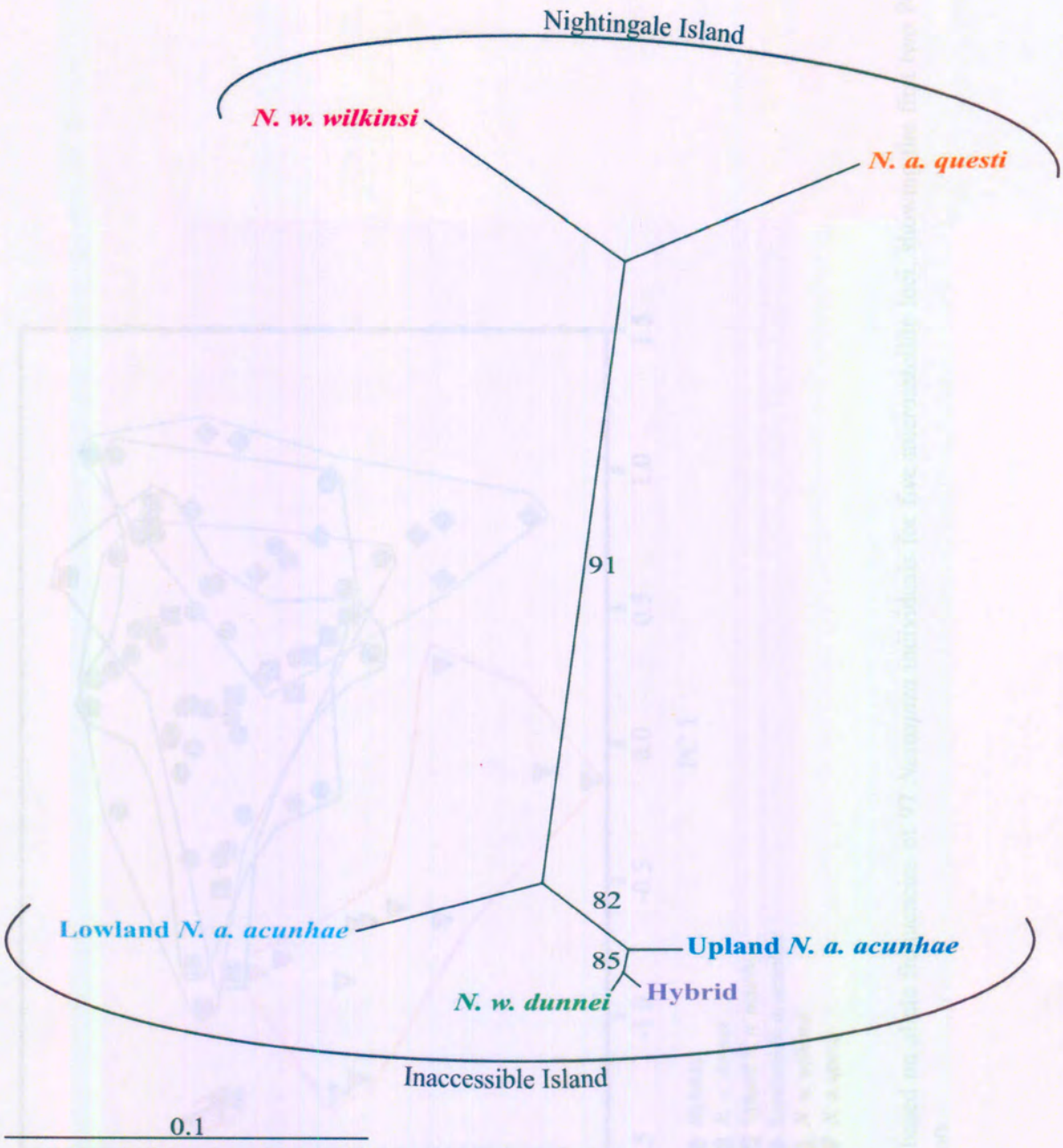


Figure 3.4: Unrooted UPGMA tree of six populations of *Nesospiza* buntings, as specified by sub-species classification and island of origin, based on Nei's standard genetic distance, D_S (Nei 1972). Bootstrap support (100 iterations) is shown at the nodes. Each branch represents a single population. Arcs represent the island upon which the populations occur.

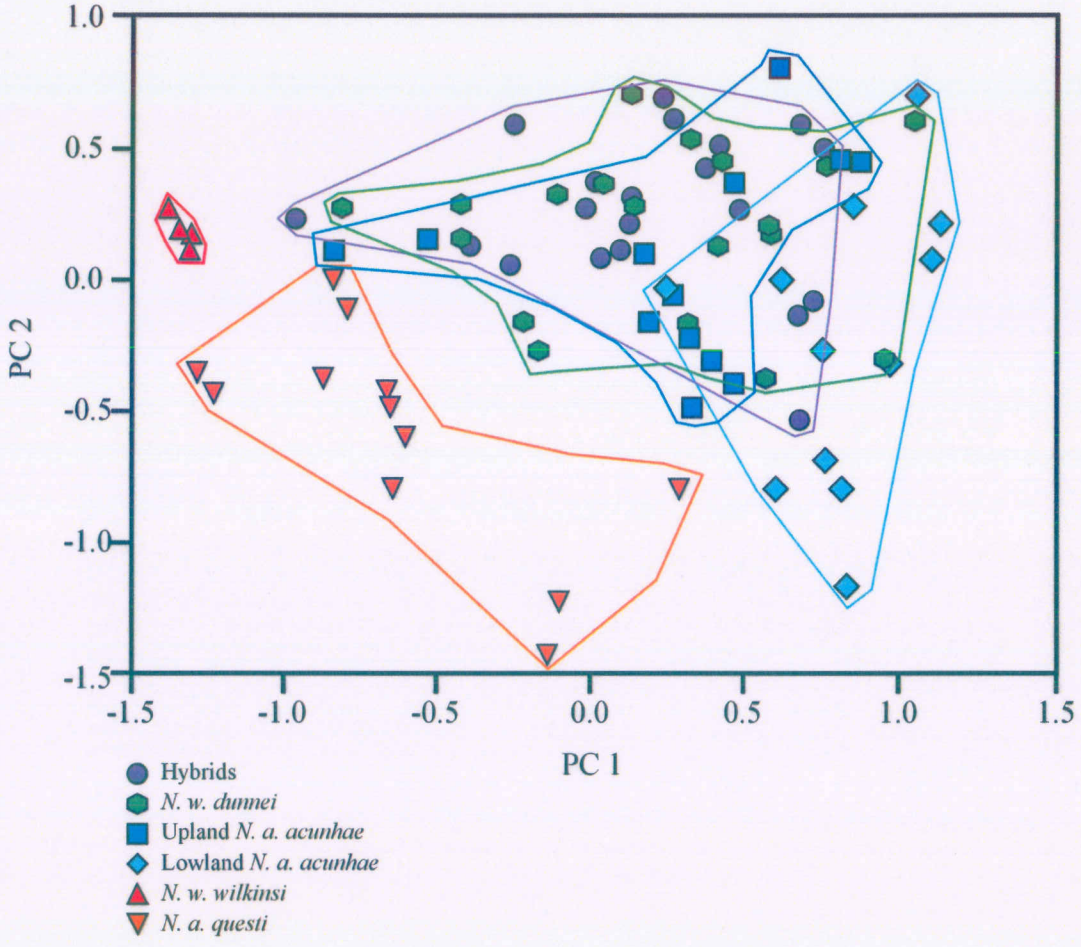


Figure 3.5: Principal components analysis based on allele frequencies of 97 *Nesospiza* individuals for five microsatellite loci, showing the first two PCs, which accounted for 52% of the total variation.

