Conservation Genetics and Hybridisation of the Forbes' Parakeet (*Cyanoramphus forbesi*) in the Chatham Islands

Chi-hang Chan

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Abstract

This study describes the isolation and characterisation of microsatellite loci in Forbes' parakeet (*Cyanoramphus forbesi*). These molecular markers are used to assess the status of interspecific hybridisation between Forbes' parakeets and Chatham Island Red-crowned parakeets (*C. novaezelandiae chathamensis*) on Mangere and Little Mangere Islands in the Chatham Islands group. The evolution of these microsatellite loci in parrots is also investigated.

Forbes' parakeets are facing the problem of genetic introgression due to extensive hybridisation with Chatham Island Red-crowned parakeets. Hybrids show a spectrum of crown morphologies between the parent species (Nixon 1982), but identification of hybrids based on morphology alone is not foolproof. Mitochondrial DNA studies by Boon (2000) showed that Forbes' parakeet is a distinct lineage, basal to all other New Zealand parakeets but several Forbes' morphotypes have Chatham Island Red-crowned parakeet haplotype. However, mitochondrial DNA markers only probe the maternally inherited lineages and cannot tell the whole story.

The microsatellite markers used in this study show that interspecific hybridisation between Forbes parakeet and Chatham Island Red-crowned parakeet is more extensive than previously expected. Microsatellite data combined with results from scoring mitochondrial DNA haplotypes show that crown morphology alone under-represents the proportion of hybrids in the population, and that a large number of cryptic hybrids (77.9%) show Forbes' parakeet morphotypes. A three factor scoring system is suggested in which a parakeet must pass both genetic (microsatellites and mitochondrial) and morphological criteria to be considered a "pure" Forbes' parakeet. Using this system, 17.8%, 1.2%, and 81.0% of the

Mangere Island parakeet population are "pure" Forbes' parakeets, "pure" Chatham Island Red-crowned parakeets, and interspecific hybrids respectively.

The results of this study have implications for the future conservation of Forbes' parakeet. Because interspecific hybridisation makes no positive contribution to the long-term survival of Forbes' parakeets, conservation measures to limit contact between the two species should be taken to control further hybridisation of parakeets on Mangere Island.

The microsatellite loci isolated in this study are found to evolve following the patterns best described by either the Stepwise Mutation Model (SMM; Ohta & Kimura 1973) or the Two-Phase Model (TPM; Di Rienzo et al. 1994) at population level. By mapping the evolutionary changes in repeat motif variations to a parrot phylogeny, it is suggested that these loci may evolve through a more complex model than sole repeat number changes.

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Chapter 1: Introduction

1.1 Overview

This thesis investigates the role of interspecific hybridisation in the recent history of Forbes' parakeet (*Cyanoramphus forbesi*), a small green parrot found on Mangere and Little Mangere Islands in the Chatham Islands group of New Zealand. Insights were gained through the study of morphological markers, microsatellite and mitochondrial DNA genetic markers in the Mangere Island parakeet population. The effect of hybridisation to the genetic diversity in the population, and the relationship between genetic makeup (biparental and female lineages) and morphologic features in parakeets were investigated. Based on the information obtained, conservation strategies to enhance the long-term genetic integrity of Forbes' parakeets are discussed. The markers developed also allowed the study of microsatellite evolution in parrots and investigation of evolution in parrots from a nuclear DNA perspective.

This chapter introduces the concepts underlying this study. Because hybridisation and the biology of microsatellite DNA have been popular topics in recent years, a large number of quality reviews about these topics have been published in the literature. The aim of this chapter is to provide a summary of findings and theories formulated to date without extensive replication of published reviews. A full reference list is provided for more detailed information about the topics.

1.2 Hybridisation between Species

1.2.1 Definition of Hybridisation

Numerous definitions exist for "hybridisation" between taxa. Due to the controversy over the definition of species (see Sites & Crandall 1997;

Avise & Walker 2000; Hendry et al. 2000), a definition of "hybridisation" that does not involve the application of particular species concepts is preferred in this study. Thus, hybridisation can be defined as "the interbreeding of individuals from what are believed to be genetically distinct populations, regardless of taxonomic status of such populations" (Rhymer & Simberloff 1996).

1.2.2 How Common Is Hybridisation Among Vertebrates?

Hybridisation, natural and human induced, is common in both plants and animals. Hybridisation is particularly common in birds: Grant & Grant (1992) estimated that 9.2% of all known bird species had bred with another species and produced hybrid offspring. In birds it was estimated the average hybridisable species pair diverged from a common ancestor about 22 million years ago (Prager & Wilson 1975), which is similar to that of frogs (21 million years), but remarkably different from placental mammals (only 2 to 3 million years; Wilson et al. 1974). A suggestion is that the very slow rate of anatomical evolution in birds might have caused the slow rate of loss of interspecific hybridisation potential (Prager & Wilson 1975).

1.2.3 Barriers to Hybridisation

For a healthy and fertile interspecific hybrid offspring to be produced successfully in nature, both pre- and post-zygotic barriers that contribute to reproductive isolation need to be overcome. Recent research involving various model organisms shows that pre-zygotic factors, such as gamete incompatibility and mating behaviour, seem to play a more important role in preventing hybridisation (Rahman et al. 2001; Wolf et al. 2001) compared to post-zygotic factors, such as offspring inviability and sterility (Coyne 1992). Presently, very little is known about how genes interact to

cause reproductive isolation, and how various genotypes determine the pattern of fitnesses.

At the population level, the barrier to hybridisation can be physical as well as genetical (reviewed by Barton & Hewitt 1985). The movement of alleles across the hybrid zone is proportional to the gradient of change in allele frequencies. A greater flux usually occurs when the gradient is shallow. When an allele flux meets a physical or genetical barrier, a sharp step builds up. Gene flow may be impeded at the barrier by: 1) environmental factors that reduce density or dispersal; or 2) reduction in density caused by lowered fitness in a cline maintained by dispersal / selection balance; or 3) the inability of an allele to recombine into a new genetic background.

1.2.4 Fitness of Hybrids

Hybrid progeny may have inferior, superior or similar fitness relative to their parents, for example, see Arnold & Hodges (1995), Barton (2001), and Burke & Arnold (2001). Hybridisation results in recombinant genotypes that are new in the environment. In some cases, these new genotypes may confer adaptive advantages to the individual, but in most cases, they have neutral or negative impacts, for example, reduced fertility (Burke & Arnold 2001). The reduction of fitness of hybrids is known as 'hybrid breakdown', and can be caused by unfavourable interactions between the genomes of parent species (Burton 1990; Breeuwer & Werren 1995; Burke et al. 1998). For instance, hybrid sterility was found to result from a large number of genetic incompatibilities in *Drosophila* flies (Coyne & Orr 1989; Cabot et al. 1994; Palopoli & Wu 1994; Perez & Wu 1995; True et al. 1996). Haldane (1922) suggested that the heterogametic sex hybrids will be more affected by genetic defects. This bias has since been found to arise from interactions between chromosomes, rather than

defects traceable to the sex chromosomes alone (Coyne 1985; Hollocher & Wu 1996). This indicates the incompatibility factors are likely to be recessive (True et al. 1996). Hybrid infertility was also found to be more common than hybrid inviability in the heterogametic sex in *Drosophila* and mammals (Orr 1993; Wu & Davis 1993).

Conversely, some hybrids can be fitter than parental species, such as between some members of the fish *Gambusia* and the finch *Geospiza* (Grant & Grant 1992; Scribner 1993; see also reviews by Arnold & Hodges 1995; Burke & Arnold 2001). Hybrid vigour often occurs if the parental species are inbred. New variants that are more adapted to new environments can sometimes be produced through hybridisation (Lewontin & Birch 1966). As Arnold & Hodges (1995) have pointed out, the fitness of hybrids is mostly estimated relative to their parents, and hybrid genotypes usually show a wide range of fitness that is difficult to estimate accurately unless hybrid classes can be precisely defined. Hybrid zones, narrow regions where genetically distinct populations meet, mate, and produce hybrids (reviewed by Barton & Hewitt 1985), have become useful situations for the study of hybridisation and gene flow across species.

1.2.5 Hybridisation, Evolution, and Conservation

Negative impacts of genetic introgression through hybridisation, especially the threat of extinction of parental species through competition with hybrids, have been well documented. For example, the Seychelles turtle dove (*Stereptopelia picturata rostrata*) and the Red-eyed dove (*S. semitorguata australis*) have both been extensively hybridised with introduced species (see Rhymer & Simberloff 1996; Huxel 1999; Wolf et al. 2001). When genetic introgression takes place from a more abundant species to a rare species, the likelihood of extinction of genotypes in the latter increases. The threat of extinction of endangered species through

hybridisation creates negative attitudes towards hybridisation, and consequently the benefits of hybridisation are less well described.

On the positive side, Lewontin & Birch (1966) suggested that introgression of genes through hybridisation could lead to rapid adaptive evolution of a population. Hybridisation can also be a source of genetic diversity that stimulates the diversification of populations (Anderson & Stebbins 1954; Dowling & Secor 1997), and / or triggers speciation (see Wiegand 1935; Orr 1995; Rieseberg et al. 1996). Hybridisation may also be a useful tool in conserving endangered populations. For example, interbreeding with the closely related Texas Puma (*Puma concolor stanleyana*) was encouraged to restore genetic diversity in the highly endangered Florida Panther (*P. c. coryi*; Hedrick 1995; Land & Lacy 2000).

1.2.6 Hybrid Morphologies

Hybrids can exhibit a wide range of morphologies. Some hybrids resemble their parental species or show phenotypes intermediate between parental species, for example, in *Colaptes* flickers and in *Cerion* snails (Moore 1987; Woodruff & Gould 1987), while others may show novel or extreme phenotypes, which are more commonly observed in plants (for examples, see Rieseberg et al. 1999; Rieseberg et al. 2003). Therefore, it is sometimes difficult to identify hybrids using phenotypic characters alone.

1.3   Birds, Parrots, and Parakeets

1.3.1 Birds (Aves)

Over 9000 avian species have been identified to date (May 1992) but the phylogenetic relationships among bird orders have not been totally resolved (Cracraft 2001; Harrison et al. 2004). While mitochondrial phylogenies generally support a bird-crocodilian relationship (Hedges

1994), discovery of fossils of feathered dinosaurs pointed at feathered, ground-living, bipedal dinosaurs as ancestors of birds (Qiang et al. 1998). It appears that birds with pronounced plumage dichromatism, generalised feeding habits, large and fragmented geographical ranges, and good dispersal capabilities usually show higher species diversity (Owens et al. 1999).

Two contrasting hypotheses have been proposed regarding the early evolution of avian species. Molecular clock studies suggested all modern avian orders originated in the Cretaceous, diverged about 100 million years ago or earlier, and many of these lineages survived the Cretaceous-Tertiary mass extinction event (Hedges et al. 1996; Cooper & Penny 1997; Rambaut & Bromham 1998). The fossil record however, suggests that most lineages of birds were exterminated at the Cretaceous-Tertiary boundary mass extinctions and that explosive evolution events followed. Thus one, or at most a few, surviving lineages may have given rise to all modern birds (Feduccia 1995, 2003).

Hybridisation and the high levels of sexual dimorphism in birds can cause biases in taxonomy, especially in earlier work that mostly relies on morphological features to identify species (Sibley 1957). One possible explanation of the observed pattern of frequent interspecific hybridisation in birds (Grant & Grant 1992) is the slow evolution of postmating isolating factors in birds, and consequently, a small scope for reinforcement of premating isolation (Grant & Grant 1997).

1.3.2 Parrots (Psittaciformes)

More than 330 species of parrots inhabit the world (Forshaw 1989). Using morphological data, Burton (1974) suggested pigeons (Columbiformes) as the closest relative to parrots. However, the phylogenetic association of

parrots remains unclear, except that they are part of the Neoaves group (Harrison et al. 2004). Based on anatomical features, Smith (1975) proposed division of parrots into four different groups: Platycercinae; Loriinae; Arinae; and Psittacinae. It was also noted by Smith (1975) that subdividing the parrot group was difficult because parrots comprised a homogenous order of birds, substantial differences among morphological characters were hard to find, and the subdivisions had to be made using features such as branching of arteries. Using DNA hybridisation techniques, Sibley & Ahlquist (1990) suggested that Australasian, African, and American parrots form three subgroups. The Australasian subgroup was found to be the ancestral lineage in mitochondrial DNA studies (Miyaki et al. 1998). The phylogeny of parrots is being gradually resolved with various genetic markers (Ovenden et al. 1987; Christidis et al. 1991; Birt et al. 1992; Triggs & Daugherty 1996; Miyaki et al. 1998; Boon et al. 2000; Kearvell et al. 2003; Groombridge et al. 2004; Ribas & Miyaki 2004; Tavares et al. 2004; de Kloet & de Kloet 2005).

1.3.3 Parrots of New Zealand

Three large parrots, the Kakapo (*Strigops habroptilus*), Kea (*Nestor notabilis*), and Kaka (*N. meridionalis*), and the smaller *Cyanoramphus* parakeets make up the parrot biota of New Zealand.

Based on data from the mitochondrial DNA control region, Boon (2000) almost completely revised the taxonomy of the *Cyanoramphus* complex. According to Boon (2000), the *Cyanoramphus* parakeets living in the New Zealand region comprise nine species and subspecies: The Antipodes Island Green parakeet (*Cyanoramphus unicolor*), Orange-fronted parakeet (*C. malherbi*), Yellow-crowned parakeet (*C. auriceps*), Forbes' parakeet (*C. forbesi*), Red-crowned parakeet (*C. novaezelandiae novaezelandiae*), Chatham Island Red-crowned parakeet (*C. n. chathamensis*), Kermadec

parakeet (*C. n. cyanurus*), Reischek's parakeet (*C. hochstetteri*), and Norfolk Island parakeet (*C. cooki*).

The New Zealand *Cyanoramphus* parakeets are believed to have radiated out from New Caledonia during the last 500,000 years. Mitochondrial cytochrome *b* sequence analysis (Boon 2000) suggested that the *Eunmyphicus* parakeets from New Caledonia shared the most recent common ancestor with *Cyanoramphus* parakeets about 2.5 million years ago.

The *Cyanoramphus* genus is unique in having dispersed successfully for long distances over water. *Cyanoramphus* parakeets have been used as subjects in a number of studies, for example, the controversial taxonomy of the Orange-fronted parakeet (Holyoak 1974; Taylor et al. 1986; Triggs & Daugherty 1996; Taylor 1998; Boon et al. 2000; Kearvell et al. 2003), the intelligence of the Yellow-crowned parakeets (Funk 2002), hybridisation between Red-crowned parakeets (*C. n. novaezelandiae*) and Yellow-crowned parakeets (Veitch 1979; Butler 1986), and between Chatham Island Red-crowned parakeets (*C. n. chathamensis*) and Forbes' parakeets (Taylor 1975). This thesis focuses on Forbes' parakeet, and its hybridisation with Chatham Island Red-crowned parakeets.

1.3.4 *Forbes' Parakeet and Chatham Island Red-crowned Parakeet*

Forbes' parakeet is a highly threatened parrot confined to Mangere and Little Mangere Islands in the Chatham Islands group (Figure 1.1). The Chatham Island Red-crowned parakeet, on the other hand, can be found on the main Chatham Island, Pitt Island, and South-East Island. Chatham Island Red-crowned parakeets are also seen visiting Mangere and Little Mangere Islands, and some breed there too.

Taylor (1975) suggested feeding habits and territoriality differences between Forbes' and Chatham Island Red-crowned parakeets based on field observations: Forbes' parakeets generally prefer forest habitats to open vegetation, while the Chatham Island Red-crowned parakeets were seen to be resident on scattered patches of grass, scrub and herbs. About 10 breeding Forbes' parakeet pairs were observed by Taylor (1975). The number of parakeets on Mangere Island was estimated at 821 (95% CI = 411 – 1231) in year 2003 (Terry Greene, NZ Department of Conservation, per. comm.).

Figure 1.1 Location of Mangere Island and South-East Island (Rangatira) in the Chatham Islands group.
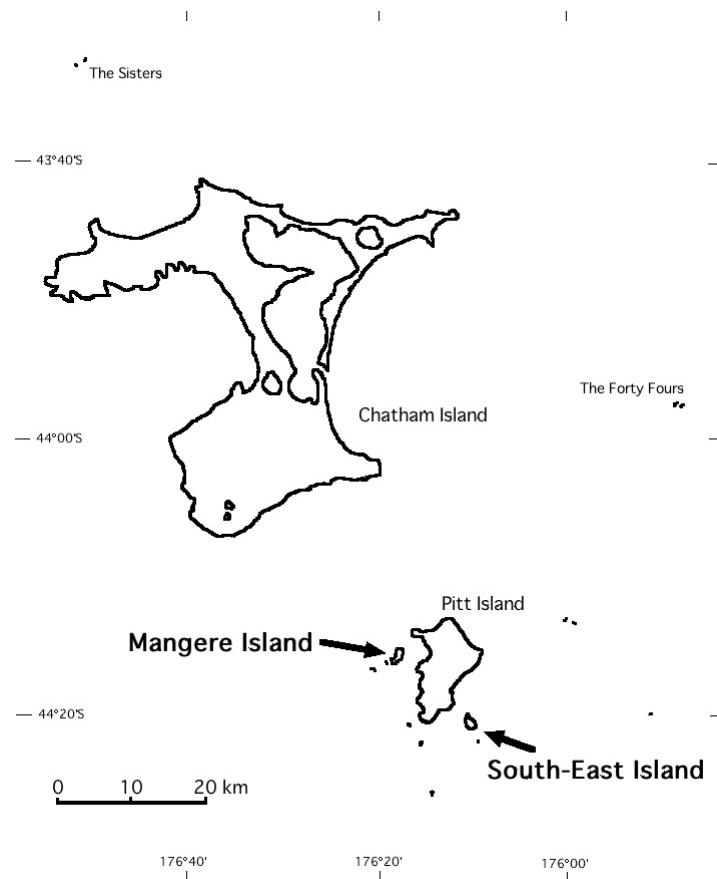
Morphologically, a Forbes' parakeet is identified by a yellow crown with red frontal band that does not extend to the eyes. Forbes' parakeets differ from Yellow-crowned parakeets by having yellowish cheeks and are generally larger in size. The Chatham Island Red-crowned parakeets have only red crown plumage and hybrids between the two species can show a range of crown colourations intermediate between the two parental species (Nixon 1982). In a 1999 census (Tertia Thurley, NZ Department of Conservation, pers. comm.) on Mangere Island, 66 parakeets were banded. Based on the morphological identification scale developed by Nixon (1982), it was estimated that 90% were Forbes' parakeets, 3% were Chatham Island Red-crowned parakeets, and 7% were hybrids.

1.3.5 *Previous Genetic Work in Forbes' Parakeets*

Using allozyme electrophoresis, Triggs & Daugherty (1996) suggested elevation of Forbes' parakeet from subspecific status of Yellow-crowned parakeet (*C. auriceps forbesi*) to full species status (*C. forbesi*). Further work with the mitochondrial control region DNA sequences (Boon 2000; Boon et al. 2001) revealed three distinct haplotypes among the Mangere Island Forbes' parakeet samples. Haplogroups 1 and 2 respectively sit within, and basal to, a Red-crowned parakeet clade. These were suggested to represent results of past hybridisations between Forbes' parakeets and Chatham Island Red-crowned parakeets (*C. n. chathamensis*) retaining Red-crowned parakeet haplotypes. Haplogroup 3 is basal to all other New Zealand parakeets, and was suggested to represent the ancestral true Forbes' parakeet lineage. Haplogroup 3 Forbes' parakeets also represent the most ancestral living *Cyanoramphus* parakeet lineage in New Zealand.

The elevation to species level of Forbes' parakeet (Triggs & Daugherty 1996; Boon 2000) has led to the increase in conservation concern of this

species. Identification of hybrids has been based purely on crown morphology, while the accuracy of morphological markers in predicting the actual genetic makeup of the birds has not been previously studied. The work presented in this thesis will explore the relationship between crown plumage colouration and the genetics of the birds via the use of molecular markers.

1.3.6 Hybridisation in the Mangere Island Parakeet Population

The history of hybridisation between Forbes' parakeets and Chatham Island Red-crowned parakeets has not been documented. It is generally believed that Forbes' parakeet was the more abundant speices on Mangere and Little Mangere Islands, while Chatham Island Red-crowned parakeets occupied the main Chatham Island, Pitt Island and South-East Island. However, there may be Chatham Island Red-crowned parakeets on Mangere and Little Mangere Islands as well. The Forbes' parakeet population is therefore surrounded by the more abundant Chatham Island Red-crowned parakeets. The proximity between Mangere Island and Pitt Island means parakeets can fly freely between these islands, and between Pitt and South-East Islands. Forbes' parakeets and Chatham Island Red-crowned parakeets can, in principal, freely interact because of a lack of a geographic barrier.

Despite Taylor (1975) suggesting that habitat preferences exist between the two species of parakeets on Mangere Island, there is no evidence of differences in mating behaviour between the two species. It is also believed that forest clearance by humans on Mangere Island may have driven Forbes' parakeets from forests and encouraged their breeding with Chatham Island Red-crowned parakeets on Mangere Island.

Genetically, there appears to be very little post-zygotic isolation between these two closely related species. The abundance of hybrids observed by Taylor (1975) also suggested hybrids and backcrosses are not inferior in fitness compared to the parent species.

The lack of pre- and post-zygotic reproductive isolation between Forbes' and Chatham Island Red-crowned parakeets could probably explain the widespread hybridisation observed at present. This widespread hybridisation, if uncontrolled, is likely to continue in the future because hybrids and backcrosses have at least equal fitness compared to the parental species. The consequence is extensive mixing and slow extinction of parental genotypes in the population, constituting a major threat to the existence of the rare Forbes' parakeet.

1.3.7 Conservation of Forbes' Parakeet

Hybridisation challenges the definition of species under the Biological Species Concept (Mayr 1942), which define species as groups of interbreeding or potentially interbreeding natural populations reproductively isolated from other such populations. While Forbes' parakeets and Chatham Island Red-crowned parakeets are considered separate species, they interbreed to form hybrids.

Aimed at identifying 'populations possessing genetic attributes significant for the present and future generations of the species in question', the Evolutionarily Significant Unit (ESU) concept was first proposed by Ryder (1986) in order to provide a rational basis for setting priorities in conservation that reflects the underlying genetic diversity, and without entering debates surrounding species concepts. Various different criteria have been suggested to define ESUs (reviewed in Fraser and Bernatchez 2001). For example, Moritz (1994) defined ESUs as 'reciprocally

monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci', whereas Crandall et al. (2000) recommended ecological data and genetic variation of adaptive significance be taken into account in establishing ESUs.