3.7 Population assignment

Levels of population differentiation (F_{ST}) strongly influenced the performance of the populations in the assignment tests. Buntings from Nightingale Island were assigned to their population of origin with high frequencies, whereas buntings from the hybrid and hybridising populations at Inaccessible Island were poorly assigned. It was thus decided to pool the three hybridising populations and rerun the analysis (Table 3.8). After combining *N. w. dunnei* and upland *N. a. acunhae* populations with the hybrid population, the frequency of assignment increased dramatically, with the exclusive assignment frequencies also increasing as a result. The assignment results also indicated shared ancestry with recent differentiation between the upland and lowland *N. a. acunhae* populations due to the fact that lowland *N. a. acunhae* genotypes were found to be similar to those from the upland population, with some lowland individuals being assigned to the upland population. Interestingly no individuals were incorrectly assigned outside of their island of origin, which lends support to the hypothesis of two island lineages. This may however be as a result of the relatively small number of loci used and the low allelic diversity of the loci in this study, which would have a direct influence on the levels of resolution obtained by the assignment tests (Cornuet *et al.* 1999).

3.8 Extra-pair parentage

The genotypes of all nestlings were compatible with those of their mother whereas five of the 43 offspring (11%) were not compatible with the composite genotypes of their putative fathers. Two of the five offspring showed a mismatch at only a single locus, these individuals are more than likely extra-pair young, however there is a chance that the mismatch was as a result of a mutation in the microsatellite repeat. This is however unlikely considering the rate at which the *Vidua* microsatellites appear to mutate. Of the five extra-pair young three of the individuals were from different nests and two were from the same nest. The same male could have sired the two extra-pair individuals from the same nest since both individuals had an identical mismatched allele. In the remaining cases each of the young had a sibling who could have been sired by the putative father. Two of the nests with extra-pair young belonged to lowland *N. a. acunhae* birds and the remaining two to hybrid pairs. The loci used in this study were not as polymorphic as loci that have been used in other extra-pair parentage studies (e.g. between eight and 75 alleles per locus at five loci used by Kempenaers *et al.* 2001), and the number of nests studied was not very high, thus the frequency of extra-pair parentage may be underestimated. However, it can be stated conclusively that extra-pair copulations and fertilisations do occur in *Nesospiza*. To further test the presence and levels of extra-pair paternity in *Nesospiza* it is suggested that more polymorphic loci be used and that a larger number of nests be tested to ensure that statistically significant sample sizes are screened.

Table 3.8 Assignment test results showing the frequency of assignment of individuals to their population of origin with the assumption that *N. w. dunnei*, the upland *N. a. acunhae* and hybrid populations form a single breeding population (Inaccessible group), using Bayesian analyses, with the leave one out option in GENECLASS. Input populations are shown in the column on the left and populations to which individuals were assigned are shown in the top row. The diagonal (in bold) shows the frequency of individuals assigned correctly to their population of origin, figures in brackets indicate the frequency at which individuals were assigned exclusively to their population of origin and sample size is indicated by n.

	Inaccessible Island		Nightingale Island	
	Inaccessible group n = 58	Lowland *N. a. acunhae* n = 13	*N. w. wilkinsi* n = 11	*N. a. questi* n = 15
Inaccessible group	**0.93** (0.43)	0.07	0.00	0.00
Lowland *N. a. acunhae*	0.38	**0.62** (0.23)	0.00	0.00
N. w. wilkinsi	0.00	0.00	**1.00** (0.00)	0.00
N. a. questi	0.00	0.00	0.13	**0.87** (0.27)

CHAPTER FOUR

DISCUSSION

4.1 Inferences from mitochondrial DNA

Several population genetic studies on passerines have shown a deficiency of genetic structuring, using mtDNA as a marker, even though morphology clearly suggested differentiation (Zink 1988, 1991, Freeland and Boag 1999, Grapputo *et al.* 1998, Sato *et al.* 1999). In all these cases recent evolution of morphological differentiation has been proposed as the reason for the contrasting molecular and morphological results, i.e. the mtDNA of these species is not yet reflective of the morphological differentiation due to the short interval since the morphological variation arose relative to the rate at which mtDNA evolves. This typically occurs in small populations that have steep selection gradients resulting in relatively rapid evolution of morphological traits (Klein *et al.* 1993, Meyer 1993, Danley and Kocher 2001).

The lack of mtDNA differentiation among *Nesospiza* populations is difficult to reconcile with their distinct morphology. The observed patterns of mtDNA variation weakly suggest the presence of two island lineages, although this is in conflict with the morphological similarity of the species. This observed pattern of variation suggests sequential island colonisation, as proposed in the stepping stone model (Kimura 1953, Kimura and Weiss 1964). The inter-island colonisation must have resulted in a severe bottleneck of the colonising population and soon after the colonisation event a new mtDNA haplotype may have arisen in one of the populations, which subsequently spread throughout that population, thus resulting in the observed distribution of haplotypes. This scenario would thus seem to favour a sympatric mode of speciation in *Nesospiza*. The low frequency of 'incorrect' haplotypes observed on the islands may be due to the retention of ancestral polymorphism within the populations subsequent to the establishment of the two mtDNA lineages or could be as a result of low levels of movement between the islands (Ryan 1992).

Although rare, inter-island movement of birds is likely to be more common among the smaller Tristan buntings (*N. acunhae*) as they are more abundant at both islands and are stronger fliers, with lower wing loadings (Ryan 1992). *Nesospiza acunhae* birds would thus have an increased probability of successfully crossing between the two islands. The distribution of 'incorrect' haplotypes among the populations could therefore be due to immigrant female birds breeding with resident males, since only females transfer mtDNA to their offspring (Lansman *et al.* 1983). Males are larger than females in most hybrid *Nesospiza* pairs (Ryan 1992). Immigrant female *N. a. acunhae* at Nightingale Island will be appreciably larger than the local male *N. a. questi* buntings and thus perhaps more likely to mate with the local male Wilkins' buntings (*N. w. wilkinsi*). Female *N. a. questi* arriving at Inaccessible Island on the other hand are more likely to mate with *N. a. acunhae* birds along the coast, since this is the habitat type closest to that found on Nightingale Island and the

males are larger than the females. This is the most likely scenario for the observed distribution of 'incorrect haplotypes' and could explain the presence thereof in the individuals where they where found (Table 3.1).

The clustering of mtDNA haplotypes by island of origin may also however, be explained by hybridisation (Freeland and Boag 1999). It has been suggested that frequent introgressive hybridisation may obscure true phylogenetic relationships between taxa due to mtDNA haplotypes, established in allopatry, spreading through sympatric populations leading to mistaken conclusions with regards to origins of populations (Grant and Grant 1992, Avise 1994). If the birds did speciate in allopatry and subsequently sympatric populations were established, hybridisation may have resulted in the complete introgression of mtDNA haplotypes within the hybridising taxa. This situation is plausible if it occurred after the birds had diverged in allopatry and shortly after sympatric populations of *N. acunhae* and *N. wilkinsi* were established on both islands. In this case immigrant birds may have had fewer mate choices and may thus have hybridised with birds from the other species. A situation similar to this has been observed in the Darwins' finch *Geospiza fuliginosa* where 73% of immigrants of this species to Daphne Major from other islands hybridise with other *Geospiza* species (Grant 1993). The resultant hybridisation may thus have led to complete introgression of mtDNA in one of the immigrant *Nesospiza* populations.

The mtDNA sequence data suggest that the island lineages at Tristan da Cunha diverged from a common ancestor relatively recently. The two lineages are thus 'young' in evolutionary terms when compared with other island passerines. By comparison, the large sequence divergence between *Rowettia* and *Nesospiza* suggests that these two genera last shared a common ancestor approximately 2.4 - 2.9 million years ago, suggesting long-term bunting presence at the Tristan da Cunha group and at Gough Island or that the birds at each island arose from different parental stocks. The large sequence divergences between *Rowettia* and *Nesospiza* also show that there is and has been no gene flow between these genera for an extended period of time and is suggestive of independent colonisations from the South American mainland. Although the *Rowettia* population at Gough Island showed low levels of haplotype diversity, this was higher than that observed at the Tristan da Cunha group. This suggests that the mtDNA of these birds has had time to evolve and build up mutations, something that the recently diverged *Nesospiza* has not had.

Radiations of other passerines at oceanic archipelagos such as the Darwin's finches (Grant 1986) and Hawaiian honeycreepers (Raikow 1976, Olson and James 1982) have been far more extensive than the radiation of *Nesospiza* at Tristan da Cunha. The fact that it appears that *Nesospiza*

has inhabited the islands for a short time relative to other island passerines could account for the limited extent of the radiation. The radiation may also have been limited due to the small island sizes, low habitat diversity and low number of islands in the group, which offer the birds fewer niches and thus opportunities to speciate. Also the fact that the climatic conditions at the islands are relatively stable all year round (Wace and Holdgate 1976, Höflich 1984), coupled with the fact that the habitat has not changed for at least 20 000 years (Wace and Dickson 1965, Preece *et al.* 1986) may have resulted in the radiation being less extensive that at other oceanic archipelagos.

4.2 Inferences of within population patterns of genetic diversity

Expected heterozygosity (H_E) for DNA microsatellites usually ranges from 0.5 – 1.0 (Jarne and Lagoda 1996); in this study mean H_E was low (0.486 for the five loci), less than the values reported for other passerine species (e.g. Reed Bunting H_E = 0.83; Grapputo *et al.* 1998). The low levels of heterozygosity observed in this study may be an artefact of using only five loci or may be due to the presence of null alleles (Callen *et al.* 1993). However, similar low levels of heterozygosity have been documented in other populations, which have undergone severe bottlenecks (e.g. bighorn sheep H_E = 0.43; Forbes *et al.* 1995, cheetah H_E = 0.39; Menotti-Raymond and O'Brien 1995). Severe bottlenecking of the founder populations of buntings at Tristan da Cunha and Gough Island may explain the low levels of genetic variability observed. The small population sizes, relative to mainland passerine populations, may also account for the low levels of heterozygosity in *Rowettia* and *Nesospiza*, especially in the *N. w. wilkinsi* population, which has the smallest population size.

4.3 Inferences of genetic divergence among populations

It is widely accepted that species specialise and adapt to new niches from generalist populations (Schoener 1965). *Nesospiza acunhae* are generalists at Tristan da Cunha, and are thus most likely closest to the ancestral form. Also generalists are more common than specialists due to more resources being available to the generalist populations (Schoener 1968). The abundance of *N. acunhae* may aid in explaining the observed patterns of differentiation at F_{ST} and R_{ST} since, due to there being more *N. acunhae* birds on both islands, based purely on probability, *N. acunhae* are more likely to cross over successfully from one island to the other without being taken by skuas. Low levels of movement of *N. acunhae* birds between the islands would promote interbreeding between the populations and could account for the observed patterns of differentiation.

Population demographics and evolutionary history play an important role in the ability of populations to adapt to changes in their environment and also directly influence the levels of genetic variability in populations (Grant and Grant 1989). Low levels of morphological variability among

island populations often occurs as a direct result of small population size and may contribute to rapid extinction due to the inability of populations to respond to environmental changes (Grant and Grant 1989, Ryan and Siegfried 1994). Larger populations are able to maintain higher levels of genetic variation and are able to endure population fluctuations with a larger probability of survival than smaller populations (Boag 1988). Small populations on the other had are less well equipped to survive population fluctuations and are profoundly affected by the effects of random genetic drift and inbreeding due to their small sizes (Hartl 1998, Carson and Templeton 1984).

The *N. w. wilkinsi* population is by far the smallest with approximately only 50 pairs (Ryan and Siegfried 1994). It is thus not surprising that this population has the lowest levels of heterozygosity ($H_O = 0.085$), allelic diversity and the largest number of monomorphic loci. Ryan and Siegfried (1994) also showed that this population had the lowest levels of morphological variability when compared with the other populations. The decreased variability in the *N. w. wilkinsi* population may be as a result of founder effects and random genetic drift, two of the key forces driving evolutionary change at oceanic archipelagos (Carson and Templeton 1984). The small size and low levels of both morphological and genetic variability of this population puts it at the highest risk for possible extinction due to its inability to respond to environmental changes.

The generalist *N. a. questi* population at Nightingale Island has greater evolutionary potential in respect of environmental changes than the specialist *N. w. wilkinsi* population. With its larger population size (*ca.* 2500 individuals) and slightly larger levels of morphological variability (Ryan and Siegfried 1994) it has the evolutionary potential to adapt to change. Levels of genetic variability in the *N. a. questi* population were also considerably greater ($H_O = 0.44$) than the *N. w. wilkinsi* population; this is however not surprising considering that its larger population size would assist in the maintenance of higher levels genetic variability.

The mtDNA data weakly suggested that the two Nightingale Island populations formed an independent lineage from the Inaccessible Island populations. This was further corroborated by the microsatellite data. Morphology however showed that *N. a. questi* and *N. w. wilkinsi* birds were respectively the smallest and largest birds in terms of body size on both islands. This seems counterintuitive since one might expect birds forming a single lineage to resemble each other more closely than they resemble birds from another lineage. Directional selection accompanied by assortative mating may have assisted in the evolution of these two species. There is also no evidence of hybridisation between *N. a. questi* and *N. w. wilkinsi* at Nightingale Island. The absence of

introgressive hybridisation coupled with the simple habitat structure of Nightingale Island may have resulted in these two populations diverging to the degree to which they have.

The situation at Inaccessible Island is far more complex than at Nightingale Island due to the occurrence of widespread hybridisation and the presence of the two morphs of *N. a. acunhae*. The two *N. a. acunhae* populations at Inaccessible Island are distinguished on plumage colouration, bill size and body size, although some degree of overlap does occur between morphological characters in these two populations (Figure 1.3). Both populations have large population numbers relative to the other populations at Tristan da Cunha (*ca* 5000 lowland *N. a. acunhae* and 2000 upland *N. a. acunhae*). Although the two morphs of *N. a. acunhae* have been shown to interbreed, this is confined to a narrow altitudinal contact zone (Ryan 1992). Genetically these two populations are only slightly differentiated from each other (pairwise $F_{ST} = 0.069$). The morphological and genetic differentiation between these two populations however highlights that upland and lowland *N. a. acunhae* share low levels of gene flow. In addition selection appears to act against upland and lowland *N. a. acunhae* hybrids, and has thus led to genetic as well as morphological differentiation of the morphs. The morphological similarity and behaviour of the two morphs further suggests that lowland *N. a. acunhae* diverged from upland *N. a. acunhae* subsequent to the divergence of *N. w. wilkinsi* which is a presumption strongly supported by the genetic data.

The *N. w. dunnei* population has surprisingly high levels of genetic variability ($H_O = 0.432$) bearing in mind that there are estimated to only be 250 individuals in this population. Considering the small size of this population one might expect similar low levels of variability as was observed in *N. w. wilkinsi* from Nightingale Island. The high levels of heterozygosity and similar high levels of morphological variability (Ryan and Siegfried 1994) in this population are likely as a result of hybridisation between this population and the upland *N. a. acunhae* population. The observation that hybridisation has assisted in maintaining genetic diversity is consistent with the low allelic diversity observed in the non-hybridising *N. w. wilkinsi* population.