In this thesis, microsatellite markers are used to examine nuclear DNA divergence between Forbes' parakeets and Chatham Island Red-crowned parakeets. Along with morphological differences examined by Nixon (1982) and mitochondrial DNA divergence established by Boon (2000), whether Mangere Island parakeets form distinct ESUs will be discussed.

1.4 Molecular Markers in Conservation Genetics Studies

1.4.1 Molecular Methods in Population Genetics

Molecular methods have been regularly employed to solve population biology problems for around 40 years. The first widely used molecular tool was electrophoresis of protein markers (Harris 1966; Hubby & Lewontin 1966; Lewontin & Hubby 1966). With recent advances in molecular biology, such as DNA sequencing (Sanger et al. 1977) and the Polymerase Chain Reaction (PCR; Saiki et al. 1985; Mullis & Faloona 1987), DNA-based markers have gained in popularity. A wide range of DNA-based techniques is now available for the study of ecological and evolutionary problems, such as parentage, individual and species identification, genetic bottlenecks, sex assignment, migration, and phylogenetic relationships etc. (see Amos & Hoelzel 1992; Burke 1994; Lambert 1995; Lambert & Millar 1995; Sunnucks 2000; Blouin 2003).

1.4.2 Molecular Genetics in Avian Biology

Molecular genetic techniques have made substantial contributions in studying ecology and evolution in birds, especially in the study of mating

systems, population structure, gene flow, and phylogenetic relationships (see Avise 1996). Techniques which use PCR allow large quantities of specific DNA product to be amplified from a small amount of DNA template (see Arnheim et al. 1990). These techniques are particularly useful in studies of species such as birds, which can be difficult to capture and handle, because non-destructive and even non-invasive samples can be taken from materials such as feathers and faeces. Non-invasive sampling has its limitations in terms of the quality and quantity of recoverable DNA, but it does cause minimal damage to the organism (Taberlet et al. 1999). A leading example of such PCR-based molecular tools is the use of microsatellite DNA markers. These have been proven to be useful in studying various aspects of genetic relationships in birds, such as the monitoring of genetic variability, and detection of extra-pair fertilisation (Ellegren 1992).

Another popular marker is mitochondrial DNA. Due to the rapid evolution of mitochondrial DNA (Brown et al. 1979), mitochondrial DNA sequencing provides a very useful tool for studying phylogenetic relationships (Barton & Jones 1983). Studies of mitochondrial DNA sequence variations can also provide useful information about the population structure, for example, the identification of four haplogroups within the Mangere Island *Cyanoramphus* parakeet population (Boon et al. 2000).

1.5   The Biology of Microsatellites

1.5.1 What are Microsatellites?

Repeat sequences in DNA were first discovered in mouse, and later found in a wide range of organisms (Waring & Britten 1966; Britten & Kohne 1968). Following recommendations by Chambers & MacAvoy (2000), repeat sequences in DNA can be classified into four classes: mononucleotide tracts, microsatellites, minisatellites, and the larger

satellites. Microsatellites are defined as short segments of 2 – 6 nucleotides repeated in more or less uniform tracts with a minimum length of 8 nucleotides.

Repeat number mutations in microsatellite loci are considered as selectively neutral (Tachida & Iizuka 1992; Michalakis & Veuille 1996; Schlötterer 2000), fitting neutral theory prediction (Kimura 1968, 1986). Microsatellites are non-randomly distributed in genomes (see Li et al. 2002, 2004), their polymorphisms can be affected by their location, and presence of other linked genes (Slatkin 1995; Thuillet et al. 2004).

1.5.2 Patterns of Microsatellite Evolution

Microsatellites evolve mostly through changes in number of repeat units. Slipped-strand mispairing, or slippage synthesis, is believed to be the major mechanism that generates the observed patterns of microsatellite evolution (Levinson & Gutman 1987; Schlötterer & Tautz 1992). Allele frequency distribution spectra of microsatellites tend to differ between loci, and there is no consensus to the best description of the distributions (Jarne & Lagoda 1996; Chambers & MacAvoy 2000). One of the most frequently used predictions of their distributions is the Stepwise Mutation Model (SMM; Ohta & Kimura 1973) which assumes that the repeat number changes through increase or decrease of one repeat unit at a time (with equal probability), and that alleles may mutate towards other allelic states already present. Some loci, however, are better described by the Infinite Allele Model (IAM; Kimura & Crow 1964), which predicts that mutations can involve any number of repeat units. Which of these models provides the best description of microsatellite allele distributions? Computer simulations by Shriver et al. (1993) showed that microsatellites generally evolve via SMM, while showing some deviations towards IAM, suggesting neither of these models accurately describes the patterns in all

microsatellite loci. Di Rienzo et al. (1994) proposed the Two-phase mutation Model (TPM) based on their survey of microsatellite allele frequencies in the human population of Sardinia. The TPM predicts that the majority of mutations are one repeat unit mutations (similar to SMM), but larger jumps of more than one repeat occur occasionally.

1.5.3 The Rise and Fall of a Microsatellite

Microsatellite repeats are believed to arise from genomic regions where simple repetitive sequences are already abundant (Tautz et al. 1986). Through phylogenetic studies, Messier et al. (1996) proposed that a microsatellite locus can be created through a single substitution event in a region of cryptic simplicity. Further studies using data in the Human Gene Mutation Database suggested that both substitution and insertion events are likely to contribute to the origin of short tandem repeats (Zhu et al. 2000b). However, the types of repeat that arise seem to be determined by the pattern of subsequent slippage events (Gordon 1997).

There is also debate regarding whether a minimum number of repeat units or nucleotides has to be reached before changes in number of repeat units can take place by slippage. Through DNA sequencing studies in the η-globin pseudogene of primates, Messier et al. (1996) suggested that the minimum number of repeat units required for slippage to occur is 4 – 5 repeats for dinucleotides, and 2 repeats for tetranucleotide units. An alternative suggestion, from studies in yeast (*Saccharomyces cerevisiae*), is that a minimum of 8 nucleotides, regardless of the type of repeat, is required (Rose & Falush 1998). Using a maximum likelihood method, Sibly et al. (2001) also showed that no slippage can occur in dinucleotide microsatellites under 5 repeats. However, another study in yeast (*S. cerevisiae*) suggested that there is no critical minimum of repeats required for microsatellite expansion (Pupko & Graur 1999).

Studies in yeast have shown that stability of microsatellite tracts is dependent on the DNA mismatch repair system (Strand et al. 1993, 1995; Sia et al. 1997). The pattern of microsatellite evolution, however, remains controversial. While some studies have suggested that longer repeat units, such as tetranucloetide repeats, have higher mutation rates compared with shorter dinucleotide units (Weber & Wong 1993; Primmer & Ellegren 1998), other studies have suggested the reverse (Chakraborty et al. 1997). It appears that, regardless of length of repeat units, mutation rate differs between loci, alleles, and species (Brinkmann et al. 1998; Kruglyak et al. 1998; Ellegren 2000a), and that these variations may be directly attributed to differences between slippage rates (Kruglyak et al. 1998). There also seems to be a general pattern that loci with larger numbers of repeat units tend to have a higher mutation rate (Wierdl et al. 1997; Primmer et al. 1998; Falush & Iwasa 1999; Ellegren 2000b; Beck et al. 2003).

Ideas concerning the pattern of microsatellite mutation also vary. The majority of observations support the hypothesis that increases in number of repeats occur more frequently than decreases (Primmer et al. 1996, 1998; Primmer & Ellegren 1998; Falush & Iwasa 1999; Twerdi et al. 1999; but see Beck et al. 2003). Microsatellites do not expand indefinitely, and there also appears to be an upper limit constraint on allele size (Garza et al. 1995; Lehmann et al. 1996; Zhivotovsky et al. 1997). The majority of studies favour the idea that loci with large numbers of repeat units tend to undergo deletions while loci with fewer repeat units tend to increase in size (Wierdl et al. 1997; Ellegren 2000b; Harr & Schlötterer 2000; Xu et al. 2000; but see Falush & Iwasa 1999; Harr et al. 2002). Long repeats were also shown to have a relatively short existence time in *Drosophila melanogaster* (Harr & Schlötterer 2000). An excellent analogy to the present consensus pattern is a model based on the "Snakes and Ladders"

board game (Chambers & MacAvoy 2000), in which ladders (increase in repeat units) are more frequently encountered at lower numbered squares (loci with few repeats), and snakes (dramatic decrease in repeat units) are more abundant at the larger numbered squares (loci with large number of repeats).

Interruptions within the repetitive tract appear to stabilise a microsatellite (Jin et al. 1996; Petes et al. 1997; Taylor et al. 1999; but see Bichara et al. 1995). These interruptions may be removed during slippage of adjacent bases and the stabilised microsatellite may only be a transition state (Harr et al. 2000). However, the "death" of a microsatellite can occur if a deletion of the repeat units follows the introduction of the interruption (Taylor et al. 1999).

1.5.4 *The Use of Microsatellites in Population Genetics*
Microsatellite markers are widely used in population genetics, especially in studies of population structure, breeding behaviour, and kinship analysis (see Ellegren 1992; Chambers & MacAvoy 2000; Blouin 2003). Recent studies have also used microsatellites to study phylogenetic relationships (see Zhu et al. 2000a).

Microsatellites appear to be very convenient tools for assessing the genetic differences between Forbes' parakeets, Chatham Island Red-crowned parakeets, and their hybrids without invasive sampling of the population. The reproducibility of microsatellite data also makes this method robust, and allows inclusion of data collected subsequent to this study.

1.6   The Aims of this Study

Through the development and use of microsatellite genetic markers in parakeets, the following questions will be addressed:

1) What is the extent of hybridisation in Forbes' parakeet as judged by nuclear genetic markers?

2) Is Forbes' parakeet in danger of losing its genetic integrity?

3) Are other genetic markers (mitochondrial DNA control region haplotypes) and morphological markers (such as crown plumage morphology) directly correlated to hybridisation in birds?

4) What is the pattern of microsatellite evolution between parrots?

5) What are the implications to future directions in conserving Forbes' parakeet?

1.7   Structure of this Thesis

1.7.1 Introduction

This chapter introduces the background information this thesis is based on, including the role of hybridisation in the evolution of a population, the biology of parrots and parakeets, and the evolution and application of microsatellite molecular markers.

*1.7.2 Chapter 2: Development of Microsatellite Loci for Forbes' Parakeet (*Cyanoramphus forbesi*) by Hybridisation Selection using Enriched Membranes*

This chapter describes the process of isolating Forbes' parakeet microsatellite loci. The microsatellite isolation work was carried out at Institute of Molecular BioSciences, Massey University, Palmerston North. The characterization of the loci was performed at the Institute of Molecular Systematics, School of Biological Sciences, Victoria University of Wellington, Wellington.

1.7.3 Chapter 3: Microsatellite DNA Studies Reveal Extensive Hybridisation between Parakeets on Mangere Island

In this chapter, microsatellite loci isolated from Forbes' parakeets are used to assess the hybridisation status between Forbes' parakeets and Chatham Island Red-crowned parakeets. A system is developed to identify true Forbes' parakeets based on morphological, nuclear and mitochondrial DNA data.

1.7.4 Chapter 4: Microsatellite Evolution in Parrots

The possible use of microsatellite flanking sequences to build phylogenies, and the evolution of microsatellites in parrots are investigated in this chapter. Implications are drawn on the evolution of microsatellite repeat motifs and the origin of microsatellites.

*1.7.5 Chapter 5: Does Crown Plumage Directly Reflect the Hybridisation Status of Parakeets? A Preliminary Study in Red-crowned Parakeets (*Cyanoramphus novaezelandiae novaezelandiae*) and Yellow-crowned Parakeets (*C. auriceps*) on the New Zealand Mainland*

The use of the isolated Forbes' parakeet microsatellite markers in other species of New Zealand parakeets is investigated in this chapter. Microsatellite and mitochondrial DNA data are used to address whether yellow feathers on the crown of a Red-crowned parakeet indicate a hybridisation history.

1.7.6 Chapter 6: Conservation of Forbes' Parakeet – Present and Outlook

Based on the data obtained in the previous chapters, implications to the conservation of Forbes' parakeets are discussed in this chapter.

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Chapter 2: Development of Microsatellite Loci for Forbes' Parakeet (*Cyanoramphus forbesi*) by Hybridisation Selection using Enriched Membranes

2.1   Introduction

Microsatellites have been reported to occur at relatively low frequency in birds (Primmer et al. 1997) compared with other organisms, such as mammals and invertebrates. A possible explanation for the low microsatellite abundance is a general reduction in genome size in avian species, which was suggested to be an adaptation to the requirements of flight (Hughes & Hughes 1995). The convenience of cross-species utility of microsatellite markers (Moore et al. 1991; Primmer et al. 1996) has made loci isolated from closely related species attractive candidates for this study. However, microsatellite loci isolated from other New Zealand parrots (Kakapo, Robertson et al. 2000; and Kaka, Sainsbury et al. 2004) did not amplify readily from Forbes' parakeet DNA (James Sainsbury and Margo van Bekkum, Victoria University of Wellington, pers. comm.), making isolation of microsatellite loci from Forbes' parakeet necessary.

One of the most commonly used methods for developing microsatellite markers is the use of hybridisation selection (see Zane et al. 2002). Briefly, this method involves fragmentation of the genome of the species of interest using restriction enzymes, cloning these fragments in competent bacterial cells through the use of plasmids, and screening resultant libraries with radioactively labelled microsatellite oligonucleotide sequences. Positive colonies are selected, plasmids isolated and primers designed based on their insert sequences.

This basic method is generally effective in species which have many microsatellites, and numerous studies using this technique have been reported over the past decade in journals such as *Conservation Genetics* and *Molecular Ecology*. However, in the case of species like birds with relatively few microsatellites, a very large number of colonies must be screened in order to find a satisfactory number of positive clones.

Enrichment techniques have been developed to increase the efficiency of the hybridisation selection technique. The enrichment steps enhance the number of microsatellites in the candidate inserts by selection prior to cloning. This increases the chance of finding microsatellite loci among the limited number of colonies that is feasible to screen.

Various enrichment techniques were developed to increase the representation of microsatellites in libraries, e.g. through the use of single-stranded uracil DNA (Ostrander et al. 1992), random amplified polymorphic DNA (RAPD; Ender et al. 1996; Lunt et al. 1999), vectorette PCR (Lench et al. 1996), and the more popular hybridisation selection methods (Kandpal et al. 1994).

For this study, a simple and straightforward hybridisation selection enrichment protocol using nylon membranes was selected to enrich Forbes' parakeet DNA templates for microsatellites. The protocol was a modification of Armour et al. (1994) and Berry et al. (2003). The enrichment process involved digesting the parakeet DNA with *Sau 3AI* restriction enzyme, selection of suitably sized fragments, ligating the fragments to synthetic SAU linkers to allow PCR amplification and hence increase template numbers, followed by hybridisation of the amplified templates to nylon membranes with bound microsatellite probes. The bound fragments of candidate microsatellites recovered from the membranes were expected to have a higher abundance of microsatellites.

These microsatellite-enriched fragment pools were used to construct libraries for screening.

2.2   Methods

2.2.1 *Extraction of DNA and Preparation of Template for Enrichment*

Samples from banded parakeets on Mangere Island were collected by Department of Conservation field staff in the form of blood stored in liquid nitrogen.

Parakeet DNA was extracted from blood samples from the populations on Mangere Island and South-East Island using the phenol / chloroform method modified from Sambrook et al. (1989). For each sample, 1 μl of blood was added to 400 μl of SET buffer (0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 1.0 mM EDTA) pre-mixed with 0.5% SDS, mixed, followed by the addition of 20 μl of Proteinase K solution (Roche) at 20 mg/ml. The mixture was incubated in a Hybaid rotating incubator at 55°C overnight.

Following incubation, 400 μl of phenol was added to the reaction, which was mixed on a rotating platform for 30 min, then centrifuged at 13000 rpm for 5 min to allow phase separation. The upper aqueous phase was transferred to a fresh plastic microcentrifuge tube, to which 400 μl of phenol:chloroform:isoamyl alcohol (25:24:1) was added, followed by 30 min mixing and a 5 min spin at 13000 rpm. The lower organic phase was removed and 400 μl of chloroform:isoamyl alcohol (24:1) was added. The reaction was allowed mixing for 30 min prior to 5 min centrifugation. To precipitate the DNA, the top phase was recovered and transferred to a new microcentrifuge tube, mixed with 1/10th volume of 3 M NaOAc pH 5.2, followed by addition of 2x volume of cold absolute ethanol. The reaction was allowed to precipitate for 3 hrs at 4°C before centrifugation at 13000 rpm for 30 min. After centrifugation, the supernatant was removed and 1

ml of 70% ethanol was added to wash the pellet. The ethanol used in this wash step was removed by a 15 min spin at 13000 rpm and the pellet was allowed to air dry at room temperature. The DNA was redissolved in 20 μl 1 mM Tris-HCl pH 8.0 buffer.

Samples from five individuals (birds ID: D172010, D172029, D172031, D175194, and D175197; Appendix 3.2) with Forbes' parakeet morphology from the Mangere parakeet population were chosen as the source of templates for enrichment.

2.2.2 Genomic DNA Fragmentation and Size Selection of Template

Fragmentation of template DNA from each sample was carried out by digesting 5 μl of purified genomic DNA with 5 U of *Sau 3AI* restriction endonuclease (Roche) in a 15 μl reaction containing 1x SuRE/Cut buffer A (Roche) and 30 μg RNase A (Sigma). The reaction was incubated at 37ºC in a water bath overnight.

Digested genomic DNA between 500 bp to 1 kb in size was selected, taking into account the ease of subsequent cloning, sequencing and primer design requirements. Size separation of fragments was performed by electrophoresis of the genomic DNA on a 1.2% LE agarose (Roche) gel with Ethidium Bromide (EtBr) in 1x TA buffer at 100 V for 30 min. Fragment sizes were determined by comparing to 1 kb DNA ladder standards (Invitrogen). Agarose slices containing the DNA of desired sizes were excised using sterile scalpel blades on an UV transilluminator. DNA was extracted from the agarose gel slice using High Pure PCR Product Purification Kit (Roche) following the manufacturer's protocol. DNA was eluted by 25 μl elution buffer provided with the kit.

The eluted DNA was quantified with a DyNA Quant 200 fluorometer (Hoefer) following manufacturer's instructions. Eluted DNA concentrations were: 11 ng/μl (sample D172010), 10 ng/μl (sample D172029), 21 ng/μl (sample D172031), 11 ng/μl (sample D175194), and 17 ng/μl (sample D175197).

2.2.3 Preparation of SAU Linkers

Synthetic oligonucleotide SAU linkers (Royle et al. 1992) were prepared in 10 replicate 10 μl reactions, each containing 5 μg of SAULA (5' GCGGTACCCGGGAAGCTTGG 3'), 5 μg of SAULB (5' GATCCCAAGCTTCCCGGGTACCGC 3'), and 1x REACT 1 buffer (Invitrogen). The solution was heated to 95ºC for 40 sec in an OmnGene thermocycler (Hybaid), followed by 65ºC – 15 min, 60ºC – 15 min, 50ºC – 15 min, 45ºC – 15 min, 40ºC – 15 min, and then held at room temperature.

2.2.4 Ligation of Template DNA to SAU Linkers

A 40 μl ligation reaction was set up containing 2.75 μg of SAU linkers, 40 ng of DNA from each of the five samples, 1x ligase buffer (Roche), 0.5 mM ATP (Roche), and 1 U T$_4$ DNA ligase (Roche). The reaction was incubated at 16ºC overnight. Unligated linkers were removed by running the reaction through a High Pure PCR Products Purification Kit (Roche) following the manufacturer's protocol. Elution of ligation products was carried out using 25 μl elution buffer.

2.2.5 Pre-enrichment PCR

To increase the amount of DNA template for enrichment hybridisation, the ligation products were PCR amplified with SAULA as primer. PCR was carried out in 12 replicates of 25 μl, each containing 1 μl of gDNA

fragments-SAU linkers, 0.5 μM SAULA primer, 1x PCR buffer (Qiagen), 3 mM MgCl$_2$, 0.8 mM dNTPs (Roche), and 1 U *Taq* DNA polymerase (Qiagen). The reaction was run on a Hybaid OmnGene thermocycler through 94°C – 4 min, 30x (58°C – 1 min, 70°C – 2 min, 94°C – 1 min), followed by a final 4 min extension at 70°C. The reaction was checked by running 2 μl aliquots of PCR products on a 1% LE agarose gel with EtBr in 1x TA buffer at 100V for 30 min, and the PCR products visualized under UV light.

The PCR products were pooled and precipitated by mixing with 30 μl of 3M NaOAc pH 5.2 and 660 μl cold absolute ethanol, allowed to stand at 4°C overnight, followed by centrifugation at 13000 rpm for 30 min. The supernatant was removed after centrifugation, and the DNA pellet was washed with 1 ml of 70% ethanol. After centrifugation at 13000 rpm for 15 min and removal of the supernatant, the pellet was air-dried. The PCR amplified size-selected DNA template was resuspended with 15 μl of 1 mM Tris-HCl pH 8.0, and quantified with a Hoefer DyNA Quant 200 fluorometer. The concentration was 350 ng/μl.

2.2.6 Preparation of Probes for Enrichment Hybridisations

The three complementary pairs of (GATA)$_7$ and (TATC)$_7$, (AAAG)$_7$ and (TTTC)$_7$, (CA)$_{15}$ and (GT)$_{15}$ were phosphorylated to facilitate their ligation to the other member in the pair. The phosphorylation reaction was carried out by heating a 50 μl mixture of 2 μg of each oligo in a pair, 1x ligase buffer (Roche), and 1 mM ATP (Roche) to 70°C for 5 min, followed immediately by chilling on ice and the addition of 30 U of T$_4$ Polynucleotide Kinase (Roche). The phosphorylation reaction was incubated at 37°C for 45 min and the enzyme was then heat inactivated at 65°C for 20 min.

The phosphorylated oligo-pairs were ligated to form "lig-concatemers" in a 65 μl reaction containing 50 μl phosphorylated oligo-pairs, 1x ligase buffer (Roche), and 1 U T$_4$ ligase (Roche). The ligation reaction was incubated at 16°C overnight.

The ligation product was then size-fractionated by electrophoresis on a 1.2% LE agarose gel with EtBr, run in 1x TA buffer at 100 V for 30 min. DNA fragments were visualized under UV light and gel slices containing fragments >200 bp were excised with scalpel blades, extracted with High Pure PCR Products Purification Kit (Roche) following manufacturer's instructions and eluted in 25 μl of the elution buffer provided in the kit.

Primer-free PCRs were set up in 8 replicates for each probe to generate giant probes. Each 25 μl reaction contained 1 μl of "lig-concatemer" template, 1x PCR buffer (Qiagen), 6.7 mM MgCl$_2$, 0.8 mM dNTPs (Roche), and 2 U of *Taq* DNA polymerase (Qiagen). The reaction was run on a Hybaid OmnGene thermocycler for 95°C – 2 min, 30x (55°C – 1 min, 72°C – 2 min, 95°C – 1 min), and 72°C – 5 min. The reaction was checked by running 2 μl aliquots on a 1% LE agarose gel with EtBr in 1x TA buffer at 100 V for 30 min. Smears extending from the wells to about 100 bp were observed. Replicates of each probe were pooled.

Each giant probe was precipitated by addition of 20 μl of 3 M NaOAc pH 5.2, 440 μl of cold absolute ethanol, and centrifuged at 13000 rpm for 30 min after an overnight incubation at 4°C. The supernatant was removed and the pellet washed with 1 ml 70% ethanol, the wash was subsequently removed after 15 min of centrifugation at 13000 rpm. The giant probe DNA pellet was resuspended in 25 μl of 1 mM Tris-HCl pH 8.0 after air-drying. The DNA solution was quantified with a DyNA Quant 200 fluorometer (Hoefer), which gave concentration estimates of

(GATA)$_7$/(TATC)$_7$ at 270 ng/μl, (AAAG)$_7$/(TTTC)$_7$ at 609 ng/μl, and (CA)$_{15}$/(GT)$_{15}$ at 560 ng/μl.

2.2.7 Membrane Enrichment

The giant probe was denatured by treatment with NaOH. For each probe, 10 replicate 9 μl reactions were set up, each containing 1 μg of giant probe, 200 mM NaOH, and 2 mM EDTA. The denaturing reaction was incubated at 37°C for 30 min, and then partially neutralised by the addition of 1 μl of 1 M Tris-HCl pH 4.8, followed by 10 min incubation at room temperature.

Each replicate of denatured giant probes was dotted on Hybond-N membranes (Amersham Pharmacia Biotech) cut into 3 mm^2 squares. The membranes were allowed to air dry, and were wrapped with plastic wrap prior to cross-linking by placing on a standard UV transilluminator for 50 sec per side.

Membranes holding the same probe were grouped together and pre-hybridised in 1.3 ml of Church and Gilbert hybridisation solution (Church & Gilbert 1984) with 1% BSA at 65°C for 3 hours in a rotating Hybaid oven.

While the membranes were pre-hybridising, PCR amplified DNA fragments-SAU linkers were denatured. Each reaction was set up in 9 μl triplicates, containing 1 μg of DNA fragments-SAU linkers, 200 mM NaOH, and 2 mM EDTA. These were allowed to incubate at 37°C for 1 hr before neutralisation with 1 μl of 1 M Tris-HCl pH 4.8, and a further incubation of 10 min at room temperature.

The pre-hybridisation solution was removed from the tube containing the membranes, and a mixture of neutralised denatured template DNA and

200 μl of fresh Church and Gilbert solution (Church & Gilbert 1984) with 1% BSA was added to the tube. The tube was incubated at 65°C overnight, rotating in a Hybaid oven.

The hybridisation solution was removed after the incubation and the membranes were washed 3 times at 65°C for 10 min with 600 μl pre-warmed 2x SSC and 0.1% SDS solution, followed by a rinse with 5x SSC at room temperature. The membranes with captured DNA fragments-SAU linkers were allowed to air dry.

Microsatellite enriched DNA was recovered from the membranes by adding 100 μl of 50 mM KOH with 0.01% SDS, followed by incubation at room temperature for 10 min with occasional flicking. After incubation, 100 μl of 50 mM Tris-HCl pH 7.5 with 0.01% SDS was added to neutralise the reactions, and the membranes were removed from the tubes.

DNA was precipitated by the addition of 20 μl of 3 M NaOAc pH 5.2, 0.5 ml of cold absolute ethanol, and 2 μl of linear polyacrylamide (Gillard & Strauss 1990), followed by a 10 min chill on ice, and a 30 min centrifugation at 13000 rpm at 4°C. The supernatant was removed from the tube and the pellet was washed with 0.5 ml 70% ethanol. The pellet was allowed to air dry after centrifugation at 13000 rpm for 15 min and the removal of the wash solution. The dried pellet was resuspended in 10 μl of 1 mM Tris-HCl pH 8.0.

2.2.8 PCR Amplification of Membrane Enriched DNA

To increase the amount of insert DNA for subsequent library constructions, PCR amplifications were set up in 4 replicates using the recovered DNA from the previous step as template. In each reaction, 1 μl of the recovered enriched DNA template was used, to which 1x PCR

buffer (Qiagen), 3 mM MgCl$_2$, 0.8 mM dNTPs (Roche), 0.5 μM SAULA primer, and 1 U *Taq* DNA polymerase (Qiagen) were added. The reaction was cycled through 95°C – 5 min, 30x (67°C – 1 min, 72°C – 2 min, 95°C – 1 min), 72°C – 4 min in an OmnGene thermocycler (Hybaid).

The reaction was checked by electrophoresis of 1 μl aliquots of products on a 1% LE agarose gel with EtBr in 1x TA buffer, run at 100 V for 30 min. Fragments of 500 bp to 1 kb were visualised under UV light.

Following manufacturer's protocol, High Pure PCR Product Purification Kit (Roche) was used to purify PCR products. The purified product was eluted with 50 μl of elution buffer provided in the kit.

2.2.9 Removal of SAU Linkers from Enriched DNA

The SAU linkers were removed from the amplified enriched DNA by an overnight digestion at 37°C with 10 U of *Sau 3AI* restriction endonuclease (Roche) in a 60 μl reaction containing 1x SuRE/Cut buffer A (Roche) and 50 μl of purified PCR products of enriched DNA. The reaction was run through High Pure PCR Products Purification Kit (Roche) following manufacturer's instructions to remove the SAU linkers, and the enriched DNA template was eluted with 25 μl of elution buffer.

The purified enriched DNA template was quantified in a Hoefer DyNA Quant 200 fluorometer. The concentrations of GATA, AAAG, and CA enriched templates were 57 ng/μl, 71 ng/μl, and 55 ng/μl respectively.

2.2.10 Preparation of Plasmids for Library Construction

Plasmid pUC18 DNA (500 ng; Roche) was digested in a 30 μl reaction with 20 U of *Bam HI* (Roche) in 1x SuRE/Cut Buffer B (Roche). The

reaction was incubated at 37ºC overnight. After the incubation, 2 U of shrimp alkaline phosphatase (Amersham Pharmacia Biotech) was added to dephosphorylate the cut ends of the plasmid, and the reaction was allowed a further 2 hours' incubation at 37ºC.

The reaction was run on a 1% LE agarose gel with EtBr in 1x TA buffer for 30 min at 100 V. A gel fragment containing the linear plasmid was cut out and the plasmid DNA was extracted with High Pure PCR Products Purification Kit (Roche), and eluted in 25 μl elution buffer. The purified plasmid DNA solution was found to have a concentration of 18 ng/μl.

2.2.11 Ligation of Inserts into Plasmids

Ligation reaction was set up for the three different types of amplified enriched DNA. Each ligation reaction was set up with 1x ligase buffer (Roche), 1 mM ATP (Roche), 50 ng of digested and dephosphorylated pUC18 DNA, 90 ng of amplified enriched DNA template, and 1 U of T_4 DNA ligase (Roche). The reaction was incubated at 16ºC overnight.

2.2.12 Transformation of Competent Cells and Making Glycerol Stocks of Transformed Cells

Insert-containing pUC18 plasmid was diluted 5-fold with TE buffer pH 7.5, and 1.5 μl of the dilution was used to transform Max Efficiency DH5α competent *Escherichia coli* cells (Invitrogen) according to manufacturer's instructions. Each of the transformation reactions was plated out onto 10 LB agar plates with 100 μg/ml of ampicillin, 40 μg/ml IPTG, 40 μg/ml X-gal. The plates were incubated at 37ºC overnight.

For each probe used, three 96 well plates were prepared with 20 μl of sterile glycerol, 80 μl of LB, and 50 ng/μl of ampicillin in each well. Using

sterile technique, white colonies were transferred from the agar plates with sterile toothpicks into the wells containing glycerol growth medium. A total of 288 colonies were transferred for each type of enriched template. The 96 well plates were incubated at 37°C overnight in a shaking incubator.

2.2.13 Transfer of Colonies, and Cross-linking of DNA onto Membranes

A sterile "hedgehog" transfer device was used to transfer colonies from the glycerol stock plates to Hybond-N membranes (Amersham Pharmacia Biotech) placed on large LB agar plates with 100 μg/ml ampicillin, and was incubated at 37°C overnight.

The membranes were then placed on a piece of 3 mm filter paper (Whatman) pre-wetted with 2x SSC and 5% SDS for 2 min to lyse colonies. The DNA liberated was cross-linked to the membrane by a 40 sec microwave treatment on high setting.

2.2.14 Hybridisation of Membranes with Radioactive Probes

For each of the three different probes, the three membranes containing cross-linked plasmid DNA from the previous step were grouped and pre-hybridised at 65°C for 2 hrs with shaking in a 125 ml solution which contained 0.3 M Na_2HPO_4 pH 7.2, 1.2 mM EDTA pH 8.0, and 0.1% SDS.

Individual reactions were set up using Megaprime DNA Labelling System (Amersham Pharmacia Biotech) to incorporate α^{32}P-dCTP into 5 ng of each type of giant probes following the manufacturer's protocol. Unincorporated radioactive nucleotides were removed by passing the reactions through 1 ml Sephadex G50 columns, with centrifugation at 2250 rpm for 4 min to help the solution move through the columns. The procedure was repeated with new columns. The eluted probes were then

boiled at 100ºC in a waterbath for 5 min and immediately put on ice for 5 min.

The radioactive probe was added to the membranes in the pre-hybridisation solution (see above), and the hybridisation reaction was incubated at 65ºC with shaking overnight.

After the overnight incubation, the membrane was rinsed twice for 30 sec with a wash solution containing 2x SSC and 0.1% SDS, and then washed with fresh wash solution at 50ºC for 15 min. The membrane was allowed to air dry, and was exposed to Fuji Super Rx film (Fujifilm) at −80ºC for 17 hrs with an intensifying screen. The autoradiograph was developed, washed and allowed to air dry.

2.2.15 Characterization of Plasmid DNA from Positive Colonies
Positive colonies were identified from the autoradiographs. The corresponding colony was picked from the glycerol stock and allowed to grow at 37ºC with shaking overnight in 10 ml liquid LB medium containing 100 μg/ml ampicillin. Plasmid DNA was extracted from these minipreps using QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. The plasmid DNA was eluted by 50 μl of elution buffer provided in the kit.

The recovered plasmid was sequenced with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) on a Perkin-Elmer model 480 thermocycler using either Forward (5' CCCAGTCACGACGTTGTAAAACG 3') or Reverse (5' AGCGGATAACAATTTCACACAGG 3') universal sequencing primers, and the fluorescently labelled products were separated on a 5% polyacrylamide gel (Long Ranger Singel Pack, BMA) by an ABI Prism 377 sequencer (Applied Biosystems). DNA sequence

data were acquired and analysed by the Sequencing Analysis software (Applied Biosystems).

2.2.16 Optimisation of PCRs for Amplification of Microsatellites Loci in Genomic DNA

Primers were designed using the software OLIGO (version 4.0 for Macintosh; Molecular Biology Insights, http://www.oligo.net) based on the insert sequences from positive clones that contained well-placed microsatellites with enough flanking region (minimum 50 bases on each side) for primers to be designed. The loci were named (*Cfor*) after the focal speices of this study, *Cyanoramphus forbesi*.

PCR reaction for amplification of microsatellite loci in genomic DNA was optimised on a Perkin-Elmer model 480 thermocycler, by modification of a protocol by Henegariu et al. (1997). The optimised reaction contained 1x PCR buffer (Qiagen), 2 mM $MgCl_2$, 50 mM KCl, 0.1 mM dNTPs (Roche), 0.4 μM of each primer (0.8 μM each for *Cfor2021*), and 1 U *Taq* DNA polymerase (Qiagen). In a 12.5 μl reaction, about 10 ng of parakeet genomic DNA was used as starting template. The reaction was cycled through 94ºC – 4 min, 30x (94ºC – 30 sec, T_a – 1 min, 65ºC – 1 min), 65ºC – 3 min (T_a are given in Table 2.1).

The PCR product was analysed on a 1% LE agarose gel containing EtBr, run at 100 V for 40 min.

2.3   Results

2.3.1 Development of Primers for Amplification of Microsatellite Loci

A total of 47 strong positive signals were detected on autoradiographs. Among these, 10 were from screening with $(GATA)_n/(TATC)_n$ probe, 13

from the $(AAAG)_n/(TTTC)_n$ probe, and 24 from the $(CA)_n/(GT)_n$ probe. All 47 inserts were sequenced, and primers were designed for 14 microsatellite loci (Table 2.1; Appendix 2.1). Among the remaining inserts sequenced, 8 were found to be false positives, 21 had microsatellite repeat arrays too close to one of the restriction sites and did not contain enough space for primers to be designed, and 4 appeared to be identical copies of other inserts identified. PCR conditions were successfully optimised in 9 loci among the 14 loci for which primers were designed (Table 2.1).

2.4   Discussion

2.4.1 Membrane Enrichment Methodology

This method was proven to be effective in isolating microsatellite loci in this study. Eight polymorphic and one monomorphic microsatellite loci were successfully developed and characterized from Forbes' parakeets. The membrane enrichment method used in this study seems to have a similar efficiency as the other enrichment methods when compared with similar studies in New Zealand parrots: Robertson et al. (2000) isolated 7 polymorphic loci in kakapo (*Strigops habroptilus*) using a protocol based on White & Powell (1997), and Sainsbury et al. (2004) isolated 6 polymorphic loci in kaka (*Nestor meridionalis*) using a protocol developed by Grist et al. (1993) and Cooper et al. (1997).

Using the same membrane enrichment protocol, Berry et al. (2003) isolated 15 polymorphic microsatellite loci in New Zealand skinks, about double that isolated from Forbes' parakeets in this study. It is likely that the low number of loci isolated in this study is due to the low abundance of microsatellites in parrots.

Table 2.1   Characteristics of microsatellite loci isolated from Forbes'
parakeet (*Cyanoramphus forbesi*). For each locus, the primers designed,
the repeat motif as first isolated, the size of the first isolated PCR product,
and the annealing temperature (T_a) of the PCR are presented.

Locus	Primer sequences 5' – 3'	Repeat motif (first isolated)	Size (bp)	T_a (°C)
Cfor0405	CS004: TCCCTGAGCAATAACCAC CS005: ATAAAACGGGAAAATAGA	$(CA)_3CG(CA)_4$	88	-
Cfor0607	CS006: GGCACGACCCAGGCAATC CS007: CATCCTTGGCAGTGTTCG	$(TTTC)_2GTTCTC(TTTC)_2$ $CTTC(TTTC)_5TTCC(TC)_4$	283	-
Cfor0809	CS008: GGATTTGTTCTAAGGGTTGT CS009: AAGGTTTTGTGGAGGCTGTG	$(GATA)_4GATG(GATA)_2$ $(GA)_2(GATA)_3$	187	63
Cfor1011	CS010: GCCTCAATGACTCCTCTC CS011: TGCCTGCTTTTGTTTATT	$(CA)_{11}CG(CA)_6$	227	-
Cfor1213	CS012: AGGCTGCCCAGAGAAGCT CS013: CTATGACATGATTACGAA	$(CTAT)_{14}CAT(CTAT)_5$ $(CTAC)_8(CTAT)_2$	287	-
Cfor1415	CS014: TTTTTGTTAGCCACTGTT CS015: GGGTATACGTTTCTTTTA	$(TG)_{16}$	215	53
Cfor1617	CS016: GGCACGACCCAGGCAATC CS017: CCCGCATCCCCACTCCAG	$(CTTT)_2CGTTCT(CTTT)_2$ $CCTT(CTTT)_5$	217	61
Cfor1819	CS018: AGGGGAGATGAACTGAGG CS019: CCCAGGTAAGTGTTTCAA	$(CA)_9$	184	58
Cfor2021	CS020: ACTTCTGCTATCCAAACC CS021: TCTGCTTGAGTGTGTCTT	$(TG)_2G(TG)_9$	239	60
Cfor2223	CS022: CTCACTTTGCTCTGTTCGTA CS023: CCTTGCTTCTTTGGATAATA	$(CA)_6CG(CA)_8$	217	60
Cfor2425	CS024: TTCAGATAAAGAGAGCAG CS025: AAGGAGCCCAGTTCATGC	$(CA)_{15}$	200	-
Cfor2627	CS126: GAATCCATCCCACCAACACA CS127: AGAAGGCAGTGAAGGGATGT	$(TG)_{20}$	170	59
Cfor2829	CS028: AATGTTTGCTCCACTTGC CS029: CCTGCGGGCTGAATGTGC	$(AAAG)_4…(AG)_4…$ $(AAAG)_4$	219	63
Cfor3031	CS030: TGCATGTGCAAAGAGTCC CS131: CATGCTCATACCCTCACTCA	$(GT)_{11}$	239	57

A number of positive clones identified in this study contained
microsatellites which were too close to the restriction site to allow
development of primers. This greatly reduced the effectiveness of the

method in obtaining amplifiable polymorphic microsatellites. Using other restriction endonuclease and plasmid combinations may help improve the yield of clones which microsatellites are centrally located in the inserts.

More detailed analyses of the Mangere and South-East Islands parakeet populations and characterization of the loci are presented in the next two chapters.

2.4.2 Is Enrichment Necessary in Isolating Microsatellite Loci from Forbes' Parakeet?

Prior to the construction and screening of enriched libraries described in this chapter, non-enriched Forbes' parakeet genomic libraries were constructed and screened for microsatellites. However, none of the 30 positive colonies selected from the non-enriched libraries contained microsatellites (see Appendix 2.2 for methods and results).

It appears an enrichment step is essential in isolating microsatellites from organisms with low microsatellite density, such as birds (Primmer et al. 1997). The difficulties in isolating microsatellites also explain why a lot of effort has been put into development of enrichment protocols (see Zane et al. 2002).

2.5 References

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Appendix 2.1   Sequences of Microsatellite Loci with PCR Primers Designed

Regions of inserts with reliable sequence are listed with primers designed set in bold.

PCR optimised loci (locus name – PCR product size):

Cfor0809 – 187 bp

CTAGCTCGGTACCCGGGGACCTTTCTGTAGCAAAACACCAACTGTCA
TTGTAT**GGATTTGTTCTAAGGGTTGT**CAGCTCTGGAAGAGTGCAGCC
ATTTCACAACCAGGAGGTGGGTGGGTGGACGGGTAGGTAGGTAGAT
GGATAGATAGATAGATAGATGGATAGATAGAGAGATAGATAGATAGA
TGGAGTGTTGGGTAGAGACAGAAATAGAGAGAG**CACAGCCTCCACA
AAACCTT**GTTTCAAGTACCCTCATTTATAACTTGGGTTTTGTAGGTTTT
CTTGCCTTTGGAAGGGGACTTCATGTATCTTCACACCGTTCCTGAGA
GTGGCAGTATTTGCACAGTGTTAATGTCTCAGGCCTGATC

Cfor1415 – 215 bp

GATCCCTGT**TTTTTGTTAGCCACTGTT**TGAAAACCTTGTTAGCTTAGT
GTCGGGCACTCATTAAACTTTCAACTTACACAAGTTGCCTCCCAGCTC
TGACAGATTGTCTGTGCCACAGTGTGTATATGCGTGAGCATGTGTTT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGACTTATTGACCTGTAT
GCAAGGCAAGTCACCG**TAAAAGAAACGTATACCC**CTTTTCACCCGA
CAGATC

Cfor1617 – 217 bp

GATCAGATACGGGGAGAGACACGGAGAAGAGAAAAGCTCCGTGCT**G
GCACGACCCAGGCAATC**GGGTGACCTCTTTCTTCCTTTCTTTCGTTC
TCTTTCTTTCCTTCTTTCTTTCTTTCTTTCTTTCTTCCTCTCTCTCCCCC
TTTCCTTCTCTTTCTCCTCGTCTCCTCGCTGCCCTCCTTCTCCTCGCC
GCTGGTGAGGCTGGTCCCTCACCCCGGTTGGTGGTGCCCCCACCGC
GGCTCTC**CTGGAGTGGGGATGCGGG**GGTCCCCAGCAGCGTCGGGG
CTGGGGGGGCTGGTGGTGCCCGCAGGAGGTTGCTTGGAGGAAGAC
AGCGTTCAGCTGGAGAGCAGGAGCCATTACACACG

Cfor1819 – 184 bp

CTAGAGGACCCCCATGTCTTGGTGTCCCCAGGGACATGGTTGCTTGC
ATCCCTTGCCCATGGTGTCC**AGGGGAGATGAACTGAGG**TTTAAGGT
GGGTGCTTGGGAATTGACCAGCTCTGAGTAGAGCTGGGCACAGCAC
AGGACCATCCCAAGGGCTGTCCATGTAGCAATCACACACACACACAC
ACACCCCTTATTTAGAATCCCTATCATTTCCCAGTCTACGGGCTGTG**T
TGAAACACTTACCTGGG**TATGCCAGGGGCATCACTGGATGCTCAGC
TCCATGCCCATCCCTCCTGGTGCTCACCTCCCTCCATCCGGAGCTGC
TCAATCCCGCTGCTTCACTTCACTCCCCATCCTGCTGCTCTGACACA
GCCACCCCATGTCACTCACCCAGCCCAGGTCACCTCAGGGGATGGC
AGAGAGCTGGCGGCTGCCTCAGGACACGGCTATTCCTGCCACCTCC
GTGATC

Cfor2021 – 239 bp

GATCTGTCAACCGAGTAGCCCTGAACTACGAAGTGAAATTACAGT**AC
TTCTGCTATCCAAACC**CCCCTGAATTGCGAGGTGTGCGGTGTGGTGT
GTGTGTGTGTGTGTTGCACGGCGGTGTAACAATGTGCTGGCACC
GCGTCAGGGCTGGAAGCGCCGCAGACCCCGTATAACGCCAAATGAG
GCTTGTCTTGTTTTGTTATTCAGATTTCTACTCTTCCATTAAAAACAGC
CTTACAATAACCAATGATAAAGGTCTGATTT**AAGACACACTCAAGCA
GA**GCAATTCAGAGCTTAGGACAGTGATC