Hybridisation between upland *N. a. acunhae* and *N. w. dunnei* is leading to large-scale introgression and removal of diagnosable genetic signatures in these two populations (even in the birds occurring outside the hybrid zone). The results suggest that upland *N. a. acunhae* and *N. w. dunnei* are actually a single panmictic population with high levels of gene flow occurring within this population. With time the large-scale hybridisation at Inaccessible Island may result in the morphological and genetic obscurity of the currently recognised taxa since F1 hybrid offspring are intermediate to their parents and appear to be fully fertile (Ryan 1992, 2001). Hybridisation has been

shown to obscure phylogenetic relationships in cases of frequent hybridisation among taxa (Grant and Grant 1992, Avise 1994). Introgressive hybridisation has further been shown to promote evolutionary change by introducing new alleles to populations, creating new combinations of alleles which may be advantageous to the individuals carrying them resulting in these individuals being favoured by natural and sexual selection (Grant and Grant 1997).

4.4 Speciation in Nesospiza

Nesospiza acunhae and *N. wilkinsi* show large and significant variation in bill and body size, song and plumage colouration (Ryan 1992). Indeed the morphological differences are so marked that the two species could be assigned to different genera (Lowe 1923). Three possible speciation scenarios; allopatric, microallopatric or sympatric speciation with parallel evolution can explain the present day distribution of taxa and observed distribution of mtDNA haplotypes and microsatellite alleles at the Tristan da Cunha Islands (Figure 4.1).

The allopatric model of speciation (Figure 4.1b) has been proposed, based on research on Darwin's finches, to apply to most bird species (Grant and Grant 1997). Grant and Grant (1997) have proposed six rules for avian speciation:

1. Speciation is initiated in allopatry
2. The sympatric phase is established after ecological divergence in allopatry
3. Evolution of postmating isolating mechanisms in allopatry or sympatry is preceded by allopatric evolution of premating isolating mechanisms
4. Premating mechanisms are controlled by additive effects of polygenes whereas postmating mechanisms are due, mainly, to nonadditive genetic effects
5. Premating mechanisms include the effects of the cultural process of sexual imprinting
6. Postzygotic incompatibilities arise in the heterogametic sex in accordance with Haldane's rule (in birds the female is the heterogametic sex)

The allopatric model assumes that a species whose range is split by a physical isolating barrier will accumulate genetic differences in each subpopulation on either side of the barrier over many generations (Mayr 1942, 1963). This is the first stage of speciation and results in ecological divergence, which in birds could result in development of different mate recognition and signalling systems (Grant and Grant 1998), due to drift and selection acting on isolated populations. The second stage of allopatric speciation involves secondary contact, by dispersal, of members of the isolated populations. If and when secondary contact occurs between previously isolated populations, the genomes of each population may have evolved to be incompatible. If the genomes are not

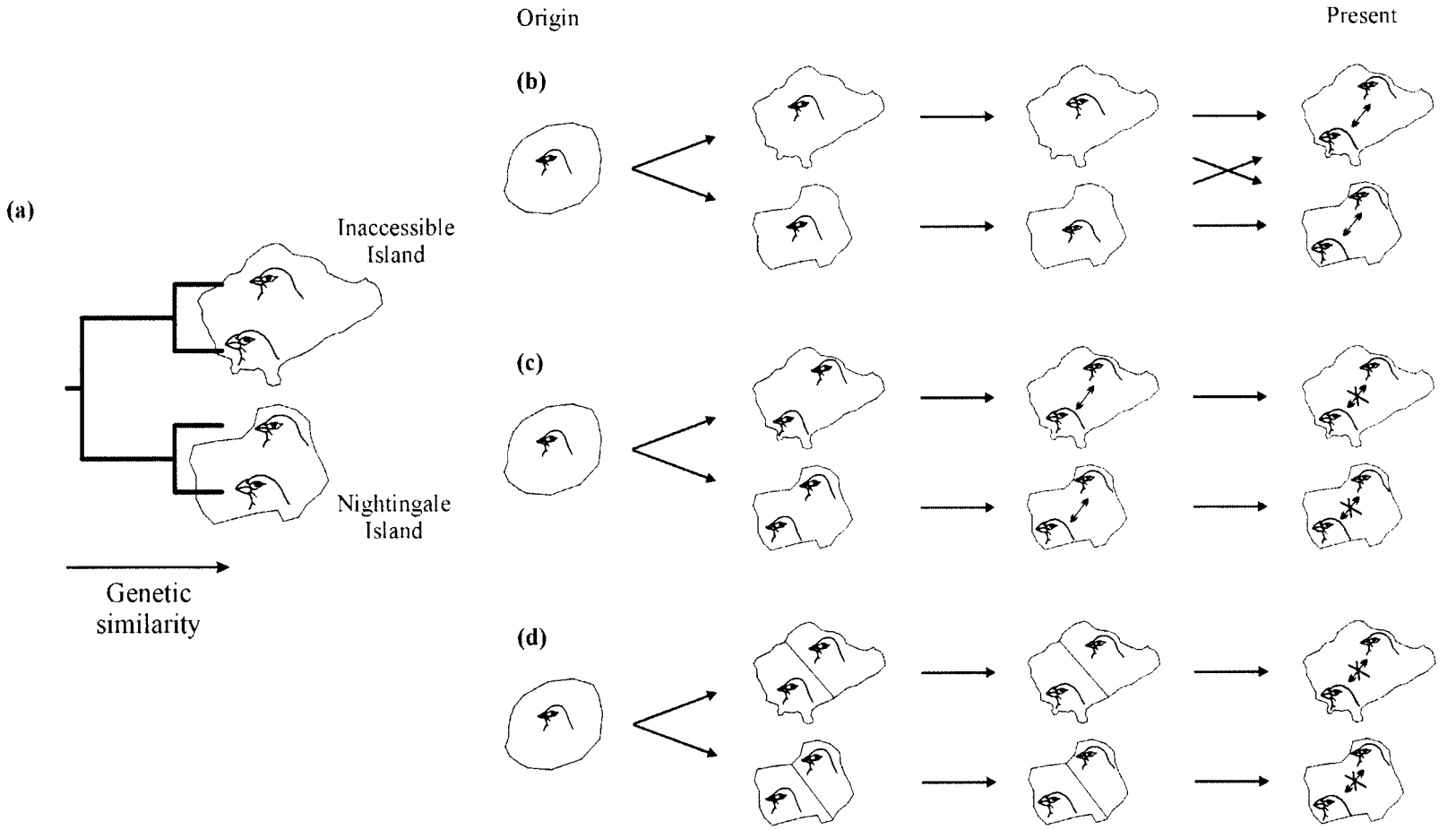


Figure 4.1: Possible scenarios explaining the distribution of *Nesospiza* buntings at Tristan da Cunha. **(a)** Large and small-billed "species" are found on Inaccessible and Nightingale Islands, but genetic markers show monophyly of species on each island. **(b)** **(c)** and **(d)** Show the three evolutionary scenarios which might explain these results: **(b)** allopatric divergence followed by secondary overlap and introgression; **(c)** parallel evolution of reproductive isolation by means of divergent selection promoting morph formation. In this case reproductive isolation may be a secondary effect of a habitat shift, changing the behaviour and morphology of the birds, or it may be as a consequence of selection favouring increasing association between fitness traits and mate preference traits; **(d)** microallopatric divergence on each island owing to earlier local barriers (modified from Johannesson 2001).

completely incompatible, reinforcement of isolating mechanisms may lead to speciation by reinforcement or may result in coalescence (Dobzhansky 1940).