Cfor2223 – 217 bp

GATAGTGGGGTATTAACGAAGCGGAAGTTGCTTCTCCTG**CTCACTTT
GCTCTGTTCGTA**AACAAAGGGACTCTGTTCCTTTATCTTATGATTTCTT
CACAGAGGAACAGGCAAACATGCATAGAAAGGAGGAGACAGGGAGA
TGGAGCTTACTGATTTTTTCAGTGAAAGGACTGATTTTCCCACCTTTT
ACATACACACACACACGCACACACACACACATTTCAATTTAG**TA
TTATCCAAAGAAGCAAGG**ACATTTCATATCAGCTGGGGACTTGGCCT
GACAAAGGTGAAGGTGATC

Cfor2627 – 170 bp

CTA**GAGGATCCATCCCACCAACACA**CATAGTTCTTTCCCTGCTGCTA
CGCTGCAGCCAGGGGTGGGGAGAGTGTGTGTGTGTGTGTGTGTGTG
TGTGTGTGTGTGTGTGCACATGTTTCCAAAGATTCGTCTGCTTTC
AAAAATCACCAGGC**ACATCCCTTCACTGCCTTCT**GGAGCCTGACCCT
TGCACGCTGCTTGGGCAAGACAAAGGAAACCTTGCAAATGCCACTGA
TC

Cfor2829 – 219 bp

CTAGTACA**AATGTTTGCTCCACTTGC**ACAGAGAGAATATTGAAAGAAA
GAAAGAAAGAGAGAGAGAGAGAAAGAGAGAGAAAGAAAGAAAAAGA
AAGAAAGGAAGAAAGAATGAAAGAAAGAAAAGAAAGAAAGGAAGGA
AGAAAGAAAGAAAGAGAAAGAAAGAAAGAAAGAGAGAAAGAAAGAGC
ATGTGGTTAGTTTTTAGAAGT**GCACATTCAGCCCGCAGG**CTCTTGCT
GGTCGCTGCCGCCCGCCCCAGTTCTGCCGGGTCCTTACCCTACAGC
TGTGGTACTGCCACAGCAGCTGGGTCAGGGCCACGATC

Cfor3031 – 239 bp

CTAGAGGAGCCCGTCGGGAGTGCGTGCAGGAGCCCGTCCACGGGG
TGCATGTGCAAAGAGTCCCTCGGAGGGGTGTGTGTGCAACAGACTG
CCCGGAGGGGGGTGTGCAAATGTCCCTGGGGCCATGTGTGAACGC
GTGCCCGAAAGCGGCGTGTGTAAGAACGTGGTGGGGTGAGTGCATG
TGCGGCGTCCCGTTGGGGTGGGTGTGTGTGTGTGTGTGTGTGTAAA
AGTGCTTGTCCAGGGGACGGGTGTGTAAGAGCCGGT**TGAGTGAGGG
TATGAGCATG**ATC

Non-optimised loci (Amplification of genomic DNA failed):

Cfor0405

AGCCAG**TCCCTGAGCAATAACCAC**CTTGGAAGCCACACACGCACAC
ACACCCCCCCCCTTTCAATTTTTCTTTCCT**TCTATTTTCCCGTTTTAT**A
TAGCTTGAAGCATGAACAATTAGAATGGCAATAAAATATTCCTTTGGC
TGATTCAAAGGGGGAACCCGGGAAGTTTGGGCACTGGGACCGGGG
GTTTCCGGGGAAGGGGAA

Cfor0607

AGATACGGGGAGAGACACGGAGAAGAGAAAAGCTCCGTGCT**GGCAC
GACCCAGGCAATC**GGGTGACCTCTTTCTTCCTTTCTTTCGTTCTCTTT
CTTTCCTTCTTTCTTTCTTTCTTTCTTTCTTCCTCTCTCTCCCCCTTTCC
TTCTCTTTCTCCTCGTCTCCTCGCTGCCCTCCTTCTCCTCGCCGCTGG
TGAGGCTGGTCCCTCACCCCGGTTGGTGGTGCCCCCACCGCGGCTC
TGTCTGACATGGCCTTCAACACCACCAGGGATGGAGCATTCACAACA
AGGCTCTGTCCGACCTGGCCT**CGAACACTGCCAAGGATG**GAGCATT
CACAACCCATTCCAGTGCCTCACCAACCTCACAGTAAATAATTTCTTC
CTTATATCTTACCTGAACTTCCCCTGTTTAAGCTTGAACCTGTTACCC
CTTGTCCTGTCACTACAGTCCCTGATGAAGAGTCCCTCCCCAGCATC
CCTATAGGCCCCCTTCAGATACTGGAAGGCTGCTATGAGGTCCCCAC
GCAGCCTTCTCTTCTCAGCTGAACAGCCCCAACTTTCTCAGCCTATCT
TCATACGGGAGTT

Cfor1011

A**GCCTCAATGACTCCTCTC**ACTCCCAAGCTATAGGACAGGCCCTGCA
ATACCAAATACAAATACTCAATCCCTTTGCTATCTTCTCCTCAATGCCC
AGACATCACACACACACACACACACACGCACACACACACAAACCC
CTTAACTCTTTACAACTCTACACGCTTTGTGCCTTCACATCTCCCAAG
CCAGCAGCAGCATGTGAAA**AATAAACAAAGCAGGCA**TGCTGAGAG
ACGGCTCAAAGATGCACCTTTAACTTACCAAGGGC

Cfor1213

CAC**AGGCTGCCCAGAGAAGCT**GTGGCTGCCCCATCCCTGGCATGCA
TGCATGCATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATC
TATCTATCTATCTATCATCTATCTATCTATCTATCTATCTACCTACCTAC
CTACCTACCTACCTACCTACCTATCTATCTAATCTATCATCTTTCTTCT
CTGAAATGTCCCAATTGTACAATGTTTAGCTGCTCCGTGTTGAAACCT
GTTGAGGATCCCCGGGTACCGAGCTCGAA**TTCGTAATCATGTCATAG**
CTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATA
CGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGA
GCTAACTCACATTAATTGCGTTGCGCTCACTGGCCGCTTTCCAGTCG
GGAAA

Cfor2425

GATTTG**TTCAGATAAAGAGAGCAG**TTTCTAGAGTTCAGGTTTGAAGA
TACACACACACACACACACACAACACACAGAGAGCTTCAAGTGTT
CAGGTTTGAAGACAAACACACACATAGACACACACACAGAGCTTC
CTCCCCTCTCTGTGTTTCCATCCCCATCAGAGGCTGGGCTCAGGCT**G**
CATGAACTGGGCTCCTTTCAGACTCACAGAATCAACCAGGTTGGTAA
AGCCCTTTAAGCTCATCCAGTCCAACCATTCCCAGCACTGCCAAGGC
CACCCCTAACCCNTGGCACTGAGGCCTCGTCTCCACGGTGTGTGAG
CACTTGCAGAGCCAGTGCCCGCAGCCCTGCCCTGGGCAGCCTGTTC
CAATGCCTGAGCACCCT

Appendix 2.2   Libraries Without Enrichment – Random Genomic DNA Sequences from Forbes' Parakeet

Methods

Preparation of Insert and Plasmid DNA

DNA was extracted from five random Mangere Island parakeet blood samples as described in section 2.2.1. From each sample, 5 μl of DNA was digested in a 15 μl reaction with 5 U of restriction enzyme *Sau3 AI* (Roche), 1x SuRE/Cut buffer A (Roche), and 0.5 μl RNase A. The reaction was incubated in a 37°C heating incubator overnight.

DNA fragments between 300 bp to 800 bp were size-selected and purified as described in section 2.2.2. The pool of size-selected fragments from the five samples were mixed.

Plasmid pBlueScript (Stratagene) was digested in a 30 μl reaction with 20 U of *Bam HI* (Roche) in 1x SuRE/Cut Buffer B (Roche). The reaction was incubated at 37°C overnight. After the incubation, 2 U of shrimp alkaline phosphatase (Amersham Pharmacia Biotech) was added, and the reaction was allowed a further 2 hours' incubation at 37°C. The digested product was separated by agarose gel electrophoresis, and the linear plasmid was excised from the gel, followed by purification with High Pure PCR Products Purification Kit (Roche).

The insert and plasmid DNA were quantified on an agarose gel. The insert was found to be 20 ng/μl. The *Bam HI* cut plasmid was at a concentration of 30 ng/μl.

Ligation of Inserts to Plasmids and Transformation

Using the protocol described in section 2.2.11, *Sau3 AI* digested insert was ligated into *Bam HI* cut plasmid. Library Efficiency DH5α competent *E. coli* cells (Invitrogen) were transformed following manufacturer's protocol. The transformed cells were plated out on 10 LB plates as described in section 2.2.12. The plates were left to incubate at 37°C overnight.

An average of 70 colonies grew on each plate. The colonies were lifted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech) following manufacturer's protocol. Each membrane was then baked 80°C for 2 hours to crosslink the DNA.

Preparation of Microsatellite Probes and Hybridisation

Hybridisation was performed as described in section 2.2.14, except the probes used were a mixture of $(CA)_{12}$ and $(GA)_{12}$ oligonucleotides radioactively labelled in a 50 μl reaction, incubated for 1 hour at 37°C, containing 25 U Terminal Deoxynucleotide Transferase (TdT; Amersham Pharmacia Biotech), 1x TdT buffer (Amersham Pharmacia Biotech), 5 μl α^{32}P-dCTP, and 25 ρmol of each of the oligonucleotides.

Isolation and Characterization of Positive Inserts

Colonies that gave positive signals on the autoradiographs were identified and picked from the agar plates. These colonies were allowed to grow overnight at 37°C with shaking in LB medium with 100 μg/ml ampicillin. Plasmid DNA was extracted from the cultures using QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. The recovered plasmid was sequenced as described in section 2.2.15. Sequences obtained were searched for homology to published sequences

at the Genbank/EMBL/DDBJ databases (http://www.ncbi.nlm.nih.gov) using the nucleotide BLAST tool.

Results

Positive Colonies

The screening of the non-enriched library yielded 30 positive colonies, of which, 17 were successfully sequenced. Only 9 of the inserts were longer than 100 bp (see **Sequences** section in this appendix).

Characterization of Insert Sequences

None of the insert sequences contained microsatellites. A search on the Genbank/EMBL/DDBJ databases did not return any matches to over 50% of the length of the insert sequences (as at January 2005). The sequences isolated represent novel random nuclear genetic fragments from *Cyanoramphus forbesi*.

Sequences

Clone #1 – 173 nt

TCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATTGGGTGGA
AGACAACTTCCTTCTGCAAGTAACAGAGGAGCCGACAAGGAGAGGT
GCCATGCTTGACCTTGTGCTCACCAACAGGGAAGGGCTCGTTGAGAA
TGTGGTACTCCAGGGCAGCCGTGGATGCAGTGA

Clone #2 – 283 nt

TCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGAATACCCAGC
CCAGCACGGGCAACTGAGGTACCAGCACGAGCATTGGTGGTTCAGT
GGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCAATTCCCG
GCCAATGCAAAGCTTTGCTTTTATTCTGTCCTGGGTTCAGCAGAAAAA
GTCTTTTTTCTCCTTCTTACCAATCATTCACAGTCTAGGAGGTTCCTC
CAGTGCATTGATGATAACTTCTTAATGCAAATGGTGGACATACCAACT

Clone #3 – 181 nt

TCAGATATTAGGAAAAATGTCTTTACTGAAAGGACTGTCAATCATTGA
AGCAGGCTGCCCAAGGAAGTGATGGCCTCACCATCCCTGGAGGTAT
TTAAAAGATGTGTAGATACGGTACTTAGGGACATGGCTTAGTGGTGG
ACTTGGCAACAGCTGGGCTCGATACCGTCGACCTCGAGGG

Clone #4 – 270 nt

TCTTGTTTTTACGAGGTGCCCGGGCAGCAGTGAGGTGCGCAGTCCA
ACAGCATCATGGTGCTGCACGGGACCTGGCTGCTAACAATGTGCCC
GAGAGGTGTGAATGCGGCAGCGTCCGGGAGGCTCCAAATGGGTAAC
AACACATGAAGGGAAAACCACCCCGGCTGCCCTCAAACCTGAGCTG
CCTCCTCCTGCTCCCCTCCTCCCCGCACCTGCCTTTCGTTTTCCTCAT
GCATGGAAGGTATTAATCACCCAGCTCGCTTTACTGGC

Clone #5 – 153 nt

TCCTTTGCTTAAACAAAGTATTGTCTGCTGTAGGCACTGTAAGCACT
GAGGAACTGTACTGTTGCCCTGCACCTCTCTCTGCACGCAAGGCTGC
AGAGCTTTCTGCTCAGTTCCTCAGCTCTGGGGAGCACATCCAGCGCA
CAACGGTGGGA

Clone #6 – 138 nt

TCATGTTACATCATGTTATAGCATCATGTCATATCATATAACCTCATTT
CATTTCCTTTTCATTGCATTTGGTTTCTTTTCTTCACCTCGTCATTTTCT
CTCCTCCTCCTCTCTCCACAAATATACCATTTCCTATCA

Clone #7 – 356 nt

TCCTGCAGGAAAGCTACTCTCTTCCTAATTACCCTGCAATTGCCAGGT
TCAAAATGTAATTTATGAGCAGCATAGATAGAATGGCCACATTAGTCC
ATTATTTCATGATAATCTTTTCTACTCTTGCTTTCAGAAAGGAAGGATT
TTGCAGCTCTCGTTGCATTGGCACTGTCAGCAGTTCCTCTGTGCAGT
GATGCTATACAACTGCACTGGAAAGATACTCCCCCAAAAATGTAGAG
CAGTCACCTAACAGCAAGGTATTACAGAAGGAGGGAGGTACTGTGAG
GTACACTTAAACAAATAAAGCATGACCTGGTGGGCTGCCTCAGACAC
TGTTGACCACATTTGGAAACAGA

Clone #8 – 140 nt

TATGATAGGAAATGGTATATTTGTGGAGAGAGGAGGAGGAGAGAAAA
TGACGAGGTGAAGAAAAGAAACCAAATGCAATGAAAAGGAAATGAAA
TGAGGTTATATGATATGACATGATGCTATAACATGATGTAACATGA

Clone #9 – 356 nt

TCTGTTTCCAAATGTGGTCAACAGTGTCTGAGGCAGCCCACCAGGTC
ATGCTTTATTTGTTTAAGTGTACCTCACAGTACCTCCCTCCTTCTGTAA
TACCTTGCTGTTAGGTGACTGCTCTACATTTTTGGGGGAGTATCTTTC
CAGTGCAGTTGTATAGCATCACTGCACAGAGGAACTGCTGACAGTGC
CAATGCAACGAGAGCTGCAAAATCCTTCCTTTCTGAAAGCAAGAGTA
GAAAAGATTATCATGAAATAATGGACTAATGTGGCCATTCTATCTATG
CTGCTCATAAATTACATTTTGAACCTGGCAATTGCAGGGTAATTAGGA
AGAGAGTAGCTTTCCTGCAGGA

Appendix 2.3   Characterization of Variable Microsatellite Loci in Forbes' Parakeet (*Cyanoramphus forbesi*) and their Use in Other Parrots

Chi-hang Chan[1], Kaye N. Ballantyne[1], David M. Lambert[2] & Geoffrey K. Chambers[1]

[1]*Institute for Molecular Systematics, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand.*
[2]*Allan Wilson Centre for Molecular Ecology and Evolution, Institute of Molecular BioSciences, Massey University, Private Bag 102904, North Shore Mail Centre, Auckland, New Zealand.*

Forbes' parakeet (*Cyanoramphus forbesi*) is endemic to Mangere and Little Mangere Islands, in the Chatham Islands group of New Zealand. They are known to hybridise with Chatham Island Red-Crowned parakeets (*C. novaezelandiae chathamensis*). Their hybrid offspring and various backcrosses exhibit a range of crown colour patterns intermediate between those of the parent species.

A previous analysis of the entire mitochondrial DNA control region divided Forbes' parakeet into three haplotype groups (Boon et al. 2001), with the major differences between the three haplogroups lying within the first 300bp from the 5'-end of the sequence. Haplogroup 3 consists of birds that are genetically distinct from other *Cyanoramphus* species whereas haplogroups 1 and 2 are much more closely related to Chatham Island Red-Crowned parakeets and believed to have arisen from past inter-

specific hybridisation. In this study, we isolated nuclear microsatellite DNA markers for measuring genetic variation in the Forbes' parakeet population to monitor its hybridisation with *C. n. chathamensis*.

Blood and feather samples were collected from a complex parakeet population on Mangere Island, which is thought to comprise of a mixture of Forbes' parakeets, Chatham Island Red-Crowned parakeets and their hybrids. DNA was extracted from blood samples using a phenol/chloroform method (Sambrook et al. 1989) or from feathers using DNeasy Tissue Kit (Qiagen). Microsatellite loci were isolated, characterised and developed from a library enriched by use of nylon membranes (Armour et al. 1994; Berry et al. 2003). Purified genomic DNA from five randomly chosen birds with Forbes' parakeets morphotype was digested with *Sau 3AI* (Roche), and the 500-1000bp gel-isolated fragments were ligated to SAU linkers (Royle et al. 1992) at 16°C for 17 hours. Pre-enrichment PCR was performed using 0.5 μM SAULA primer (5' GCGGTACCCGGGAAGCTTGG 3'), 1x PCR buffer (Qiagen), 3 mM MgCl$_2$, 0.8 mM dNTPs, 1 U *Taq* polymerase (Qiagen). The reactions were cycled through 94°C – 4 min; 30 x (58°C – 1 min, 70°C – 2 min, 94°C – 1 min); 70°C – 4 min on a Hybaid OmnGene thermocycler. Hybond-N membranes (Amersham Pharmacia Biotech) cut into 3mm^2 squares, saturated with (CA/GT)$_n$, (AAAG/TTTC)$_n$, or (GATA/CTAT)$_n$ solutions were used to select for pre-enriched fragments containing microsatellite DNA as described in Berry et al. (2003).

The enriched fragments were PCR amplified under the same conditions as the pre-enrichment PCR except that the annealing temperature was increased to 67°C and the number of cycles was increased to 35. The SAU linkers were removed by digestion with *Sau 3AI* (Roche) and products recovered by purification with High Pure PCR Products Purification kit (Roche). The *Sau 3AI* digest fragments were ligated into

pUC18 plasmids and transformed into competent *Escherichia coli*, strain DH5a cells. Colonies were lifted on Hybond-N membranes (Amersham Pharmacia Biotech) and screened with α^{32}P-labelled $(CA)_n$, $(AAAG)_n$, or $(GATA)_n$ probes following the protocol in Berry et al. (2003). The membranes were exposed on Fuji Super RX film (Fujifilm) with an intensifying screen for 17 h. Plasmid were isolated from 47 positive candidate colonies identified from autoradiographs using QIAprep Miniprep kit (Qiagen) and their avian inserts were cycle sequenced with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and products were run on an ABI Prism 377 sequencer (Applied Biosystems).

Fourteen sets of PCR primers were designed based on recovered DNA sequences using OLIGO (version 4.0 for Macintosh; Molecular Biology Insights, http://www.oligo.net), and we were able to optimise amplification conditions for 9 sets of primers in 12 randomly chosen parakeet DNA samples. Candidate loci were amplified in 1x PCR buffer (Qiagen), 2 mM $MgCl_2$, 50 mM KCl, 0.1 mM dNTPs, 1 μM Fluorescein-12-dUTP (Roche; for genotyping reactions only), 0.4 μM each primer (0.8 μM each for *Cfor2021*), and 1 U *Taq* DNA polymerase (Qiagen). The reactions were run on a Perkin-Elmer model 480 thermocycler for an initial denaturation at 94ºC – 4 min, followed by 30x (94ºC – 30 sec, T_a – 1 min, 65ºC – 1 min), and a final extension of 65ºC – 3 min (T_a are given in Table 1). Genotyping and sequencing were performed on an ABI Prism 377 sequencer (Applied Biosystems).

A total of 250 parakeets from Mangere Island were screened to examine variability at each of the loci. All loci, except *Cfor2223*, were found to be polymorphic in the Mangere Island parakeet population (Table 1). The monomorphic locus, *Cfor2223*, could be amplified with primers (F: CTCACTTTGCTCTGTTCGTA and R: CCTTGCTTCTTTGGATAATA) at T_a

= 60ºC, and a 217bp allele with repeat motif $(CA)_6CG(CA)_8$ was observed in Forbes' parakeet.

Observed and expected heterozygosity values were calculated using the Microsatellite Analyser software (version M3.15; Dieringer & Schlötterer 2002; see Table 1). Deviation from Hardy-Weinberg equilibrium (HWE) was tested by the Markov chain method (Guo & Thompson 1992) as implemented in GENEPOP (version 3.4; Raymond & Rousset 1995). *Cfor1415*, *Cfor1617*, *Cfor1819*, *Cfor2627*, and *Cfor3031* all showed significant deviations from HWE (P<0.05). Possible explanations for these observations include non-random mating in the population as suggested by mitochondrial control region studies (Boon et al. 2001; Ballantyne 2003) and the presence of null alleles. The latter is likely to be the explanation for deviation from HWE in *Cfor2627* as 59 individuals were apparently homozygous for the null allele.

Loci isolated were also tested for linkage disequilibrium using GENEPOP (version 3.4; Raymond & Rousset 1995). Evidence for significant linkage disequilibrium was detected between *Cfor0809* and *Cfor2829*, and between *Cfor1415* and *Cfor2829* (both with P=0.00) after Bonferroni correction (Rice 1989). However, we did not find evidence for linkage disequilibrium at these loci in three other New Zealand *Cyanoramphus* parakeet populations (unpublished data). Therefore, it is unlikely these loci are physically linked on the same chromosomes.

The loci were also tested across a wide range of other parrot and cockatoo species, the results are shown in table 2. We believe that the loci described here, in conjunction with other primers for parrots previously published (for example, Robertson et al. 2000; Sainsbury et al. 2004), will provide useful markers for conservation studies of parrots worldwide.

Acknowledgements

Table 1. Microsatellite loci isolated from Forbes' parakeet: N = number of alleles, T_a = annealing temperature of PCR, H_O = observed heterozygosity, H_E = expected heterozygosity. Loci indicated with asterisks (*) showed significant heterozygote deficiencies (all with P=0.00). The DNA sequences of microsatellite loci isolated in this study have been deposited in DDBJ under accession numbers AB181800-AB181808.