In *Nesospiza,* however, sympatric heterospecific populations have the same mtDNA haplotype and a different haplotype from their allopatric (and presumed conspecific) island neighbours. It would thus appear that there has been complete lineage sorting of mtDNA haplotypes on each island. Allopatric speciation is therefore unlikely as a model for explaining the observed pattern of monophyly of mtDNA haplotypes by island in *Nesospiza*, since under these assumptions it would require large-scale introgression of mtDNA, in at least one of the populations. This situation is unlikely considering that presently hybridisation only occurs on the plateau of Inaccessible Island. However if the large and small billed forms did indeed speciate in allopatry, hybridisation shortly after the establishment of sympatric populations of the two species may have resulted in complete introgression of mtDNA in the recolonising population if this population had very few birds, a situation similar to that observed in *Geospiza fuliginosa* (Grant 1993).

The allopatric model may thus be followed with regards to speciation between *N. acunhae* and *N. wilkinsi,* considering that hybridisation between birds from the same island has resulted in ambiguous signals in terms of the nuclear and mitochondrial genetic signatures of these populations. This has resulted in them appearing to be more closely related to their sympatric, heterospecific neighbours. The allopatric model of speciation however does not provide an adequate explanation for the presence of the two sympatric *N. a. acunhae* morphs at Inaccessible Island, and the fact that these two morphs appear to be speciating in sympatry.

The second and most likely speciation scenario for *Nesospiza* would involve sympatric speciation (Figure 4.1c). Sympatric speciation has been found to occur in brood parasitic indigobirds by means of a simple host switch (Sorenson *et al.* 2003). Sympatric speciation does not require physical isolation of populations to result in genetic divergence. Subpopulations may have overlapping distributions but still diverge due to different selection pressures, leading to the formation of new species (Bush 1969, 1975). Bush (1969) advocated that speciation could and does occur due to traits evolving in response to divergent selection on subpopulations, co-occurring in the same area, resulting in them adapting to different habitats or niches. Such subpopulations become genetically distinct from each other if a niche shift occurs in some (but not all) of the organisms. This shift in habitat can be reinforced by means of individuals mating within the preferred habitat. If mating is restricted to preferred habitats it results in the establishment of genetically distinct sympatric habitat lineages, which over time evolve into distinct species (Bush 1969, 1975).

Sympatric speciation is thus driven by divergent selection on the individuals in a subpopulation leading to the evolution of different ecotypes or lineages. This divergent selection is reinforced by means of assortative mating in the presence of gene flow (Johannesson 2001). Assortative mating thus acts as a cohesion mechanism driving the evolution of species in sympatry.

The results of this study seem to support a sympatric mode of speciation in *Nesospiza*. The mtDNA data shows that sympatric populations of *Nesospiza* carry the same mtDNA suggesting that birds on each island arose from the same mitochondrial lineage. The observed pattern of variation from the partial cytochrome b sequences does not allow genetic discrimination between the designated species and subspecies at the mtDNA level. Interestingly though the two *Nesospiza* haplotypes observed differ from each other by a single silent transition with each haplotype occurring at very high frequency on either Inaccessible or Nightingale Island thus allowing genetic discrimination between birds based on their island of origin. The division of mtDNA haplotypes based on the island of origin suggests that birds from the same island arose from a parental stock with the same haplotype. As a result of this, interspecific relatedness within islands is lower than the intraspecific relatedness between subspecies, according to the current species and subspecies designations (Lowe 1923, Hagen 1952). The current observed distribution of haplotypes thus suggests that large- and small-billed forms arose in sympatry and there were thus independent origins of large- and small-billed forms on each of the islands. The most plausible explanation for this observed distribution of mtDNA haplotypes is that *Nesospiza* is a case of recent sympatric speciation with parallel evolution on each island.

The microsatellite DNA data further support a sympatric model of speciation in *Nesospiza* since sympatric populations at each island have similar allele frequencies and are more closely related to each other in the individual and population level analyses, suggesting closer relationships between sympatric heterospecific populations than between allopatric conspecific populations. This paraphyletic relationship between birds of the same 'apparent' morphological subspecies at mitochondrial and nuclear level is best explained by considering parallel speciation.

Parallel speciation occurs when reproductive isolation evolves independently, more than once, in several different areas. Cases of parallel evolution are the key to explaining sympatric speciation because these are cases where the evolutionary process has had a similar outcome at more than one location, in the absence of any obvious barriers to gene flow (Schluter and Nagel 1995). Several recent studies on parallel evolution have shown that characters affecting reproductive isolation can diverge rapidly in sympatry as a result of the forces of natural selection (Johannesson *et al.* 1993,

Schliewen *et al.* 1994, Taylor *et al.* 1996, Lu and Bernatchez 1999, Rundle *et al.* 2000). These studies, based on the phylogenetics and ecology of natural populations, reveal much about the timescales, patterns and processes that lead to speciation in sympatry (reviewed in Johannesson 2001). In *Nesospiza* parallel evolution of reproductive isolation on each island would thus have been established by divergent selection acting on the sympatric populations, resulting in a similar evolutionary outcome at both Inaccessible and Nightingale Islands. This could have arisen due to a possible niche shift (some of the birds starting to feed on *Phylica* fruits), which resulted in changes in the morphology and behaviour of a subset of the population, with a strong selective advantage for individuals with these new traits. The morphological and nuclear DNA differentiation of the upland and lowland morphs of *N. a. acunhae* lends further support to the hypothesis that sympatric speciation has occurred at Tristan da Cunha, since these birds appear to be in the process of speciation at Inaccessible Island and neither appears to have arisen on one of the other islands.

Finally, parapatric (microallopatric) divergence (Figure 4.1d) could have occurred at Tristan da Cunha given a temporary barrier separating the populations on each island and they would thus have diverged in partial isolation. The islands however have a relatively stable environment and due to the small size of the islands it is unlikely that local barriers acted as isolating mechanisms in the past.

4.5 The most likely speciation scenario

Although low levels of variation were observed using mtDNA as a genetic marker it was found that the mtDNA haplotypes clustered the birds into two island lineages. These two lineages were further strongly supported by the microsatellite data in all of the analyses, even in the face of low levels of allelic diversity at most of the loci. The genetic data are thus not concordant with the morphological classification of *Nesospiza*. The literature describes *Nesospiza* as a simple two species adaptive radiation at an oceanic archipelago (Lack 1947). Standard theory based on speciation of avian taxa at islands requires populations to be allopatric, at least during the early stages of speciation (Lack 1947, Mayr 1963, Grant 1986, Grant and Grant 1997). For allopatric speciation to be plausible in *Nesospiza* secondary invasions of both islands associated with complete introgression in at least one of the populations after speciation would have been required to support the current observed distributions of mtDNA haplotypes and nuclear alleles.

The molecular data refute these assumptions and suggest that the islands were colonised by island hopping. Each island was only colonised once and once a population of buntings was established on each island sympatric speciation occurred, independently in each of these populations, due to a subset of each population specialising on the fruits of the *Phylica* trees thus leading to

divergent selection for bill size. The close association with *Phylica* trees could have led to divergent selection for bill size being reinforced by assortative mating even though sympatric congeners were present. Strong directional selection for larger bills in the two *N. wilkinsi* populations coupled with genetic drift would have resulted in large and small-billed forms diverging into distinct species on each island. However, due to the similarity in available habitat and selection pressures at the two islands and due to shared ancestry of the birds the evolutionary process had a similar outcome and led to sympatric speciation with parallel evolution of the different bill morphs. This proposed mechanism for speciation for *Nesospiza* is concordant with the observed patterns of morphological variation at the Tristan da Cunha group and with the observed patterns of nuclear and mtDNA variation.

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APPENDIX

Appendix 1: Compound microsatellite genotypes of all specimens of *Nesospiza* and *Rowettia* buntings genotyped for this study, numbers indicate allele sizes as scored with GENOTYPER software (PE Applied Biosystems). Individuals were numbered according to the blood sample used to obtain the genotype and question marks indicate missing data.

Island of origin	Species and individual number	INDIGO 8		INDIGO 15		INDIGO 30		INDIGO 31		INDIGO 40		INDIGO 41	
Inaccessible	Hybrid 8	158	158	193	197	232	232	308	308	235	239	357	361
	Hybrid 9	158	158	193	197	232	232	304	308	235	239	341	357
	Hybrid 10	158	158	193	197	232	232	304	308	235	239	?	?
	Hybrid 11	158	158	193	197	232	232	308	308	235	235	353	361
	Hybrid 12	158	158	197	197	232	232	304	308	235	235	361	365
	Hybrid 13	158	158	197	197	232	232	308	308	235	235	357	361
	Hybrid 15	158	158	193	197	232	232	304	308	235	239	361	361
	Hybrid 16	158	158	193	197	232	232	308	308	235	239	361	361
	Hybrid 24	158	158	197	197	232	232	308	308	235	235	341	361
	Hybrid 25	158	158	197	197	232	232	308	308	?	?	?	?
	Hybrid 26	158	158	193	197	232	232	308	308	235	239	361	365
	Hybrid 27	158	158	197	197	232	232	308	308	235	235	357	369
	Hybrid 31	158	158	193	197	232	232	304	308	235	235	341	353
	Hybrid 41	158	158	197	197	228	232	304	308	239	239	353	357
	Hybrid 42	158	158	197	197	232	232	308	308	235	235	361	361
	Hybrid 43	158	158	197	197	232	232	308	308	235	235	341	369
	Hybrid 44	158	158	193	197	232	232	304	308	235	235	?	?
	Hybrid 45	158	158	197	197	232	232	308	308	235	239	357	361
	Hybrid 47	158	158	197	197	232	232	308	308	235	235	361	361
	Hybrid 48	158	158	197	197	232	232	304	308	235	235	341	365
	Hybrid 49	158	158	197	197	232	232	308	308	235	239	341	361
	Hybrid 50	158	158	197	197	232	232	308	308	?	?	?	?
	Hybrid 53	158	163	197	197	232	232	308	308	235	239	353	361
	Hybrid 54	158	158	197	197	232	232	308	308	235	239	345	361
	Hybrid 58	158	158	197	197	228	232	308	308	235	235	357	361
	Hybrid 67	158	158	197	197	232	232	304	308	235	235	341	361
	Hybrid 71	158	158	197	197	228	232	308	308	235	239	345	357
	Hybrid 73	158	158	197	197	232	232	308	308	235	235	357	357
	Hybrid 74	158	158	193	197	228	232	308	308	235	239	357	357
	Hybrid 75	158	158	193	193	232	232	308	308	235	239	353	357
	Hybrid 77	158	158	193	197	232	232	304	304	235	235	353	361
	Hybrid 79	158	158	193	197	232	232	308	308	235	239	345	369
	Hybrid 80	158	158	193	197	228	232	304	304	239	239	353	361
	Hybrid 83	158	158	193	197	232	232	304	308	235	239	361	361
	Hybrid 84	158	158	197	197	232	232	308	308	235	239	357	361
	Hybrid 85	158	158	197	197	232	232	308	308	239	239	353	353
	Hybrid 88	158	158	197	197	232	232	308	308	235	239	361	361
	Hybrid 89	158	158	197	197	232	232	308	308	235	235	345	353
	Hybrid 90	158	158	193	197	232	232	308	308	235	235	341	361
	Hybrid 91	158	163	197	197	232	232	308	308	235	235	357	361
	Hybrid 92	158	158	193	197	220	232	308	308	235	239	357	369
	Hybrid 93	158	158	197	197	228	228	308	308	235	235	345	357

Island of origin	Species and individual number	INDIGO 8		INDIGO 15		INDIGO 30		INDIGO 31		INDIGO 40		INDIGO 41	
Inaccessible	Hybrid 94	158	158	193	197	228	232	308	308	235	235	357	361
	Hybrid 95	158	158	193	197	232	232	304	308	235	239	345	361
	Hybrid 96	158	158	197	197	232	232	308	308	239	239	353	353
	Hybrid 97	158	158	193	197	232	232	308	308	?	?	357	357
	Hybrid 98	158	158	197	197	232	232	308	308	235	235	353	361
	Hybrid 99	158	158	197	197	232	232	308	308	235	239	341	361
	Hybrid 100	158	158	197	197	232	232	308	308	235	235	357	361
	Hybrid 101	158	158	193	197	232	232	308	308	235	239	357	357
	Hybrid 102	158	158	193	193	232	232	308	308	235	235	357	361
	Hybrid 105	158	158	193	197	232	232	308	308	235	239	341	357
	Hybrid 123	158	158	197	197	232	232	308	308	235	239	341	369
	Hybrid 124	158	158	193	197	220	228	308	308	235	235	357	369
	Hybrid 125	158	158	197	197	232	232	308	308	235	239	361	361
	Hybrid 126	158	158	193	193	232	232	304	304	235	239	345	357
	Hybrid 127	158	158	197	197	232	232	308	308	235	239	341	365
	Hybrid 128	158	158	197	197	232	232	308	308	239	239	353	357
	Hybrid 130	158	158	193	197	232	232	304	308	235	239	357	361
	Hybrid 131	158	158	193	197	228	232	304	304	?	?	357	361
	Hybrid 132	158	158	197	197	232	232	308	308	?	?	341	341
	Hybrid 133	158	158	197	197	228	232	308	308	235	239	341	341
Inaccessible	*N. w. dunnei* 5	158	158	193	197	232	232	308	308	235	239	353	369
	N. w. dunnei 7	158	163	193	197	232	232	308	308	235	235	357	357
	N. w. dunnei 8	158	158	193	197	232	232	308	308	235	239	353	361
	N. w. dunnei 9	158	158	193	193	232	232	304	308	235	235	345	361
	N. w. dunnei 11	158	158	197	197	232	232	304	308	235	239	345	357
	N. w. dunnei 12	158	158	193	197	232	232	304	308	235	239	357	361
	N. w. dunnei 13	158	158	193	197	232	232	304	308	235	235	353	357
	N. w. dunnei 14	158	163	193	197	232	232	304	308	239	239	341	353
	N. w. dunnei 15	158	158	193	193	232	232	304	308	235	239	357	357
	N. w. dunnei 16	158	158	193	197	232	232	308	308	235	235	361	369
	N. w. dunnei 17	158	158	193	197	232	232	308	308	235	235	361	361
	N. w. dunnei 18	158	158	197	197	232	232	308	308	235	235	341	361
	N. w. dunnei 19	158	158	197	197	232	232	308	308	235	235	361	369
	N. w. dunnei 20	158	158	193	197	232	232	304	308	235	235	357	357
	N. w. dunnei 21	158	158	193	193	232	232	304	308	235	235	345	361
Inaccessible	Upland *N. a. acunhae* 7	158	158	193	197	228	232	308	308	235	235	341	357
	Upland *N. a. acunhae* 9	158	158	193	197	228	232	308	312	235	239	357	361
	Upland *N. a. acunhae* 12	158	158	197	197	228	232	304	308	235	239	357	361
	Upland *N. a. acunhae* 15	158	158	197	201	228	232	308	308	235	235	361	361
	Upland *N. a. acunhae* 16	158	158	193	197	232	232	308	308	235	235	357	361
	Upland *N. a. acunhae* 19	158	158	193	197	232	232	308	308	235	235	353	357
	Upland *N. a. acunhae* 22	158	158	193	197	220	228	308	308	235	239	357	361
	Upland *N. a. acunhae* 23	163	163	197	197	228	232	308	308	235	239	345	357
	Upland *N. a. acunhae* 25	158	158	193	197	232	232	304	304	235	235	341	361
	Upland *N. a. acunhae* 26	158	158	189	193	232	232	304	308	235	235	365	369
	Upland *N. a. acunhae* 27	158	158	193	193	220	232	308	308	235	239	357	357
	Upland *N. a. acunhae* 28	158	158	193	197	220	232	308	308	235	239	357	361