Locus	Primer Sequences 5' – 3'	Repeat Motif as First Isolated	N	Allele Size (bp)	T_a (°C)	H_O	H_E
Cfor0809	F: GGATTTGTTCTAAGGGTTGT R: AAGGTTTTGTGGAGGCTGTG	$(GATA)_4GATG$ $(GATA)_2(GA)_2(GATA)_3$	4	183-203	63	0.08	0.09
Cfor1415	F: TTTTTGTTAGCCACTGTT R: GGGTATACGTTTCTTTTA	$(TG)_{16}$	7	211-227	53	0.76	0.73
Cfor1617*	F: GGCACGACCCAGGCAATC R: CCCGCATCCCCACTCCAG	$(CTTT)_2...(CTTT)_2CCTT$ $(CTTT)_5$	2	217-225	61	0.10	0.28
Cfor1819*	F: AGGGGAGATGAACTGAGG R: CCCAGGTAAGTGTTTCAA	$(CA)_9$	4	176-188	58	0.45	0.60
Cfor2021	F: ACTTCTGCTATCCAAACC R: TCTGCTTGAGTGTGTCTT	$(TG)_2G(TG)_9$	2	233-239	60	0.26	0.26
Cfor2627*	F: GAATCCATCCCACCAACACA R: AGAAGGCAGTGAAGGGATGT	$(TG)_{20}$	4	160-172	59	0.35	0.79
Cfor2829	F: AATGTTTGCTCCACTTGC R: CCTGCGGGCTGAATGTGC	$(AAAG)_4...(AG)_4...$ $(AAAG)_4$	3	219-231	63	0.38	0.40
Cfor3031*	F: TGCATGTGCAAAGAGTCC R: CATGCTCATACCCTCACTCA	$(GT)_{11}$	7	233-247	57	0.41	0.65

Table 2. Amplification of *Cfor* loci in other parrot and cockatoo species. + = amplification of a microsatellite, - = no amplification. Sequences from amplified microsatellites have been deposited in DDBJ under accession numbers listed.

	Species	DDBJ Accession numbers	Cfor0809	Cfor1415	Cfor1617	Cfor1819	Cfor2021	Cfor2223	Cfor2627	Cfor2829	Cfor3031
Australasian	*Cyanoramphus novaezelandiae chathamensis*	AB182657-65	+	+	+	+	+	+	+	+	+
	Cyanoramphus novaezelandiae novaezelandiae	AB182684-92	+	+	+	+	+	+	+	+	+
	Cyanoramphus auriceps	AB182666-74	+	+	+	+	+	+	+	+	+
	Cyanoramphus malherbi	AB182675-83	+	+	+	+	+	+	+	+	+
	Cyanoramphus hochstetteri	AB183203-11	+	+	+	+	+	+	+	+	+
	Cyanoramphus unicolor	AB181982-90	+	+	+	+	+	+	+	+	+
	Cyanoramphus saisetti	AB185424-32	+	+	+	+	+	+	+	+	+
	Eunymphicus cornutus	AB183212-20	+	+	+	+	+	+	+	+	+
	Eunymphicus uvaeensis	AB183221-29	+	+	+	+	+	+	+	+	+
	Barnardius barnardi	AB185433-39	+	+	+	+	-	+	+	+	-
	Glossopsitta pusilla	AB183241-46	+	-	+	+	+	-	+	-	+
	Micropsitta pusio	AB183230-35	+	-	+	+	+	-	+	-	+
	Platycercus eximius	AB185132-37	+	+	+	+	-	+	-	-	+
	Trichoglossus haematodus	AB183236-40	+	-	+	-	+	-	+	-	+
	Psephotus varius	AB185138-41	-	+	+	-	-	-	+	-	+
	Melopsittacus undulatus	AB185142-43	-	-	-	+	-	-	+	-	-
	Strigops habroptilus	AB182693-94	-	-	-	+	-	+	-	-	-
	Nestor notabilis	AB182695-96	+	-	+	-	-	-	-	-	-
	Nestor meridionalis	AB182697-98	+	-	+	-	-	-	-	-	-
African	*Coracopsis nigra barkyli*	AB181960-64	+	-	-	+	+	-	+	-	+
	Poicephalus senegalus	AB181969-75	+	-	+	+	+	-	+	+	+
	Poicephalus meyeri	AB181965-68	+	-	+	+	-	-	+	-	-
	Psittacus erithacus	AB181976-81	+	-	+	+	+	-	+	-	+
Cacatuidae	*Nymphicus hollandicus*	AB185416-23	+	+	+	+	+	+	+	+	-
	Eolophus roseicapillus	AB185413-15	+	-	+	-	-	-	+	-	-
	Cacatua haematuropygia	AB185130-31	+	-	-	-	-	-	+	-	-

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Chapter 3: Microsatellite DNA Studies Reveal Extensive Hybridisation between Parakeets on Mangere Island

3.1 Introduction

Hybridisation may bring both advantages and disadvantages to a population. On the positive side, hybridisation can be a source of new genetic variation that enhances adaptation (Lewontin & Birch 1966), and a stimulus for evolution and diversification (Anderson & Stebbins 1954; Dowling & Secor 1997). On the negative side hybridisation may threaten the very existence of a species, especially when genetic introgression is from an abundant species to a rare species (Rhymer & Simberloff 1996). For example, hybridisation between American black ducks (*Anas rubripes*) and mallards (*A. platyrhynchos*) has led to a significant reduction in genetic differentiation and breakdown of species integrity (Mank et al. 2004). In some cases, hybrid progeny are less fit than their parents, but the reverse can also be true (Arnold & Hodges 1995; Barton 2001). When hybrids are of about equal fitness to one or both parental species, hybrid zones can establish and may remain in equilibrium for long periods of time (Barton & Hewitt 1985). Although interspecific hybridisation events are common, the processes involved are empirically well understood in only a very few systems (Stone 2000).

The main aims of this chapter are to investigate: 1) the level of hybridisation taking place in the Mangere Island Forbes' parakeet population; 2) methods for classifying parents and hybrids through the use of genetic markers; and 3) the correlation between the genetic status of individual birds and their morphological features. Insights are gained on the genetic structure of the populations through the use of the

microsatellite markers isolated for Forbes' parakeet. Mutation modes of these loci are also studied.

Two parakeet populations are studied in this chapter: 1) The Mangere Island population, which is a mixed population made up of Forbes' parakeets, Chatham Island Red-crowned parakeets and hybrids; and 2) The South-East Island population, which is largely made up of Chatham Island Red-crowned parakeets. The three different types of markers investigated are microsatellites (MS), mitochondrial DNA control region (MT), and crown plumage morphology (MP).

3.2 Methods

3.2.1 Screening of Parakeets at Cfor loci

Parakeet DNA was extracted from a total of 285 parakeet blood and feather samples collected between 1999 and 2002 (250 from Mangere Island, 35 from South-East Island). DNA extraction from blood was performed as described in section 2.2.1. To extract DNA from various types of feather samples, the tips of 1 – 7 feathers were used as the source of DNA. Higher success of feather DNA extraction is usually obtained from feathers with more skin tissue attached. Extraction was performed using DNeasy Tissue Kit (Qiagen) following manufacturer's protocol.

The DNA samples were genotyped at 8 polymorphic loci (*Cfor0809*, *Cfor1415*, *Cfor1617*, *Cfor1819*, *Cfor2021*, *Cfor2627*, *Cfor2829*, and *Cfor3031*). To set up genotyping reactions, 1 μM of Fluorescein-12-dUTP (Roche) was added to the PCR reaction set-up (see section 2.2.16), labelling the PCR products with a fluorescent tag. The amplified allelic products were separated on 5% polyacrylamide gels (Long Ranger Singel

Pack, BMA) by an ABI Prism 377 sequencer (Applied Biosystems), and analysed by the GeneScan Analysis software (Applied Biosystems).

The DNA sequence identity of each observed allele type was confirmed by cycle sequencing on a Perkin-Elmer model 480 thermocycler with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), using double stranded PCR product (section 2.2.16) purified by High Pure PCR Products Purification Kit (Roche) as template. The cycle sequencing reaction was analysed on a 5% polyacrylamide gel (Long Ranger Singel Pack, BMA) using an ABI Prism 377 sequencer (Applied Biosystems), and analysed by the Sequencing Analysis software (Applied Biosystems).

3.2.2 *Hardy-Weinberg Equilibrium and Linkage Disequilibrium Tests*

The Microsatellite Analyser (MSA) software (version M3.15; Dieringer & Schlötterer 2002) was used to calculate the observed and expected heterozygosity values for all variable loci across the Mangere Island and South-East Island parakeet populations. Allele frequencies at these loci were also calculated using the MSA software, and were compiled with the Appleworks spreadsheet software (version 6; Apple Computer).

Deviation from Hardy-Weinberg equilibrium (HWE) was tested by the Markov chain method (Guo & Thompson 1992) as implemented in GENEPOP (version 3.4; Raymond & Rousset 1995). The programme was run for 300 batches with 1000 interations per batch, and dememorization was set at 1000. Linkage disequilibrium between loci was also tested using the GENEPOP software.

3.2.3 Models of Microsatellite Evolution

Loci were evaluated for fit in the infinite allele model (IAM; Kimura & Crow 1964) versus the one-step mutation model (SMM; Ohta & Kimura 1973) and the two-phase model (TPM; Di Rienzo et al. 1994) of microsatellite evolution based on observed allele size distributions.

A likelihood test (Nielsen 1997) was performed to assess the goodness-of-fit to SMM versus TPM models of evolution. The estimator of variance in allele size θ (= $4N_e\mu$; where N_e is the effective population size, and μ the mutation rate; Valdes et al. 1993), the estimated proportion of multi-step mutations p, and the likelihood value $L(\theta)$ were estimated using a Markov chain recursion method implemented in the MISAT software (Nielsen 1997). The Markov chain was set for 10^5 runs.

The null hypothesis that the loci evolve via strict SMM was tested for each locus by calculation of the likelihood ratio $-2\log\lambda$, following Nielsen & Palsbøll (1999), where

$$\lambda = \frac{\max\ [L(\theta,\ p = 0)]}{\max\ [L(\theta,\ p)]}$$

A critical value of this test statistics can be obtained by comparing $-2\log\lambda$ to the χ^2 distribution with degree of freedom (df) = 1 (Nielsen 1997). In other words, the null hypothesis can be rejected at $P = 0.05$ when $-2\log\lambda$ ≥ 3.84 (χ^2 distribution critical value at df = 1, $P = 0.05$).

At present, the software MISAT does not have the capability to calculate likelihood values for loci with more than one repetitive theme. Therefore, the statistics could not be calculated for *Cfor2829*, which consists of both dinucleotide and tetranucleotide repeat motifs (see Chapter 2, Table 2.1).

3.2.4 Genetic Differentiation Between Mangere and South-East Islands Populations

Six loci, *Cfor0809*, *Cfor1415*, *Cfor1617*, *Cfor2021*, *Cfor2829*, and *Cfor3031* were selected for further analyses (see sections 3.3.1 and 3.3.3). Estimator θ_{ST} (Weir & Cockerham 1984) of F_{ST} (Wright 1951) and the inbreeding coefficient F_{IS} were calculated using the MSA software. The estimator ρ_{ST} (Rousset 1996) of R_{ST} (Slatkin 1995) was calculated using the GENEPOP software.

Differentiation between Mangere Island and South-East Island parakeet populations was also assessed by Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) as implemented in the software GeneticStudio (version 2.01; Dyer & Sork 2001).

3.2.5 Testing for Genetic Bottleneck

In a bottlenecked population, there is a reduction in allelic diversity (allele numbers) and heterozygosity at polymorphic loci. Allelic diversity is reduced faster than heterozygosity in a bottlenecked situation (Cornuet & Luikart 1996). Therefore, assuming mutation drift-equilibrium, recently bottlenecked populations are expected to show observed heterozygosity (H_O) values larger than those expected from the observed number of alleles in the populations (Cornuet & Luikart 1996). The Mangere Island and South-East Island parakeet populations were evaluated for evidence of recent genetic bottlenecks using the software Bottleneck (version 1.2.02; Cornuet & Luikart 1996). This programme tests for significant differences between observed and expected heterozygosities under mutation drift-equilibrium conditions through implementation of three statistical tests for heterozygosity excess, a "sign test", a "standardised differences test", and a "Wilcoxon sign-rank test", each under the mutational models IAM, SMM, and TPM. The statistical power of these

tests was evaluated by Luikart & Cornuet (1998) and Luikart et al. (1998). The "sign test" suffered from low statistical power, and the "standardised differences test" requires 20 or more polymorphic loci. The "Wilcoxon sign-rank test" provides relatively high power with as few as four polymorphic loci, and thus is most appropriate for this study. Because most microsatellite loci mutate in a mode between IAM and SMM, the use of both models for the test was recommended (Luikart & Cornuet 1998). The populations were also tested for recent bottlenecks under the TPM with 5% multi-step changes. The programme was run for 10^6 replications under the mutation models IAM, SMM, and TPM.

3.2.6 Bayesian Clustering and Population Assignment

Assignment tests were performed with the software NewHybrids (version 1.1b3; Anderson & Thompson 2002). Compared with other available assignment methods, NewHybrids is closely related to that of Rannala & Mountain (1997), but differs substantially in that NewHybrids treats all individuals in a population simultaneously rather than on an individual basis. NewHybrids is more similar to the Bayesian method, Structure (Pritchard et al. 2000), for analysing structured populations. The method of Pritchard et al. (2000) focuses on populations with unknown underlying structure, whereas when some basic information in the population structure is available, NewHybrids provides a more detailed analysis using an inheritance model defined by genotype. Assignment tests using fully Bayesian methods (Pritchard et al. 2000) are found to perform better than partial Bayesian exclusion tests (Rannala & Mountain 1997), provided that representative genotypes of the true population origin are sampled (Manel et al. 2002).

Accuracy of NewHybrids in assigning an individual to the correct population was tested using a dataset of two simulated populations generated with the software Easypop (version 1.8; Balloux 2001).

To determine the best strategy, a hypothetical population that contained parental species, hybrids and various backcrosses with known genotypes and identities was constructed, and tested with different settings of the NewHybrids software (see Table 3.4).

The contribution of individual loci to the final assignment outcome was assessed by running the programme with one locus removed at a time using the Mangere Island data set.

The NewHybrids assignment test using genotypic data from the Mangere Island and South-East Island populations was run with six loci with a setting of four genotypic classes (2 parental classes, F_1 and F_2 hybrids; Table 3.1). The run was repeated for a total of 30 times, each with 10^6 sweeps after a burn in period of 10^5 sweeps. Each run required 3.75 hours on a Macintosh computer with a PowerPC 900 MHz G3 processor.

Table 3.1   Genotypic classes assumed for assignment testing using NewHybrids (Anderson & Thompson 2002). The symbols $G_{g,2}$, $G_{g,1}$, and $G_{g,0}$ represent the expected frequencies of loci having 2, 1, or 0 genes originated from species A (Anderson & Thompson 2002).

Genotypic Class	$G_{g,2}$ (A, A)	$G_{g,1}$ (A, B) or (B, A)	$G_{g,0}$ (B, B)
Parent A	1.00	0.00	0.00
Parent B	0.00	0.00	1.00
F_1 Hybrid	0.00	1.00	0.00
F_2 Hybrid	0.25	0.50	0.25

3.2.7 Genetic Distances Between Individuals

A genetic distance measure based on the proportion of shared alleles (Bowcock et al. 1994), which is independent of the models of microsatellite evolution, was used to evaluate the relationship between Forbes' and Chatham Island Red-crowned parakeets through Principal Coordinates Analysis (Gower 1966). A genetic distance matrix was constructed using the MSA software, and was plotted onto two-dimensional space using the software PCO (Anderson 2003).

Because some of the *Cfor* loci evolve closely to strict SMM (Table 3.3) in the Mangere Island and South-East Island parakeet populations, the pattern of individual genetic differences was also investigated using a second genetic distance measure: $(\delta\mu)^2$ (Goldstein et al. 1995), which incorporates features of the SMM. The distance measure $(\delta\mu)^2$ is also found to be appropriate for estimating evolutionary divergent times (Takezaki & Nei 1996) because $(\delta\mu)^2$ has a linear relationship with time. Genetic distance matrices based on the $(\delta\mu)^2$ statistic were calculated using the MSA software with 100 bootstrap replicates. The distance matrices were used to construct 100 neighbour-joining trees (Saitou & Nei 1987) by the Neighbor module in the Phylip software package (version 3.61; Felsenstein 1989). A majority-rule consensus of the 100 trees was obtained by using the Consense module in the package, and the resulting consensus tree was plotted using the Drawtree module.

3.2.8 Correlation Between Genetic and Morphological Variables

The relationship between microsatellite assignment, mitochondrial haplogroups, and crown plumage variations was explored through Multiple Correspondence Analysis (MCA; Tenenhaus & Young 1985) using the MCA module of the ADE-4 software (Thioulouse et al. 1997).

Based on mitochondrial control region studies, Boon et al. (2001) classified Forbes' parakeet into three mtDNA control region sequence haplogroups. Haplogroup 3 represented the pure Forbes' parakeet and haplogroups 1 and 2 represented Chatham Islands Red-crowned parakeet haplogroups present in Forbes' Parakeet morphotypes due to past hybridisations. The Chatham Island Red-crowned parakeets were represented by a separate haplogroup. A PCR-RFLP assay has been developed to distinguish between the three Forbes' parakeet haplogroups and the Chatham Islands Red-crowned parakeet haplogroup (Ballantyne et al. 2004). Haplogroup memebership of samples used in this study was studied by Ballantyne et al. (2004).

The morphology of parakeets was scored by crown plumage (Hilary Aikman, NZ Department of Conservation, pers. comm.) using a five point scale based on Nixon (1982). A clear Forbes' parakeet morphotype was scored as MP1 on the scale and a Chatham Island Red-crowned parakeet morphotype was scored MP5 on the scale. Hybrids were scored on the scale MP2 (fewer red feathers on crown) to MP4 (more red feathers on crown).

3.3 Results

3.3.1 Locus Characteristics of Cfor Loci in Mangere and South-East Islands Populations

DNA extracted from samples from the parakeet populations on Mangere Island and South-East Island was screened at the 9 optimised loci. Individual genotype profiles of the 8 polymorphic loci were tabulated (Appendix 3.1), and allele frequencies were calculated (Figure 3.1, Table 3.2).

Figure 3.1   Allele frequency distributions of *Cfor* loci in two populations of parakeets. Black solid bars represent the Mangere Island population (250 individuals), Grey shaded bars represent the South-East Island population (35 individuals), and the numbers above bars represent the number of times an allele was observed.
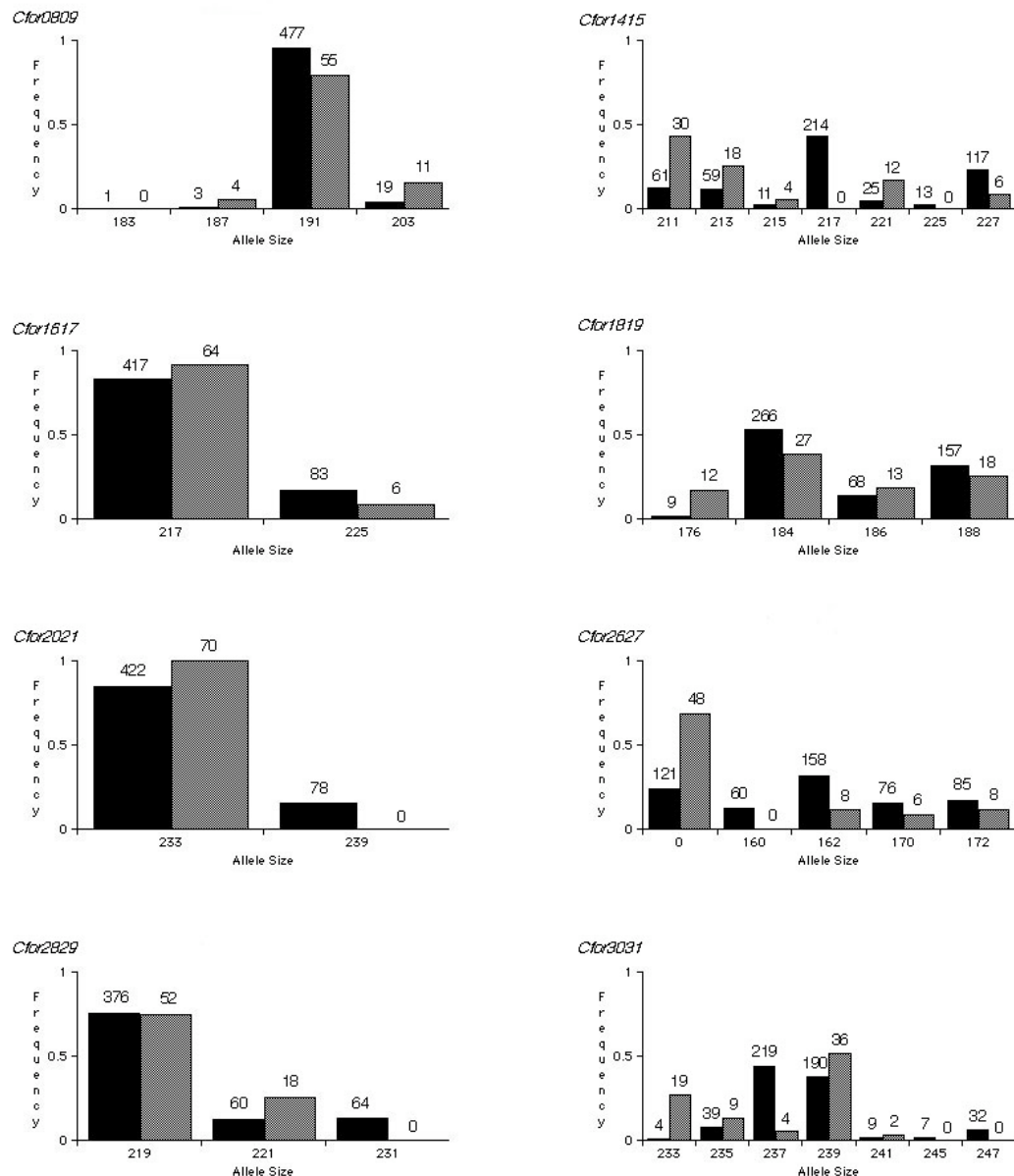
Eight loci (*Cfor0809*, *Cfor1415*, *Cfor1617*, *Cfor1819*, *Cfor2021*, *Cfor2627*, *Cfor2829*, and *Cfor3031*) were found to be polymorphic while one locus

(*Cfor2223*) was monomorphic (allele size = 217 bp) across all of the samples tested. *Cfor2021* was fixed for the 233 bp allele in the South-East Island population.

3.3.2 Hardy-Weinberg Equilibrium and Linkage Disequilibrium

Observed and expected heterozygosities were calculated and Hardy-Weinberg equilibrium (HWE) was tested across all the polymorphic loci (Table 3.2). In the Mangere Island population, *Cfor0809*, *Cfor2021* and *Cfor2829* were in HWE while *Cfor1415*, *Cfor1617*, *Cfor1819*, *Cfor2627* and *Cfor3031* showed significant deviations ($P < 0.05$). In the South-East Island population, all loci except *Cfor1819* and *Cfor2627* conformed to expectation under HWE. In the Mangere Island population, *Cfor1617*, *Cfor1819*, *Cfor2627* and *Cfor3031* showed significant heterozygote deficiencies ($P = 0.00$ for all 4 loci). In the South-East Island population, only *Cfor1819* and *Cfor2627* showed significant heterozygote deficiencies ($P = 0.00$ for both loci).

Evidence for apparent linkage disequilibrium was found between *Cfor0809* and *Cfor2829*, and between *Cfor1415* and *Cfor2829* in the Mangere Island population after sequential Bonferroni correction (Rice 1989). However, tests for linkage disequilibrium in other populations suggested the observed linkage disequilibrium in the Mangere Island population may be an artefact caused by the underlying mixed population structure (see section 3.4.3).

It is worth noting that the mixed species nature and overlapping generations in the population samples may affect the reliability of HWE and linkage disequilibrium tests.

3.3.3 Size Homoplasy and Null alleles

Sequencing of 184 bp alleles of *Cfor1819* revealed size homoplasy at this locus. Two motifs, $(CA)_9$ and $(CA)_5C_3(CA)_2C$, (contributing respectively 33% and 67% of the 54 sequenced 184 bp alleles) were observed. The 186 bp allele at this locus had a repeat motif of $(CA)_{10}$, and the 188 bp allele had a repeat motif of $(CA)_4C(CA)_2C_9$. Size homoplasy at this locus has apparently increased the observed frequency of the 184 bp allele.

Table 3.2   Loci characteristics and Hardy-Weinberg equilibrium. The number of alleles observed is represented by *n*; H_O and H_E represent observed and expected heterozygosities respectively. In locus *Cfor2627*, the frequencies of the null allele, indicated by asterisks (*), represent the observed frequencies in homozygotes.

Locus	Population	*n*	Allele Sizes (bp)	Allele Frequency	H_O	H_E	HWE (*P*)	HWE (Std.er.)
Cfor0809	Mangere	4	181	0.002	0.0840	0.0886	0.4079	0.0080
			187	0.006				
			191	0.954				
			203	0.038				
	South-East	3	187	0.057	0.3714	0.3598	1.0000	0.0000
			191	0.786				
			203	0.157				
Cfor1415	Mangere	7	211	0.122	0.7560	0.7311	0.0000	0.0000
			213	0.118				
			215	0.022				
			217	0.428				
			221	0.050				
			225	0.026				
			227	0.234				
	South-East	5	211	0.429	0.7429	0.7205	0.5761	0.0050
			213	0.257				
			215	0.057				
			221	0.171				
			227	0.086				
Cfor1617	Mangere	2	217	0.834	0.1000	0.2774	0.0000	0.0000
			225	0.166				
	South-East	2	217	0.914	0.1714	0.1590	1.0000	0.0000
			225	0.086				

Cfor1819	Mangere	4	176	0.018	0.4520	0.6008	0.0000	0.0000
			184	0.532				
			186	0.136				
			188	0.314				
	South-East	4	176	0.171	0.4000	0.7317	0.0000	0.0000
			184	0.386				
			186	0.186				
			188	0.257				
Cfor2021	Mangere	2	233	0.844	0.2640	0.2639	1.0000	0.0000
			239	0.156				
	South-East	1	233	1.000	0.0000	0.0000	-	-
Cfor2627	Mangere	5	0	0.242*	0.3480	0.7923	0.0000	0.0000
			160	0.120				
			162	0.316				
			170	0.152				
			172	0.170				
	South-East	4	0	0.686*	0.2286	0.7354	0.0000	0.0000
			162	0.114				
			170	0.086				
			172	0.114				
Cfor2829	Mangere	3	219	0.752	0.3840	0.4045	0.0568	0.0020
			221	0.120				
			231	0.128				
	South-East	2	219	0.743	0.4571	0.3876	0.3939	0.0014
			221	0.257				
Cfor3031	Mangere	7	233	0.008	0.4120	0.6543	0.0000	0.0000
			235	0.078				
			237	0.438				
			239	0.380				
			241	0.018				
			245	0.014				
			247	0.064				
	South-East	5	233	0.271	0.5429	0.6505	0.1569	0.0045
			235	0.129				
			237	0.057				
			239	0.514				
			241	0.029				

Homozygotes for a null allele at *Cfor2627* were observed in both populations. The null allele occurred more frequently in the South-East Island population (allele frequency = 0.686) than in the Mangere population (allele frequency = 0.242). Presence of null alleles generates apparently significant shifts in HWE (Pemberton et al. 1995), creating a bias towards increased proportions of homozygotes. The loci *Cfor1819* and *Cfor2627* were excluded from further population analyses.

3.3.4 Mutation Behaviour of Cfor *Loci*

The proportion of multi-step mutations p, and the likelihood ratio $-2\log\lambda$ (Nielsen & Palsbøll 1999) was estimated for loci *Cfor0809*, *Cfor1415*, *Cfor1617*, *Cfor2021*, and *Cfor3031* (Table 3.3). Because size homoplasy and the presence of null alleles respectively at loci *Cfor1819* and *Cfor2627* compromise estimation of p, the calculations were not run for these two loci. Locus *Cfor2829* was also excluded form the analysis because it is a complex microsatellite locus made up of di- and tetra-nucleotide repeat units. In this case, the present design of the software MISAT will assume a tetranucleotide repeat as jumps of two dinucleotide repeats, thus causing biases towards increase in the number of multi-step mutations.

The proportion of multi-step mutations at maximum likelihood of θ, calculated by MISAT using the method described in Nielsen (1997), ranged from 0.000 (in *Cfor3031*) to 0.475 (in *Cfor0809* and *Cfor1617*). For *Cfor0809* and *Cfor1415*, the SMM was rejected in the Mangere Island population, but the model could not be rejected in the South-East Island population at the statistical confidence level of $P = 0.05$. The discrepancies between the two populations may be caused by the mixed population structure on Mangere Island, or the incomplete sampling of alleles due to the small sample size from South-East Island. In locus *Cfor1617*, the proportion of multi-step mutations (p) at maximum likelihood of θ is 0.475, but the SMM could not be rejected at this locus because there are only two observed alleles, and the differences between the 217 bp and 225 bp alleles could be accounted for by either two steps of tetranucleotide jumps, or one 8 nucleotide mutation. Analyses revealed that *Cfor2021* mutates by TPM and *Cfor3031* mutates by SMM. The mutational behaviour of microsatellites will be discussed in more detail in the next chapter.

Table 3.3　　Mutation behaviour of *Cfor* loci.　*p* indicates the proportion of multi-step mutations at max $L(\theta)$.

Locus	Population	*p*	-2logλ	Mutation Model
Cfor0809	Mangere	0.475	7.50	TPM
	South-East	0.400	1.26	SMM
Cfor1415	Mangere	0.025	14.68	TPM
	South-East	0.025	0.21	SMM
Cfor1617	Mangere	0.475	0.99	SMM
	South-East	0.475	0.29	SMM
Cfor2021	Mangere	0.475	3.84	TPM
	South-East	-	-	-
Cfor3031	Mangere	0.000	0.00	SMM
	South-East	0.000	0.00	SMM

3.3.5　Genetic Differentiation Between Populations and Bottlenecks

F_{ST} between the Mangere and South-East Islands parakeet population was 0.1225 ($P = 0.0001$), and R_{ST} was 0.2014.　The higher R_{ST} value suggested that the two populations differed slightly more in distribution of allele size than allele frequency (Slatkin 1995).　The variance between the two populations was 0.175 ($P = 0.01$; AMOVA).　The inbreeding coefficient F_{IS} was 0.1534, indicating a moderate level of inbreeding in the populations studied.

Results of the bottleneck tests showed that the one tailed "Wilcoxon sign-rank test" for heterozygosity excess in the Mangere Island parakeet population was not significant under the IAM ($P = 0.22$), SMM ($P = 0.78$), or TPM ($P = 0.58$) models.　In the South-East Island parakeet population, the test was also not significant under the IAM ($P = 0.05$), SMM ($P = 0.50$), or TPM ($P = 0.41$) models.　The results suggested the absence of a detectable genetic bottleneck in both populations.

3.3.6 Bayesian Assignment of Individuals using Microsatellites

Testing of the NewHybrids software with an Easypop simulated data set of two populations and 285 individuals showed 284 (99.6%) of the samples were assigned to the correct population with a posterior probability ≥ 0.95. The remaining individual assigned to the correct population with $P = 0.85$. None of the samples was misassigned. When tested with a hypothetical population consisting of parental types, hybrids and various backcrosses, all parental type individuals were assigned a posterior probability of ≥ 0.95 to their respective classes (Table 3.4). While all hybrids had posterior probabilities < 0.90 in the parental classes, some backcrosses could be assigned probabilities between 0.88 to 0.91 to the parental classes. Figure 3.2 shows the effects to the final assignment score of 6 chosen parakeet samples by removal of one locus in the NewHybrids assignment test. The assignment of some individuals remained the same regardless of the removal of any locus. However, the removal of *Cfor1617* and *Cfor3031* caused a shift in assignment in some individuals. It appears that the assignment methodology relies more heavily on loci that show large differences in genotypes between the two parental types. Increasing the number of loci used in assignments would certainly help in increasing the accuracy and power, but this would be difficult in parrots as it appears difficult to isolate a larger number of microsatellite loci from parrot species (Hughes et al. 1998; Robertson et al. 2000; Russello et al. 2001; Caparroz et al. 2003; Sainsbury et al. 2004).

Parakeets in the Mangere Island and South-East Island populations were scored according to their posterior probabilities in either parental classes (Appendix 3.2; Table 3.5). Birds with P(Forbes') ≥ 0.95 were given a score of MS1 (Forbes' parakeet; 67 birds, 23.5%), those with P(Red-crowned) ≥ 0.95 were given a score of MS3 (Red-crowned parakeet; 25 birds, 8.8%), all other birds which did not fit either criteria were assigned a score of MS2 (hybrids or unassigned; 193 birds, 67.7%). At present, the

programme does not have the ability to distinguish unassigned birds from hybrids. The Mangere Island parakeet population contained birds of all three types (67 Forbes' parakeets; 171 hybrids; 12 Chatham Island Red-crowned parakeets). The South-East Island population was solely made up of Chatham Island Red-crowned parakeets and hybrids (13 Chatham Island Red-crowned parakeets; 22 hybrids).

The assignment results based on multi-locus microsatellite genotypes were compared to data from mitochondrial control region and morphological studies (Appendix 3.2).

Table 3.4   Posterior probabilities of NewHybrids assignment in a hypothetical population of 20 individuals consisting of parents A and B, F_1 and F_2 hybrids, F_1 x parent A and F_1 x B backcrosses, with genotypic classes described in Table 3.1. P refers to the probability of being assigned into a genotypic class.

Genotypic Class	P(A)	P(B)	P(F_1)	P(F_2)
Parent A	0.9970	0.0000	0.0002	0.0028
Parent A	0.9965	0.0000	0.0002	0.0033
Parent A	0.9976	0.0000	0.0001	0.0023
Parent B	0.0000	0.9979	0.0001	0.0020
Parent B	0.0000	0.9988	0.0001	0.0011
Parent B	0.0000	0.9964	0.0002	0.0034
F_1 Hybrid	0.0001	0.0147	0.9048	0.0804
F_1 Hybrid	0.0047	0.0005	0.9230	0.0718
F_2 Hybrid	0.0039	0.8953	0.0041	0.0967
F_2 Hybrid	0.0005	0.0004	0.8850	0.1141
F_2 Hybrid	0.8005	0.0033	0.0080	0.1882
F_2 Hybrid	0.0049	0.7415	0.0913	0.1623
F_2 Hybrid	0.0017	0.0000	0.7992	0.1991
F_2 Hybrid	0.8595	0.0039	0.0133	0.1233
F_1 x A Backcross	0.0386	0.0007	0.7131	0.2476
F_1 x A Backcross	0.8950	0.0000	0.0225	0.0825
F_1 x A Backcross	0.0172	0.3741	0.4600	0.1487
F_1 x B Backcross	0.0000	0.8812	0.0388	0.8000
F_1 x B Backcross	0.0003	0.0056	0.8849	0.1092
F_1 x B Backcross	0.0017	0.9148	0.0033	0.0802

Figure 3.2 Contribution of individual locus to the final Bayesian assignment probabilities in 6 chosen samples. P(FB) refers to the probability of assignment as a pure Forbes' parakeet.
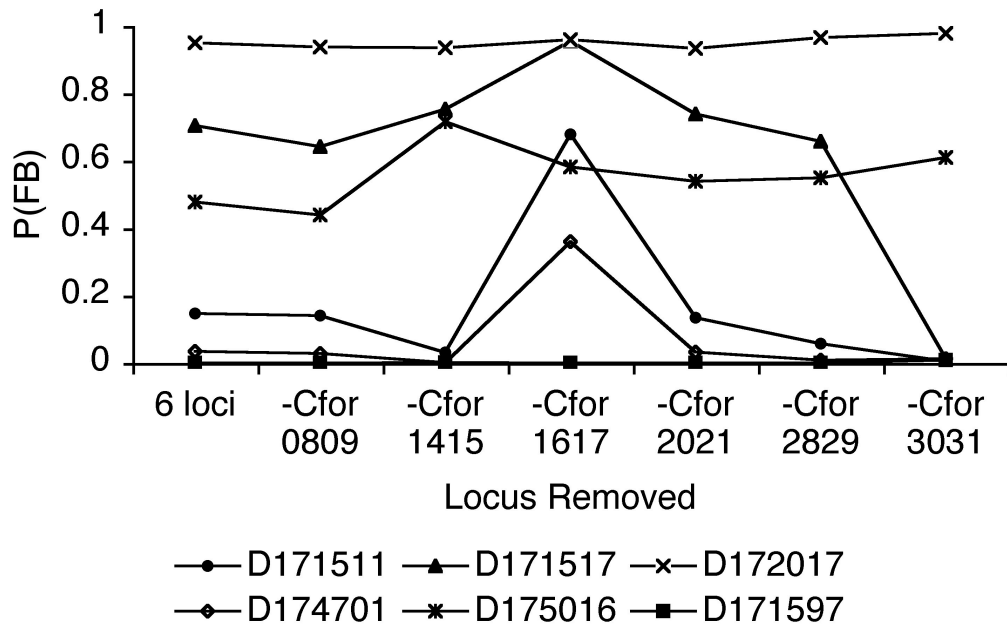
The pure Forbes' parakeets could be defined as birds with microsatellite (NewHybrids) assignment score MS1, mitochondrial haplogroup MT3, and morphological score MP1. Birds with microsatellite assignment score MS3, mitochondrial haplogroup MT4, and morphological score MP5 were classified as Chatham Island Red-crowned parakeets. All other birds were assigned as hybrids.

Among the 285 samples screened with microsatellites in this study, 203 (169 from Mangere Island, and 34 from South-East Island) also had data available on both mitochondrial haplotypes and morphological groups. On Mangere Island, pure Forbes' parakeets made up 30 (17.8%) of the 169 birds, 2 (1.2%) were pure Chatham Island Red-crowned parakeets and

137 (81.0%) were hybrids or unassigned birds. On South-East Island, 12 (35.3%) of the 34 birds were pure Chatham Island Red-crowned parakeets, and 22 (64.7%) were hybrids or unassigned birds.

Table 3.5   Classification of parakeets using genotypic and phenotypic markers.

Type of Marker	Classes	Description
Microsatellites	MS1	Pure Forbes' parakeet
	MS2	Hybrids or unassigned
	MS3	Pure Chatham Island Red-crowned parakeet
Mitochondrial DNA	MT1	Hybrid (haplogroup 1)
	MT2	Hybrid (haplogroup 2)
	MT3	Pure Forbes' parakeet
	MT4	Pure Chatham Island Red-crowned parakeet
Crown Plumage	MP1	Pure Forbes' parakeet
	MP2	Hybrids (appearance like Forbes' parakeet)
	MP3	Hybrids
	MP4	Hybrids (appearance like Chatham Island Red-crowned parakeet)
	MP5	Pure Chatham Island Red-crowned parakeet

3.3.7 Genetic Distances Analysis

Principal Coordinate Analysis of a 203 x 203 distance matrix based on the proportion of shared microsatellite alleles allowed graphical comparison between microsatellite assignment and assignment based on both genetic and morphological variables (Figure 3.3). Axis PCO 1 represented 31.5% of the total variation, while axis PCO 2 represented 21.1% of the variation. Cumulatively, 52.6% of the total variability is represented by the two axes. While Chatham Island Red-crowned parakeets cluster as a group to the right of the plot, Forbes' parakeets are more spread out. A large swarm of hybrid or unresolved individuals was scattered mainly towards the Forbes' parakeet side and the centre of the plot. This suggested that a number of birds that were similar to Forbes' parakeet (in terms of microsatellite alleles frequencies) had a hybridisation history detectable by mitochondrial haplotypes or crown plumage.

Figure 3.3   Principal Coordinate Analysis of genetic distance based on proportion of shared microsatellite alleles. A cumulative 52.6% of total variation was represented by the two PCO axes. Birds were separated into three categories (Forbes' parakeets, Hybrids, and Red-crowned parakeets) based on assignment using genetic and morphological data.

● Forbes' Parakeet ▲ Hybrids ✗ Red-crowned Parakeet
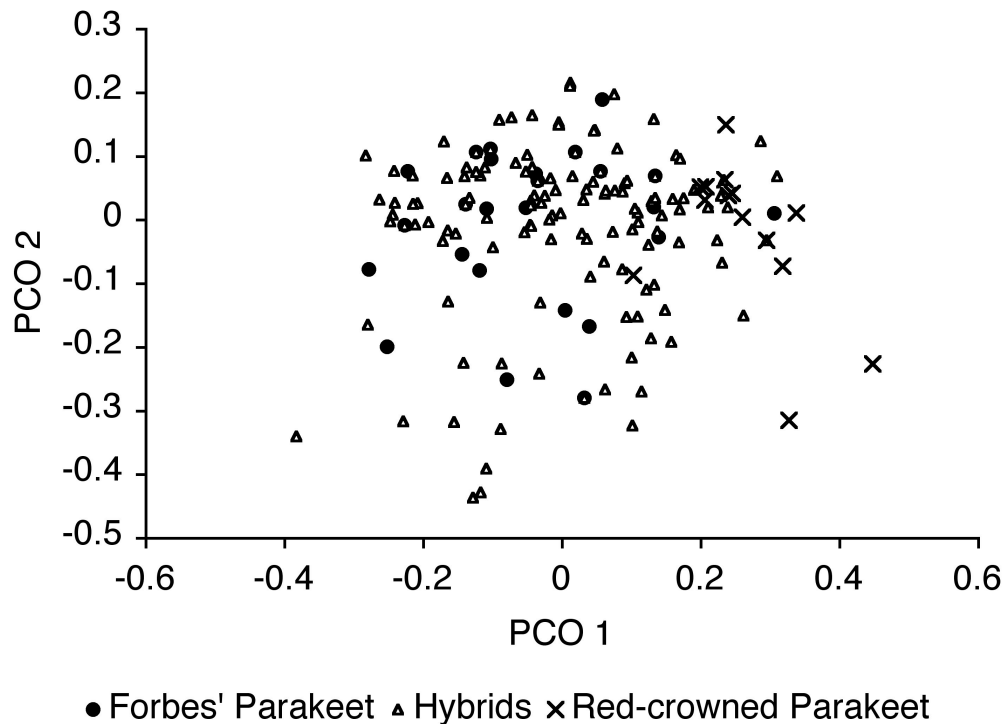
A neighbour-joining consensus tree constructed from 285 x 285 $(\delta\mu)^2$ matrices of genetic distance between individuals showed that the majority of birds were clustered into a group that originated from about the same point (Figure 3.4). A clear branch was observed which contained mostly Forbes' parakeets (classified using the three available genetic and morphological variables), some unknown Mangere Island parakeets (with mtDNA and / or morphological data missing), and three hybrids or unresolved birds. The pattern of the tree suggested birds which are classified as Forbes' parakeets can be a very variable group genetically, with some individuals very close to Chatham Island Red-crowned

parakeets and hybrids, while some individuals are more distantly related to all other birds.

Figure 3.4   Neighbour-joining tree built from $(\delta\mu)^2$ genetic distances with 100 bootstraps. F, R, and H represent respectively pure Forbes' parakeet, pure Chatham Island Red-crowned parakeet, and hybrids or unresolved birds; M and S respectively represent Mangere Island and South-East Island parakeets with no mitochondrial DNA and / or morphological data available.
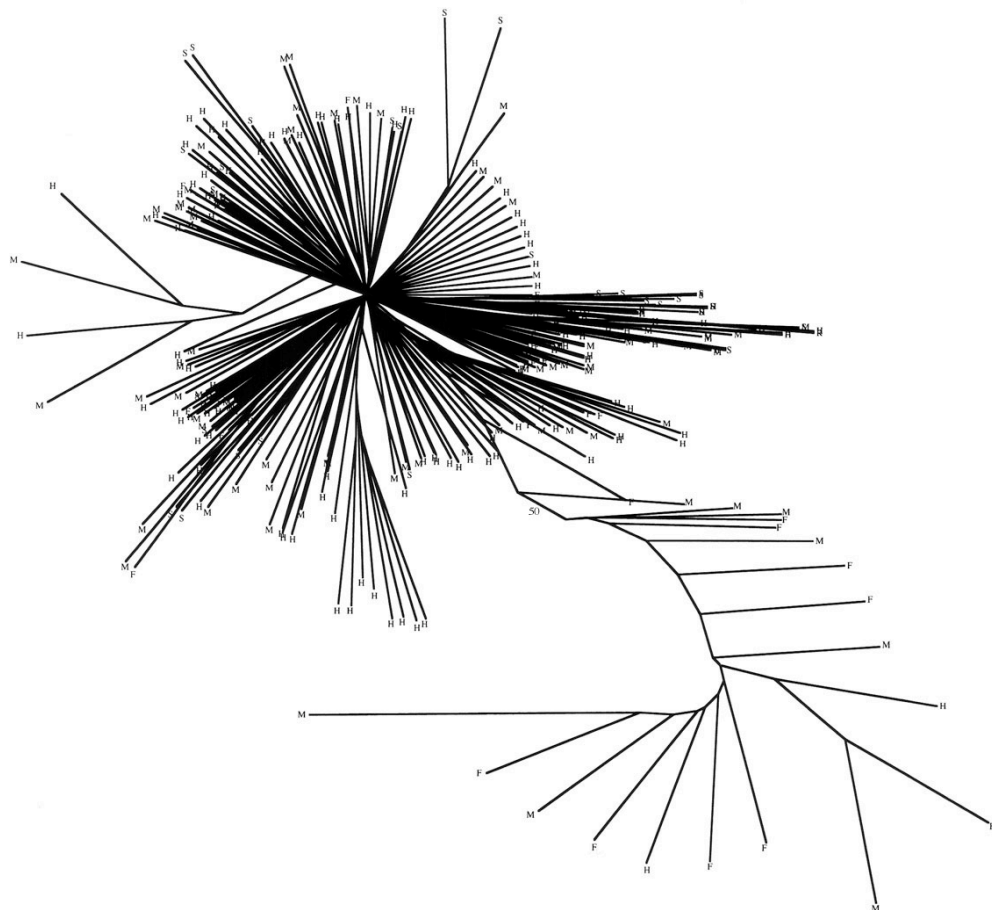
The data also showed discrepancies between Bayesian and distance-based methods for assignment. However, results from both methods

suggested there had been extensive hybridisation in the Mangere Island parakeet population.