Island of origin	Species and individual number	INDIGO 8		INDIGO 15		INDIGO 30		INDIGO 31		INDIGO 40		INDIGO 41	
Inaccessible	Upland *N. a. acunhae* 29	158	158	193	197	232	232	308	308	239	239	341	353
	Upland *N. a. acunhae* 30	158	158	193	197	232	232	308	308	235	239	341	357
	Upland *N. a. acunhae* 31	158	158	193	197	228	232	304	308	235	239	357	361
	Upland *N. a. acunhae* 32	158	158	193	197	232	232	304	308	235	239	341	369
	Upland *N. a. acunhae* 33	158	158	193	197	232	232	304	308	235	235	341	365
Inaccessible	Lowland *N. a. acunhae* 1	158	158	197	197	228	232	304	308	235	239	361	361
	Lowland *N. a. acunhae* 2	158	158	197	197	232	232	308	308	235	235	353	353
	Lowland *N. a. acunhae* 4	158	158	197	197	228	232	308	308	235	235	353	357
	Lowland *N. a. acunhae* 16	158	158	197	197	228	232	308	308	239	239	353	353
	Lowland *N. a. acunhae* 18	163	163	197	197	228	232	308	308	239	239	369	369
	Lowland *N. a. acunhae* 24	158	158	197	197	228	232	308	308	235	239	357	373
	Lowland *N. a. acunhae* 28	163	163	197	197	232	232	308	308	235	239	353	353
	Lowland *N. a. acunhae* 39	158	158	197	197	228	228	308	308	235	235	361	361
	Lowland *N. a. acunhae* 41	158	158	197	197	228	232	308	308	235	235	353	357
	Lowland *N. a. acunhae* 42	158	158	197	197	228	232	304	308	235	239	361	361
	Lowland *N. a. acunhae* 44	158	158	197	197	228	232	308	308	235	239	353	357
	Lowland *N. a. acunhae* 45	158	158	197	197	228	232	308	308	235	235	357	357
	Lowland *N. a. acunhae* 46	158	158	197	197	228	232	308	308	235	235	357	357
	Lowland *N. a. acunhae* 47	158	158	197	197	232	232	308	308	235	235	357	357
	Lowland *N. a. acunhae* 48	158	158	197	197	228	228	308	308	235	235	357	373
	Lowland *N. a. acunhae* 52	158	158	197	197	232	232	308	308	235	239	353	353
	Lowland *N. a. acunhae* 53	158	158	197	197	232	232	308	308	235	235	357	361
	Lowland *N. a. acunhae* 54	158	163	197	197	228	228	308	308	235	239	345	373
	Lowland *N. a. acunhae* 55	158	158	197	197	228	228	308	308	239	239	353	373
	Lowland *N. a. acunhae* 56	158	158	197	197	228	232	308	308	235	235	357	357
	Lowland *N. a. acunhae* 57	158	158	197	197	232	232	308	308	239	239	373	373
	Lowland *N. a. acunhae* 60	158	158	197	197	228	232	308	308	235	235	357	357
	Lowland *N. a. acunhae* 61	158	158	197	197	228	232	308	308	239	239	373	373
	Lowland *N. a. acunhae* 62	158	158	197	197	232	232	308	308	239	239	373	373
Nightingale	*N. w. wilkinsi* 4	158	158	193	197	232	232	304	304	235	235	?	?
	N. w. wilkinsi 6	158	158	193	193	232	232	304	304	235	235	329	361
	N. w. wilkinsi 7	158	158	193	193	232	232	304	304	235	235	361	361
	N. w. wilkinsi 8	158	158	193	193	232	232	304	304	235	235	361	361
	N. w. wilkinsi 10	158	158	193	193	232	232	304	304	235	235	361	361
	N. w. wilkinsi 12	158	158	193	193	232	232	304	304	235	235	365	365
	N. w. wilkinsi 14	158	158	193	193	232	232	304	304	235	235	361	365
	N. w. wilkinsi 15	158	158	193	193	232	232	304	304	235	235	365	365
	N. w. wilkinsi 16	158	158	193	193	232	232	304	304	235	235	361	361
	N. w. wilkinsi 17	158	158	193	193	232	232	304	304	235	235	361	365
	N. w. wilkinsi 18	158	158	193	193	232	232	304	304	235	235	?	?
	N. w. wilkinsi 21	158	158	193	193	232	232	304	304	235	235	?	?
	N. w. wilkinsi 23	158	158	193	193	232	232	304	304	235	235	?	?
	N. w. wilkinsi 24	158	158	193	193	232	232	304	304	235	235	361	361
	N. w. wilkinsi 25	158	158	193	193	232	232	304	304	235	235	361	365
	N. w. wilkinsi 26	158	158	193	193	232	232	304	304	235	235	?	?
Nightingale	*N. a. questi* 7	158	158	193	193	228	232	304	308	235	235	353	353
	N. a. questi 8	158	158	193	193	228	232	304	304	235	235	353	361

APPENDIX

Island of origin	Species and individual number	INDIGO 8		INDIGO 15		INDIGO 30		INDIGO 31		INDIGO 40		INDIGO 41	
Nightingale	*N. a. questi* 9	158	158	193	193	228	232	304	308	235	235	353	353
	N. a. questi 13	158	158	193	197	228	232	304	304	235	235	353	357
	N. a. questi 14	158	158	193	197	228	232	304	304	235	235	361	361
	N. a. questi 15	158	158	193	197	232	232	304	304	235	235	353	361
	N. a. questi 16	158	158	193	193	228	232	304	304	235	235	361	365
	N. a. questi 19	158	158	193	197	232	232	304	304	235	235	357	361
	N. a. questi 21	158	158	197	197	228	228	304	304	235	235	353	357
	N. a. questi 25	158	158	193	193	228	232	304	304	235	235	353	361
	N. a. questi 27	158	158	193	197	228	228	304	308	235	235	353	357
	N. a. questi 31	158	158	193	193	228	232	304	308	235	235	345	353
	N. a. questi 34	158	158	197	197	228	232	304	308	235	235	353	357
	N. a. questi 35	158	158	193	193	228	232	304	308	235	235	353	357
	N. a. questi 40	158	158	193	193	232	232	304	304	235	235	357	361
Gough	*Rowettia goughensis* 1	148	148	232	240	216	216	316	324	235	235	333	333
	Rowettia goughensis 2	148	148	240	240	216	216	316	324	235	235	333	333
	Rowettia goughensis 3	148	148	232	236	216	216	324	324	235	235	333	333
	Rowettia goughensis 4	148	148	220	240	216	216	328	332	235	235	333	333
	Rowettia goughensis 5	148	148	224	232	216	216	320	324	235	235	333	333
	Rowettia goughensis 6	148	148	228	232	216	216	316	324	235	235	333	333
	Rowettia goughensis 7	148	148	232	240	216	216	320	332	235	235	333	333
	Rowettia goughensis 8	148	148	232	240	216	216	320	324	235	235	333	333
	Rowettia goughensis 9	148	148	228	244	216	216	316	328	235	235	333	333
	Rowettia goughensis 10	148	148	228	240	216	216	316	316	235	235	333	333
	Rowettia goughensis 12	148	148	236	240	216	216	320	320	235	235	333	333
	Rowettia goughensis 13	148	148	236	240	216	216	316	324	235	235	333	333
	Rowettia goughensis 14	148	148	244	244	216	216	328	332	235	235	333	333
	Rowettia goughensis 15	148	148	240	248	216	216	332	332	235	235	333	333
	Rowettia goughensis 16	148	148	236	240	216	216	320	332	235	235	333	333