3.3.8 Relationships Between Genetic and Morphological Variables

Looking at the 203 samples for which there are data available of all three variables, pairwise comparisons of the variables were made in Table 3.6.

Comparing between microsatellite assignments (MS) and mitochondrial DNA control region haplogroups (MT; Table 3.6a), birds assigned MS1 were largely MT3 individuals. While MS2 birds could have quite variable MT assignments, MS3 birds were mainly MT4 individuals. This indicated that hybridisations that took place in the past have led to extensive genetic mixing between the two species. The existence of MS1 + MT3 individuals suggested that some genetically pure or near-pure Forbes' parakeets still remain in the Mangere Island population. Because the MS2 category consist of individuals which were either hybrids or individuals which were unable to be assigned in the NewHybrid assignment test, it is difficult to precisely determine the relationship between this class of birds and other variables.

Between microsatellite assignment (MS) and crown morphology (MP; Table 3.6b), it is clear that MS1 birds are largely MP1 (with clear Forbes' parakeet crown), and MS3 birds are mostly MP5 (red-crowned). The majority of MS2 birds also have clear Forbes' parakeet crown morphology, indicating the presence of a large number of cryptic hybrids which look like Forbes' parakeets.

Less clear relationships were observed between mitochondrial DNA (MT) and crown morphology (MP; Table 3.6c). MP5 (red-crowned) birds belong to either MT2 or MT4, while most MP1 (Forbes' parakeet crown) birds are

MT3. Existence of 9 birds with MP1 and MT4 clearly showed hybridisation has taken place between female Chatham Island Red-crowned parakeets and male Forbes' parakeets. The presence of a number of birds with MT3 lineage and MP3 or MP4 crown morphology suggests male Chatham Island Red-crowned parakeets also hybridise with female Forbes' parakeets.

Table 3.6   Relationship between variables analysed by number of individuals in each category (indicated by numbers in brackets).   a) microsatellites versus mitochondrial haplotypes; b) microsatellites versus morphology; c) mitochondrial haplotypes versus morphology.

a) Microsatellites	Mitochondrial haplotypes			
	MT1 (21)	MT2 (22)	MT3 (112)	MT4 (48)
MS1 (43)	3	4	32	4
MS2 (140)	16	16	78	30
MS3 (20)	2	2	2	14

b) Microsatellites	Morphology scores				
	MP1 (136)	MP2 (10)	MP3 (11)	MP4 (9)	MP5 (37)
MS1 (43)	38	2	3	0	0
MS2 (140)	97	8	7	6	22
MS3 (20)	1	0	1	3	15

c) Mitochondrial haplotypes	Morphology scores				
	MP1 (136)	MP2 (10)	MP3 (11)	MP4 (9)	MP5 (37)
MT1 (21)	20	0	0	1	0
MT2 (22)	11	2	2	2	5
MT3 (112)	96	8	7	1	0
MT4 (48)	9	0	2	5	32

Comparing crown morphology with the combined genetic data of microsatellites and mitochondrial DNA (Table 3.7), it is clear that the majority of genetic Forbes' parakeets have a clear Forbes' parakeet morphology (MP1). A hybrid, on the other hand, can display variable crown morphology which can be very similar to, or intermediate between, the parent species.

Correlations among the three variables were also analysed with Multiple Correspondence Analysis (Figure 3.5). The first two axes of MCA represented a cumulative 37.1% of the variation between the three variables. The x-axis sampled 24.1% of the total variation between the three variables, and the y-axis sampled 13.0%. The scatter plot showed correlation between Red-crowned parameters (microsatellites class MS3, mitochondrial haplogroup MT4, morphological scales MP4 and MP5), as well as close associations between Forbes' parakeet genetic characters MS1 and MT3. However, Forbes' parakeet crown plumage MP1 is almost equally distant from MS1 and MS2. The results suggest that crown plumage is a reasonably good indicator of "Red-crownedness", but birds with Forbes' parakeet crown patterns are as likely to be hybrids as to be pure Forbes' parakeets.

Table 3.7   Relationship between genotype and crown morphology. The number of individuals in each category is indicated by numbers in brackets. a) Mangere Island population; b) South-East Island population.

a) Genotype	Morphology scores				
	MP1 (136)	MP2 (10)	MP3 (11)	MP4 (9)	MP5 (3)
Forbes' (MS1 + MT3; 33)	30	1	2	0	0
Red-crowned (MS3 + MT4; 2)	0	0	0	0	2
Hybrids (all others; 134)	106	9	9	9	1

b) Genotype	Morphology scores				
	MP1 (0)	MP2 (0)	MP3 (0)	MP4 (0)	MP5 (34)
Forbes' (MS1 + MT3; 0)	0	0	0	0	0
Red-crowned (MS3 + MT4; 12)	0	0	0	0	12
Hybrids (all others; 22)	0	0	0	0	22

Figure 3.5 Multiple Correspondence Analysis of genetic and morphological variables. The ellipses, from the centre, represented 50, 70, 90, and 99% of samples. MS, MT and MP abbreviated respectively for microsatellites, mitochondrial control region, and morphology. The number after the marker types corresponded to the classification using that marker (Table 3.5).
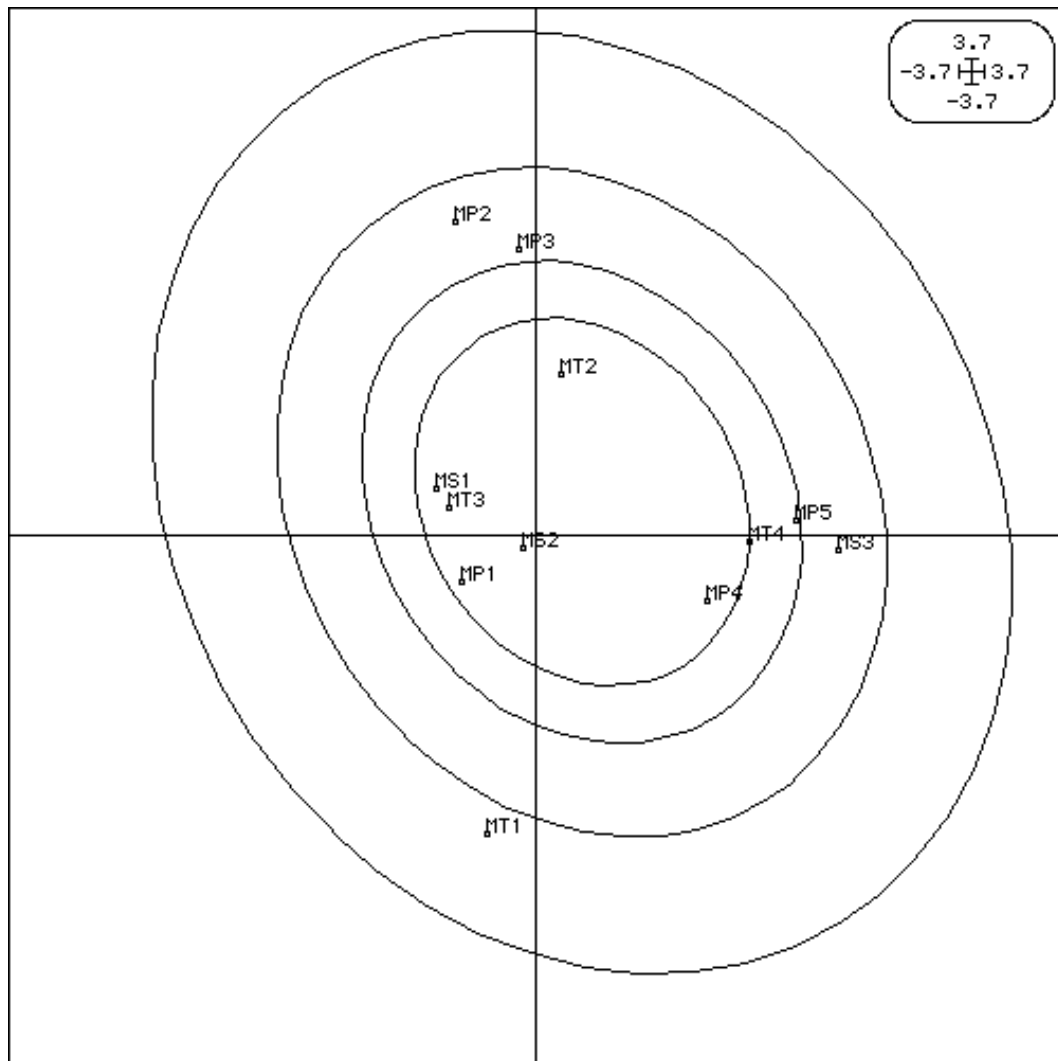
3.4   Discussion

3.4.1 Identification of "Pure" Forbes' Parakeets

Using microsatellites and mitochondrial DNA genetic markers, a large proportion (77.9%) of phenotypic Forbes' parakeets were found to be

cryptic hybrids. To supplement the previous identification scheme based solely on morphological features, a new system has been developed in this study using nuclear and mitochondrial genetic markers and crown morphology to identify "pure" or near-"pure" Forbes' parakeets. A "pure" Forbes' parakeet needs to satisfy three criteria: 1) being assigned as Forbes' parakeet using microsatellite markers in the NewHybrids assignment (MS1); 2) having mitochondrial control region haplotype 3 (MT3; Boon et al. 2001); and 3) showing a clear Forbes' parakeet crown morphology (MP1; Nixon 1982). Any bird that fails one of these tests is likely to be the progeny of hybridisation between Chatham Island Red-crowned parakeet or other hybrids or backcrosses.

Analyses of microsatellite data with both Bayesian and genetic distance methods have shown extensive interspecific hybridisation in the Mangere Island parakeet population. Calculations of F_{ST} and R_{ST} showed that there was a clear genetic differentiation in the Mangere and South-East Islands populations, and suggested differences in genetic makeup of these two populations.

F_{ST} and R_{ST} often differ substantially, and there is no clear consensus over their relative accuracy (Balloux & Lugon-Moulin 2002). Under a strict stepwise mutation model, no statistic was found to be best overall, and the level of differentiation and gene flow determines whether F_{ST} or R_{ST} performed better (Balloux & Goudet 2002). Although some studies comparing microsatellites with other markers did not detect strong discrepancies in estimating population genetic structure (Barker et al. 1997; Estoup et al. 1998; Lynch et al. 1999; Ross et al. 1999) there were also possibilities of underestimating divergence between populations when highly polymorphic genetic markers, such as microsatellites, were used in the analysis (Nauta & Weissing 1996; Hedrick 1999; Balloux et al. 2000). Despite the issues raised concerning the accuracy of assessing population

differentiation using microsatellites, F_{ST} remains a reasonable basic descriptor of population structure (Neigel 2002; Pearse & Crandall 2004).

3.4.2 Interspecific Hybridisation in the Mangere Island Parakeet Population

Cyanoramphus is a relatively recently evolved group (Boon et al. 2001), and the possible lack of reproductive isolation between its taxa promotes the possibility of hybridisation (Bigelow 1965; Prager & Wilson 1975; Grant & Grant 1992). Little is known about the genetic basis of post-mating isolation mechanisms in birds, such as factors that affect viability and fertility of hybrid embryos (Grant & Grant 1997). Information about pre-mating barriers, such as morphological and behavioural differences between the two species of parakeets, is also very limited. The abundance of hybrids seen here suggests that pre-mating isolation may be non-existant between the two parakeet species.

The existence of "pure" and near-"pure" Forbes' parakeet types in the population suggests that hybridisation in the past has not driven Forbes' parakeets to complete extinction. Instead, hybridisation may have helped the Mangere Island population survive a severe bottleneck in the past, possibly induced by habitat clearance. However, the presence of a large number of individuals with intermediate genotypes indicated that hybridisation is acting to gradually bring the genetics of the parent species to an intermediate form, and the genetic introgression from Chatham Island Red-crowned parakeets may cause a slow and protracted extinction of Forbes' parakeets.

3.4.3 Characteristics of Cfor Loci

Linkage disequilibrium was detected between *Cfor0809* and *Cfor2829*, and between *Cfor1415* and *Cfor2829* in the Mangere Island parakeet population. However, linkage disequilibrium was not detected in South-East Island samples, mainland Red-crowned parakeets (*C. novaezelandiae novaezelandiae*) or Yellow-crowned parakeets (*C. auriceps*; see Chapter 5). Therefore, it is unlikely that these loci are actually physically linked on the same chromosomes. The linkage disequilibrium observed in the Mangere Island samples may be caused by the mixed population structure. It is also worth noting that the power to detect linkage disequilibria is correlated with heterozygosity, and greater heterozygosity generally results in greater power in the estimation (Ott & Rabinowitz 1997). The relatively low heterozygosities of loci *Cfor0809* and *Cfor2829* in the Mangere Island population (see Table 3.2) suggest a limited power in the estimation of linkage disequilibria involving these loci.

A large proportion of birds from the South-East Island population were found to be homozygotes for a non-amplifying allele, or null allele, at locus *Cfor2627*. The occurrence of null alleles may be explained by a large deletion in the locus, or small regions of deletion or nucleotide substitution mutations at the primer annealing sites (Callen et al. 1993). Two sets of primers were designed for *Cfor2627*. In addition to primers CS126 and CS127 (Table 2.1), primers CS026 (5' AGTTCTTTCCCTGCTGCTAC 3') and CS027 (5' ATGTGCCTGGTGATTTTTGA 3') were designed which were 23 bases and 16 bases towards the 3'-end of CS126 and CS127 respectively (see Chapter 2, Appendix 2.1). In birds which primers CS126 and CS127 failed to amplify the locus *Cfor2627*, amplifications with primers CS026 and CS027, CS026 and CS127, CS126 and CS027 also failed. All samples which failed to amplify at locus *Cfor2627* produced PCR products at all other loci (Appendix 3.1), suggesting that errors in the PCR, such as insufficient starting DNA template, were unlikely. The null

allele at *Cfor2627* was mostly likely to be caused by a large mutation or deletion at the flanking region of the microsatellite, perhaps even deletion of the entire locus. Null alleles could be a tool to investigate the evolutionary constraints on microsatellite loci if alternative primers were available to amplify the allele, and the cause of the non-amplification could be deduced (Lehmann et al. 1996). However, attempts in obtaining sequences from *Cfor2627* null alleles have so far been unsuccessful.

Because detection of null alleles in heterozygotes is difficult, loci with null alleles generally show an artefactually higher than expected number of homozygotes. Null alleles can create confusions in population studies, especially in linkage or parentage work (Koorey et al. 1993; Pemberton et al. 1995), thus these loci were not included in further population analyses in this study. A software package, Microchecker, has been developed to estimate the frequency of null alleles and subsequently adjusts allele genotype frequencies of the amplified alleles, permitting their use in some further population analyses (van Oosterhout et al. 2004). However, since this study involved assignment of individuals based on their allelic profiles, such adjustments of allele genotypes may be unsuitable, and the conservative approach of not including the *Cfor2627* locus was employed.

Size homoplasy (reviewed in Estoup et al. 2002) refers to microsatellite alleles that are identical in state (i.e. those having identical size), but may not be identical by decent. Homoplasy is a commonly observed phenomenon in molecular evolution, occuring in both allozymes (Coyne et al. 1979) and DNA sequences (Hassanin et al. 1998), and could be widespread at microsatellite loci (for example, van Oppen et al. 2000; Culver et al. 2001; Chambers & MacAvoy 2004). Size homoplasy in microsatellite data causes reduced observed number of alleles, heterozygosities and gene diversity, and thus could lead to larger than expected numbers of homozygotes in genetic analyses.

3.4.4 Accuracy of Assignment Test

The power and resolution of assignment tests are dependent on the methods used and the number of loci included in the analysis. The relationship between the power to identify different hybrid classes and the number of loci used has been modelled for molecular markers, such as restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), and protein electrophoresis (Boecklen & Howard 1997). These authors suggested as few as four or five markers should be sufficient to classify individuals into parental, F_1 hybrids, and simple backcross categories. However, to distinguish more complex classes of backcrosses, upwards of 50 markers would be required (Floate et al. 1994; Boecklen & Howard 1997). Using 6 loci, the probability of misclassifying a first generation backcross as a parental species was 0.03, but the probability of misassigning a fifth generation backcross as a parent species could be as high as 0.83 (Boecklen & Howard 1997). The model also suggested an individual's genotype and allele frequencies would be very similar to the parent species after generations of backcrossing.

Using simulation methods, Cornuet et al. (1999) showed that the mutational process of microsatellites played an important role in determining the efficiency of assignment methods. In a study comparing highly variable microsatellite markers with moderately variable allozymes (Estoup et al. 1998), higher assignment scores were obtained using more variable microsatellite markers.

Bayesian and genetic distance based assignment tests are the most commonly used methods for assigning individuals to populations using microsatellite genotypic data. The accuracy of the two methods varies with genetic differentiation between populations (Cornuet et al. 1999). With 6 loci and at $F_{ST} = 0.1$, a Bayesian method can result in 70% (Cornuet et al. 1999) to 97% (Berry et al. 2004) correct assignment. In comparison,

genetic distance-based methods using shared allele distance (Bowcock et al. 1994) and $(\delta\mu)^2$ distance (Goldstein et al. 1995) would give 65% and 25% accurate assignment respectively (Cornuet et al. 1999). Discrepancies between Bayesian and distance-based methods could explain the observation that some Forbes' parakeets (assigned by the Bayesian method, mitochondrial control region, and crown plumage) appear in the Chatham Island Red-crowned parakeet cluster in Figures 3.3 and 3.4. On the other hand, existence of a large number of hybrids in the analyses using distance-based methods (Figures 3.3 and 3.4) suggested that a large proportion of MS2 (hybrids or unresolved) category parakeets in the NewHybrids assignment are actual hybrids. A survey by Choisy et al. (2004) of various assignment methods (gene identities, least square regression, private alleles, maximum likelihood, coalescence times, and Monte Carlo Markov chain) showed that, while all methods performed well in populations with recent hybridisation and high differentiation between parental populations, a Monte Carlo Markov chain method (such as NewHybrids) performed better than other methods under more restricted (marginal admixture) proportions and with low differentiation of parental populations, and hence it is more robust.

Another consideration is the nature of the samples collected. The Mangere Island samples came from a mixed population with overlapping generations. There exists a major problem in defining "pure" Forbes' parakeet genotypes, leading to a reduced accuracy of assignment tests.

In summary, through analyses of microsatellite, mitochondrial DNA and morphology data, a new and improved system has been developed to identify Forbes' parakeets, hybrid parakeets and Chatham Island Red-crowned parakeets in the Chatham Islands. Although Forbes' parakeets have hybridised extensively with Chatham Island Red-crowned parakeets,

the ability to identify existing "pure" or near-"pure" Forbes' parakeets will certainly assist the future conservation of this species.

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Appendix 3.1 Individual Profiles at Eight Polymorphic *Cfor* Microsatellite Loci

Birds were banded with colour bands and / or metal numbered bands. In the case of colour bands the codes are presented in the format:

Left leg band(s) – Right leg band(s)

The colour codes are:

B – Blue

Bk – Black

G – Green

M – Metal

O – Orange

R – Red

W – White

Y – Yellow

The allelic genotypes are presented in two-column format for each locus, with one allele size (bp) in each column. 0 indicates a null allele.