Appendix 2A: Matrix of pairwise comparisons among five microsatellite loci in *Nesospiza* bunting hybrids, from Inaccessible Island, Tristan da Cunha, showing the probabilities of genotypic linkage disequilibrium for each pairwise comparison.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41
INDIGO 15	-				
INDIGO 30	1.000	-			
INDIGO 31	0.272	0.144	-		
INDIGO 40	0.070	0.010	0.178	-	
INDIGO 41	0.685	0.819	0.587	0.333	-

Appendix 2B: Matrix of pairwise comparisons among five microsatellite loci in *Nesospiza wilkinsi dunnei* buntings from Inaccessible Island, Tristan da Cunha, showing the probabilities of genotypic linkage disequilibrium for each pairwise comparison.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41
INDIGO 15	-				
INDIGO 30	1.000	-			
INDIGO 31	0.080	0.487	-		
INDIGO 40	1.000	0.370	1.000	-	
INDIGO 41	0.250	1.000	0.355	0.034	-

Appendix 2C: Matrix of pairwise comparisons among five microsatellite loci in upland *Nesospiza acunhae acunhae* buntings from Inaccessible Island, Tristan da Cunha, showing the probabilities of genotypic linkage disequilibrium for each pairwise comparison.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41
INDIGO 15	-				
INDIGO 30	0.622	-			
INDIGO 31	0.399	0.813	-		
INDIGO 40	0.195	1.000	1.000	-	
INDIGO 41	0.173	0.962	0.820	0.511	-

Appendix 2D: Matrix of pairwise comparisons among five microsatellite loci in lowland *Nesospiza acunhae acunhae* buntings from Inaccessible Island, Tristan da Cunha, showing the probabilities of genotypic linkage disequilibrium for each pairwise comparison. N.P = not possible meaning one of the loci in the pairwise comparison is monomorphic in the population.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41
INDIGO 15	-				
INDIGO 30	N.P	-			
INDIGO 31	N.P	1.000	-		
INDIGO 40	N.P	1.000	0.461	-	
INDIGO 41	N.P	0.926	1.000	0.567	-

Appendix 2E: Matrix of pairwise comparisons among five microsatellite loci in *Nesospiza wilkinsi wilkinsi* buntings from Nightingale Island, Tristan da Cunha, showing the probabilities of genotypic linkage disequilibrium for each pairwise comparison. N.P = not possible meaning one of the loci in the pairwise comparison is monomorphic in the population.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41
INDIGO 15	-				
INDIGO 30	N.P	-			
INDIGO 31	N.P	N.P	-		
INDIGO 40	N.P	N.P	N.P	-	
INDIGO 41	N.P	N.P	N.P	N.P	-

Appendix 2F: Matrix of pairwise comparisons among five microsatellite loci in *Nesospiza acunhae questi* buntings from Nightingale Island, Tristan da Cunha showing the probabilities of genotypic linkage disequilibrium for each pairwise comparison. N.P = not possible meaning one of the loci in the pairwise comparison is monomorphic in the population.

	INDIGO 15	INDIGO 30	INDIGO 31	INDIGO 40	INDIGO 41
INDIGO 15	-				
INDIGO 30	0.152	-			
INDIGO 31	0.777	0.328	-		
INDIGO 40	N.P	N.P	N.P	-	
INDIGO 41	0.816	0.382	0.120	N.P	-

Appendix 3A: Allele frequency distribution, expected (H_E) and observed (H_O) heterozygosity, sample size for each population and number of alleles observed for locus INDIGO 15.

	Inaccessible Island				Nightingale Island	
			Upland	Lowland		
Allele size	Hybrid	*N. w. dunnei*	*N. a. acunhae*	*N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
189	-	-	0.031	-	-	-
193	0.300	0.421	0.344	-	0.969	0.700
197	0.700	0.579	0.594	1.000	0.031	0.300
201	-	-	0.031	-	-	-
H_E	0.42	0.49	0.53	0.00	0.06	0.42
H_O	0.44	0.52	0.75	0.00	0.06	0.33
Sample size	25	19	16	13	16	15
No. Alleles	2	2	4	1	2	2

Appendix 3B: Allele frequency distribution, expected (H_E) and observed (H_O) heterozygosity, sample size for each population and number of alleles observed for locus INDIGO 30.

	Inaccessible Island				Nightingale Island	
			Upland	Lowland		
Allele size	Hybrid	*N. w. dunnei*	*N. a. acunhae*	*N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
220	-	-	0.063	-	-	-
228	0.060	0.053	0.219	0.423	-	0.467
232	0.940	0.947	0.719	0.577	1.000	0.533
H_E	0.11	0.10	0.43	0.49	0.00	0.50
H_O	0.12	0.11	0.44	0.53	0.00	0.67
Sample size	25	19	16	13	16	15
No. Alleles	2	2	3	2	1	2

Appendix 3C: Allele frequency distribution, expected (H_E) and observed (H_O) heterozygosity, sample size for each population and number of alleles observed for locus INDIGO 31.

	Inaccessible Island				Nightingale Island	
			Upland	Lowland		
Allele size	Hybrid	*N. w. dunnei*	*N. a. acunhae*	*N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
304	0.220	0.211	0.125	0.039	1.000	0.800
308	0.780	0.789	0.844	0.962	-	0.200
312	-	-	0.031	-	-	-
H_E	0.34	0.33	0.27	0.07	0.00	0.32
H_O	0.28	0.42	0.19	0.08	0.00	0.40
Sample size	25	19	16	13	16	15
No. Alleles	2	2	3	2	1	2

Appendix 3D: Allele frequency distribution, expected (H_E) and observed (H_O) heterozygosity, sample size for each population and number of alleles observed for locus INDIGO 40.

	Inaccessible Island				Nightingale Island	
			Upland	Lowland		
Allele size	Hybrid	*N. w. dunnei*	*N. a. acunhae*	*N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
235	0.688	0.684	0.688	0.654	1.000	1.000
239	0.313	0.316	0.313	0.346	-	-
H_E	0.43	0.43	0.43	0.45	0.00	0.00
H_O	0.46	0.42	0.50	0.23	0.00	0.00
Sample size	24	19	16	13	16	15
No. Alleles	2	2	2	2	1	1

Appendix 3E: Allele frequency distribution, expected (H_E) and observed (H_O) heterozygosity, sample size for each population and number of alleles observed for locus INDIGO 41.

	Inaccessible Island				Nightingale Island	
			Upland	Lowland		
Allele size	Hybrid	*N. w. dunnei*	*N. a. acunhae*	*N. a. acunhae*	*N. w. wilkinsi*	*N. a. questi*
329	-	-	-	-	0.046	-
341	0.109	0.053	0.219	-	-	-
345	0.065	0.105	0.031	-	-	0.033
353	0.109	0.184	0.063	0.231	-	0.433
357	0.217	0.316	0.313	0.346	-	0.233
361	0.413	0.263	0.219	0.192	0.636	0.267
365	0.044	-	0.063	-	0.318	0.033
369	0.044	0.079	0.094	0.077	-	-
373	-	-	-	0.154	-	-
H_E	0.75	0.78	0.79	0.76	0.49	0.68
H_O	0.78	0.68	0.94	0.38	0.36	0.80
Sample size	23	19	16	13	11	15
No. Alleles	7	6	7	5	3	5