Mangere Island Population:

Metal Band	Colour Band	Microsatellite Loci														
		Cfor0809		Cfor1415		Cfor1617		Cfor1819		Cfor2021		Cfor2627		Cfor2829		Cfor3031	
-	BR-G	191	203	217	217	217	217	184	188	233	233	0	0	219	231	237	237
-	BW-G	191	191	225	227	217	217	184	188	233	233	162	172	219	219	245	247
-	G-BG	191	191	225	227	217	217	184	188	233	233	170	172	219	219	237	237
-	G-BO	191	191	213	227	217	217	184	188	233	239	162	162	219	219	237	237
-	G-BR	191	191	217	227	217	217	184	188	233	233	170	172	219	219	237	237
-	G-BW	191	191	213	217	217	217	184	188	233	239	170	172	219	231	237	239
-	G-BY	191	191	225	227	217	217	184	188	233	233	170	172	219	221	237	237
-	G-GW	191	191	213	217	217	217	184	188	233	239	162	162	219	219	237	239
-	G-OW	191	191	211	217	217	217	184	184	233	233	170	172	219	231	239	239
-	GO-W	191	191	217	227	217	217	184	184	233	233	170	172	219	219	235	237
-	G-RB	191	191	225	227	217	217	184	184	233	233	170	172	219	221	237	237
-	G-RG	191	191	217	227	217	217	184	188	233	233	170	172	219	221	237	237
-	G-RO	191	191	225	227	217	217	184	188	233	233	170	172	219	221	237	237
-	G-RW	191	191	211	221	217	217	184	184	233	233	160	160	221	231	239	239
-	G-RY	191	191	213	217	217	217	184	184	233	239	170	170	219	219	237	237
-	G-WB	191	191	217	221	217	217	184	188	233	239	0	0	219	231	237	237
-	G-WG	191	191	217	221	217	217	184	188	233	239	0	0	219	231	237	237
-	GW-G	191	191	211	213	217	217	184	188	233	239	160	160	219	221	239	239
-	G-WO	191	191	211	217	217	217	184	188	233	239	0	0	219	221	239	239
-	G-WR	191	191	217	227	217	217	188	188	233	233	170	172	219	219	235	237
-	GW-W	191	191	217	227	217	217	184	188	233	233	162	162	219	219	237	237
-	G-WY	191	191	211	217	217	217	184	186	233	233	160	160	219	231	239	239
-	G-YR	191	191	211	213	225	225	184	186	233	233	162	172	219	219	237	239
-	OW-G	191	191	217	217	217	217	184	184	233	233	170	172	219	231	237	239
-	OY-G	191	191	217	217	217	217	186	188	233	239	160	160	219	219	235	237
-	RB-G	191	191	211	213	225	225	184	184	233	233	162	162	219	219	237	239
-	RG-G	191	191	213	227	217	217	184	188	239	239	162	162	219	219	237	237
-	RO-G	191	191	217	227	217	217	186	188	233	233	0	0	219	221	237	237
-	RW-G	191	191	217	227	217	217	188	188	233	233	162	172	219	219	237	237
-	RW-W	191	191	217	227	217	217	186	186	233	233	162	162	219	219	237	237
-	RY-G	191	203	211	217	217	225	184	184	233	233	162	162	219	219	237	239

-	WB-G	191	191	217	227	217	217	184	184	233	233	162	162	219	219	239	239
-	WG-G	191	191	213	217	217	217	184	188	233	233	0	0	219	219	237	239
-	WO-G	191	191	225	227	217	217	184	186	233	233	162	172	219	219	245	247
-	WR-G	191	191	217	217	217	217	188	188	233	233	162	162	219	219	237	237
-	W-RW	191	203	217	217	217	217	176	184	233	233	172	172	219	231	239	239
-	W-WB	191	191	217	217	217	217	184	186	233	239	172	172	219	231	237	237
-	W-WR	191	203	215	215	217	217	186	188	233	233	170	172	231	231	237	239
-	WY-G	191	191	217	227	217	217	184	186	233	233	162	162	219	219	239	239
-	YW-G	191	203	213	217	217	217	176	184	233	233	170	172	219	231	237	239
-	Y-YG	191	203	211	211	217	225	184	184	233	233	0	0	221	221	239	239
D171508	GG-M	191	191	217	227	217	217	184	186	233	233	170	172	219	219	237	237
D171511	BW-M	191	191	213	217	225	225	184	184	233	233	0	0	219	219	237	239
D171514	M-RW	191	191	213	217	217	217	184	188	233	233	0	0	219	219	247	247
D171515	M-RB	191	191	215	225	217	217	184	188	233	233	162	162	219	219	237	239
D171516	M-RY	191	191	217	227	217	217	184	188	233	239	170	172	219	219	235	237
D171517	M-RG	191	191	217	227	225	225	186	186	233	233	162	172	219	219	235	237
D171518	M-RO	191	191	211	217	217	217	184	184	233	239	172	172	219	219	237	237
D171519	M-WR	191	191	213	217	217	225	184	184	233	239	0	0	219	231	237	237
D171520	M-WB	191	191	217	227	217	217	184	184	233	239	162	162	219	231	239	239
D172001	M-WY	191	191	215	217	217	217	184	186	233	233	162	162	219	219	237	239
D172002	M-WG	191	191	225	227	217	217	184	184	233	233	162	162	219	231	247	247
D172003	M-WO	191	191	213	217	217	217	186	188	233	239	0	0	219	231	239	239
D172005	M-BW	191	191	217	227	217	217	186	188	233	239	172	172	219	219	237	239
D172006	M-BY	191	191	213	217	217	225	184	186	233	233	0	0	219	219	237	239
D172007	M-YR	191	191	213	217	225	225	184	186	233	233	162	172	219	221	233	233
D172008	M-YG	191	191	211	213	225	225	184	186	233	239	172	172	219	219	237	237
D172010	M-YB	191	191	221	227	217	217	184	184	233	233	160	162	219	219	237	237
D172011	M-GR	191	191	213	217	217	217	186	188	233	239	162	162	219	219	237	237
D172012	M-GW	191	191	213	227	225	225	186	186	233	233	160	172	219	219	241	247
D172013	M-GB	191	191	217	227	217	217	184	188	233	239	170	172	219	219	237	239
D172015	M-GO	191	191	217	217	225	225	184	184	233	239	162	162	219	219	239	239
D172016	RW-M	191	191	213	217	217	217	184	188	233	233	170	172	219	219	235	239
D172017	RB-M	191	191	217	227	217	217	188	188	233	239	0	0	219	231	235	237
D172018	RY-M	191	191	217	227	217	217	184	188	233	239	0	0	219	219	235	235
D172019	RG-M	191	191	217	217	217	217	188	188	233	233	0	0	219	219	235	237

D172020	RO-M	191	191	215	217	217	217	186	188	233	233	170	172	219	219	237	237
D172021	WR-M	191	191	213	217	217	217	184	184	233	239	0	0	219	219	235	237
D172022	WB-M	191	191	213	217	217	217	184	184	233	233	0	0	219	221	237	239
D172023	WY-M	191	191	217	217	217	225	184	188	233	239	160	172	219	219	237	237
D172024	WG-M	191	191	213	217	225	225	184	188	239	239	162	172	219	219	235	237
D172025	BR-M	191	191	217	217	217	217	188	188	233	239	0	0	219	219	235	237
D172026	WO-M	191	191	217	227	217	217	184	188	233	239	170	170	219	221	237	237
D172027	BW-M	191	191	211	213	217	225	184	188	233	239	162	172	219	231	237	237
D172028	BY-M	191	191	213	217	217	217	184	184	233	239	170	170	219	219	235	237
D172029	BG-M	191	191	217	217	217	217	186	186	239	239	170	172	219	219	237	239
D172030	BO-M	191	191	227	227	217	217	184	184	233	233	162	172	219	219	245	247
D172031	YR-M	191	191	221	227	225	225	188	188	233	239	162	162	219	221	237	237
D172032	YW-M	191	191	217	217	217	217	184	184	233	239	170	172	219	219	239	239
D172033	YB-M	191	191	217	217	217	217	184	186	233	239	170	172	219	219	237	239
D172034	YG-M	191	191	217	217	217	217	186	188	233	233	0	0	219	221	239	239
D172035	YO-M	191	191	217	217	217	217	184	184	233	233	0	0	219	231	237	239
D172036	GR-M	191	191	213	227	217	217	188	188	233	233	160	160	219	219	245	247
D172037	GW-M	191	191	217	227	217	217	186	188	233	233	162	162	219	231	237	239
D172038	GB-M	191	191	221	227	217	217	184	188	233	233	160	162	219	231	239	239
D172040	M-BO	191	191	221	227	217	217	184	186	233	233	162	162	219	219	247	247
D172042	M-YO	191	191	217	227	217	217	188	188	233	233	162	162	219	219	237	237
D172043	GO-M	191	191	217	227	217	217	188	188	233	233	170	170	219	219	237	239
D172044	M-OO	191	191	213	217	217	217	184	188	233	239	162	162	219	219	235	239
D172046	M-BB	191	191	213	227	217	217	184	184	239	239	0	0	219	219	237	237
D172047	M-	191	191	213	227	217	225	184	188	233	233	170	170	219	219	245	247
D172048	OW-M	191	191	221	227	225	225	184	184	233	233	160	160	219	219	239	239
D172049	OR-M	191	191	217	227	225	225	184	188	233	233	170	172	219	219	235	237
D172050	OY-M	191	191	217	227	217	217	186	188	233	233	170	170	219	219	237	237
D172051	OG-M	191	191	211	221	217	225	184	184	233	233	160	162	219	219	239	239
D172052	OB-M	191	191	211	217	217	217	184	188	233	233	160	160	219	219	235	237
D172053	M-OW	187	191	217	217	225	225	184	188	233	233	162	162	219	221	237	239
D172054	M-OR	191	191	211	227	217	217	184	184	233	239	0	0	219	219	245	247
D172055	M-OY	191	191	213	217	217	217	184	184	233	239	0	0	219	219	235	237
D172056	M-OG	191	191	211	217	217	217	184	188	233	233	162	162	219	219	235	237
D172057	M-OB	191	191	211	217	217	217	184	184	233	233	162	162	219	219	237	237

D172058	M-YY	191	191	211	213	217	225	188	188	233	233	160	160	219	219	239	239
D172059	M-GG	191	191	215	217	217	217	186	188	233	233	0	170	219	221	235	237
D172061	M-WW	191	191	213	217	217	217	184	184	233	233	0	170	219	231	237	239
D174701	R-GO	191	191	217	227	225	225	184	184	233	233	160	162	219	219	239	239
D174702	R-RW	191	191	217	227	217	217	184	184	233	233	160	160	219	219	239	239
D174703	R-BY	191	191	217	227	217	217	184	188	233	239	162	162	219	219	237	239
D174704	R-RB	191	191	217	217	217	225	184	184	233	233	0	0	219	219	237	239
D174705	R-OR	191	203	213	217	217	217	176	184	233	233	170	172	219	219	237	239
D174706	R-OY	203	203	217	217	217	225	176	184	233	233	0	0	219	219	237	239
D174707	R-GW	191	191	217	217	225	225	184	184	233	233	170	172	219	231	239	241
D174708	R-RY	191	191	211	227	217	217	188	188	233	233	0	0	219	221	237	239
D174709	R-BO	191	191	217	227	217	217	184	184	233	233	0	0	219	219	235	235
D174710	R-OG	191	191	211	217	217	217	188	188	233	239	0	0	219	221	237	237
D174711	R-GY	191	191	217	227	217	217	184	188	233	239	170	170	219	219	237	239
D174712	R-RG	191	191	221	227	217	217	184	184	233	233	170	170	219	219	237	239
D174713	R-WO	191	191	221	227	225	225	184	184	233	233	170	172	219	219	237	239
D174714	R-RO	191	191	217	217	217	217	184	188	233	233	170	170	219	219	235	237
D174715	R-YB	191	191	221	227	217	217	184	184	233	233	160	162	219	219	237	239
D174716	R-WY	191	191	213	227	217	217	184	188	233	233	0	0	219	219	235	235
D174717	R-YR	191	191	217	227	217	217	184	188	233	233	0	0	219	219	235	237
D174718	R-GB	191	191	217	227	217	217	186	188	233	239	160	160	219	219	237	237
D174719	R-OB	191	191	217	227	217	217	184	188	233	233	0	0	219	219	235	237
D174774	R-YG	191	191	211	217	217	217	184	184	233	233	0	0	219	231	237	239
D175001	BY-G	191	191	217	227	217	217	188	188	233	233	160	160	219	231	235	235
D175002	G-YW	191	191	213	217	217	217	184	184	233	239	160	162	221	231	235	237
D175003	G-YB	191	191	217	217	217	217	184	188	233	233	162	172	219	219	237	247
D175004	YB-G	191	191	213	217	217	217	184	184	233	239	160	162	219	219	237	237
D175005	G-YO	191	191	211	227	217	217	184	188	233	233	0	0	219	219	237	239
D175006	BG-G	191	191	217	227	217	217	184	188	233	233	160	162	219	219	237	239
D175007	G-YG	191	191	213	217	225	225	186	188	233	239	0	0	221	231	237	239
D175009	YG-G	191	191	217	217	217	217	188	188	233	233	0	0	219	219	235	237
D175010	BO-G	191	191	217	221	217	217	184	184	233	233	162	162	219	219	239	239
D175013	GR-G	191	191	213	227	217	217	186	186	233	233	162	162	219	219	245	247
D175014	G-GY	191	191	211	211	225	225	188	188	233	233	162	162	219	221	239	239
D175015	G-GO	191	191	217	227	217	217	184	188	233	233	162	162	219	221	241	241

D175016	G-OR	191	191	211	217	217	217	188	188	233	233	162	172	219	231	237	237
D175017	G-OB	191	191	215	227	217	225	184	184	233	233	162	162	219	221	239	239
D175018	GO-G	191	191	217	227	217	217	188	188	233	233	0	0	219	219	235	235
D175019	GB-G	191	191	211	213	217	225	186	186	233	233	162	162	219	219	235	237
D175020	GY-G	191	191	227	227	225	225	184	188	233	233	0	0	219	221	247	247
D175021	G-OG	191	191	217	227	217	217	188	188	233	233	170	172	219	219	237	239
D175022	G-OY	191	191	211	211	217	217	186	186	233	233	0	0	219	221	239	239
D175023	OR-G	191	191	217	227	217	217	184	184	233	233	170	172	219	221	239	239
D175101	RW-B	191	191	215	217	217	217	184	188	233	233	160	172	219	231	239	239
D175102	RB-B	191	191	211	217	217	225	184	188	233	233	170	172	219	231	239	239
D175103	RY-B	191	191	225	227	217	217	184	184	233	233	162	172	219	219	247	247
D175104	RG-B	191	191	217	227	217	217	186	188	233	233	170	170	219	219	247	247
D175105	B-RW	191	203	213	217	225	225	176	188	233	239	162	172	219	231	237	239
D175106	B-RB	191	191	213	221	217	217	184	184	233	239	162	162	219	231	239	239
D175107	B-RY	191	191	217	227	225	225	184	188	233	233	160	162	219	231	239	239
D175108	B-RG	191	191	211	217	225	225	184	186	233	233	0	0	219	221	237	237
D175109	B-RO	191	203	211	211	225	225	176	188	233	239	170	172	219	231	237	237
D175110	B-WR	191	191	217	227	225	225	184	184	233	233	162	172	219	219	237	237
D175111	B-WB	191	191	217	217	217	217	186	188	233	233	160	162	219	219	237	237
D175112	RO-B	191	191	213	217	217	217	184	186	233	239	162	172	219	219	237	237
D175113	B-WY	191	191	217	217	217	217	186	186	233	233	162	162	219	219	237	239
D175114	WR-B	191	191	217	227	217	217	184	184	233	239	162	162	219	219	247	247
D175115	WB-B	191	191	215	217	217	217	176	176	233	233	170	172	231	231	239	239
D175116	B-WG	191	191	213	217	225	225	184	184	233	233	162	162	219	231	239	239
D175117	WY-B	191	191	217	217	217	217	184	184	233	239	162	162	219	219	237	239
D175118	BW-B	191	191	213	217	217	217	184	184	233	233	170	172	219	221	237	247
D175119	B-WO	191	191	211	217	225	225	184	188	233	233	162	172	219	219	239	239
D175120	B-BR	191	191	217	227	217	217	188	188	233	233	160	160	219	231	239	239
D175121	B-BW	183	191	211	217	217	217	186	188	233	233	170	172	219	231	237	237
D175122	B-BY	191	191	211	217	217	217	176	188	233	233	160	172	219	231	239	241
D175123	WG-B	191	191	217	227	217	217	184	184	233	239	170	172	219	219	239	239
D175124	B-BG	191	191	213	227	217	217	184	186	233	233	0	172	219	231	237	237
D175125	B-BO	191	191	213	227	217	217	186	188	239	239	162	162	219	231	235	235
D175126	WO-B	191	191	211	227	217	217	184	188	233	233	162	172	219	221	239	239
D175127	BR-B	191	191	217	227	217	217	184	184	233	233	172	172	221	221	239	239

D175128	B-YR	191	191	217	217	217	217	188	188	233	239	162	162	219	219	237	237
D175129	BY-B	191	191	213	217	217	225	188	188	233	239	162	162	219	219	237	237
D175130	B-YW	191	191	217	227	217	217	184	188	233	233	160	160	219	231	237	237
D175131	B-YB	191	191	217	217	217	217	186	188	233	239	0	0	219	219	233	237
D175132	BG-B	191	191	227	227	217	217	184	188	233	233	162	162	219	231	247	247
D175133	B-YG	191	191	217	221	217	217	184	184	233	239	160	160	231	231	239	241
D175134	BO-B	191	191	211	213	217	225	186	188	233	233	162	162	219	221	237	239
D175135	YR-B	191	191	213	217	217	217	184	188	233	239	170	172	219	219	237	237
D175136	YW-B	191	191	217	227	217	217	186	188	233	239	0	0	219	219	237	237
D175139	B-GR	191	191	217	217	217	217	184	188	233	239	162	162	219	219	237	239
D175140	B-GW	191	191	211	215	217	217	184	184	233	233	162	162	219	221	239	239
D175141	YG-B	191	191	225	227	217	225	184	184	233	233	162	172	219	219	239	239
D175142	B-GB	191	191	213	217	217	217	184	184	233	233	172	172	219	231	237	237
D175143	B-GY	191	191	211	211	217	217	188	188	233	233	162	162	219	219	239	239
D175144	YO-B	191	191	211	225	217	225	184	184	233	233	162	162	219	221	237	239
D175145	B-GO	191	191	211	211	217	217	186	186	233	233	0	0	219	221	233	239
D175146	B-BkW	191	191	217	217	225	225	188	188	233	233	162	172	219	219	237	239
D175173	GR-B	187	191	211	213	217	217	184	184	233	233	0	0	219	221	239	239
D175174	GG-B	191	191	217	221	217	217	184	184	233	233	160	160	219	221	239	239
D175175	GW-B	191	191	225	227	217	217	188	188	233	233	170	172	219	219	237	237
D175176	GY-B	191	191	211	217	217	225	186	188	233	233	170	172	219	219	239	239
D175177	OO-B	191	203	217	217	217	217	186	188	233	233	0	0	219	221	237	237
D175178	YY-B	191	191	217	217	217	217	184	184	233	233	0	0	219	219	237	239
D175179	RR-B	191	191	217	227	217	217	184	184	233	233	170	172	219	219	235	237
D175180	B-OR	191	191	217	227	217	217	188	188	233	233	0	0	219	219	239	239
D175181	B-	191	191	227	227	217	217	184	188	233	233	162	162	219	219	247	247
D175182	B-OW	191	191	221	221	217	217	188	188	233	233	162	172	219	231	239	239
D175183	B-OB	191	191	211	225	217	217	184	184	233	233	162	162	219	219	237	237
D175184	B-OG	191	191	217	217	225	225	188	188	233	239	0	0	219	219	239	241
D175186	OR-B	191	191	227	227	217	225	184	188	233	233	160	162	219	219	237	237
D175187	GO-B	191	191	217	217	217	217	184	184	233	233	0	0	219	219	237	237
D175190	OY-B	191	191	217	227	217	217	184	184	233	233	170	172	219	219	237	237
D175191	OY-B	191	191	213	217	217	217	184	184	233	239	0	0	221	221	237	241
D175192	OG-B	191	191	217	227	217	217	184	188	233	233	170	170	219	219	237	239
D175193	WW-B	191	191	217	227	217	225	188	188	233	239	160	162	219	219	237	237

ID	Code																
D175194	BkR-B	191	191	221	227	217	217	184	188	233	239	160	162	219	231	239	239
D175197	B-BkR	191	191	217	227	217	217	184	184	233	233	162	162	219	221	237	237
D175198	B-BkO	191	191	211	213	217	217	184	184	233	233	162	162	219	221	239	239
D175199	B-BkG	191	203	211	213	217	217	184	184	233	233	0	0	219	221	237	239
D175200	B-BkY	191	191	213	217	217	217	184	184	233	233	162	162	219	219	237	239
D175201	-	191	191	213	227	217	217	186	186	233	233	160	160	221	231	239	239
D175202	-	191	191	217	227	217	217	184	188	233	239	162	162	219	219	239	239
D175203	-	191	191	217	217	217	217	184	184	233	233	162	162	219	221	237	237
D175204	-	191	191	217	227	217	217	184	184	233	233	0	0	231	231	239	239
D175205	-	191	191	217	227	217	217	184	188	239	239	162	172	219	219	237	237
D175206	-	191	191	217	227	217	217	184	188	233	233	162	172	219	219	239	239
D175207	-	191	191	217	217	217	217	184	186	233	233	160	160	219	219	237	237
D175208	-	191	191	217	227	217	217	184	188	233	233	170	170	219	221	239	239
D175209	-	191	191	213	217	217	217	184	186	233	239	162	162	219	219	239	241
D175210	-	191	191	217	217	217	217	184	184	233	233	162	162	219	219	237	239
D175211	-	191	191	217	227	217	217	184	184	233	239	162	172	219	219	237	237
D175212	-	191	191	217	217	217	217	184	188	233	233	170	170	219	231	237	239
D175213	-	191	191	213	217	217	217	184	184	233	239	0	0	219	219	235	237
D175214	-	191	191	217	217	217	217	184	184	233	233	0	0	219	219	237	237
D175215	-	191	191	217	227	217	217	188	188	233	233	170	170	219	219	239	239
D175216	-	191	191	221	227	217	217	184	184	233	233	160	160	219	231	247	247
D175218	-	191	191	217	217	217	217	184	188	233	233	0	0	219	231	237	239
D175219	-	191	191	217	227	217	225	186	186	233	233	170	170	219	219	239	239
D175223	-	191	191	227	227	217	217	184	184	233	233	170	170	219	221	237	237
D175225	-	191	191	217	227	217	217	186	186	233	233	0	0	219	221	239	239
D175226	-	191	191	213	217	217	217	184	184	233	233	170	172	219	219	237	237
D175227	-	191	191	221	227	217	217	184	184	233	239	162	162	219	231	239	239
D175228	-	191	191	217	217	217	217	184	184	233	239	170	172	219	231	237	237
D175230	-	191	191	211	227	217	225	184	188	233	233	170	172	219	231	239	239
D175231	-	191	191	211	211	217	217	184	184	233	233	160	160	219	231	239	239
D175232	-	191	191	217	221	217	217	186	186	233	233	0	0	219	221	239	239
D175234	-	191	191	211	217	217	217	184	186	233	239	160	160	219	231	239	239
D175237	-	191	191	211	227	217	217	184	188	233	233	160	160	219	231	239	239
D175243	-	191	191	221	227	217	217	188	188	233	233	160	162	219	231	237	239
D175248	-	191	191	221	221	217	217	188	188	233	233	162	172	219	231	239	239

		Cfor0809		Cfor1415		Cfor1617		Cfor1819		Cfor2021		Cfor2627		Cfor2829		Cfor3031	
D175250	-	191	203	217	217	217	217	184	184	233	233	162	162	219	219	237	239
D175255	-	191	191	227	227	217	217	184	184	233	233	170	172	219	219	247	247
D175267	-	191	203	217	227	217	217	184	184	233	233	162	162	219	219	237	239
D175271	-	191	191	217	217	217	217	186	186	233	233	162	162	219	219	235	237
D175272	Y-GR	187	191	211	215	217	217	184	184	233	233	0	0	221	221	237	239
D175273	Y-OR	191	203	211	217	225	225	184	184	233	233	0	0	221	221	237	239
D175275	YB-Y	191	203	211	211	217	225	188	188	233	233	0	0	221	221	237	239
D175276	YO-Y	191	203	211	217	217	225	184	184	233	233	162	162	221	221	237	239
D175278	YW-Y	191	203	211	211	217	217	184	184	233	233	162	162	221	221	239	239

South-East Island Population:

Metal Band	Colour Band	Microsatellite Loci															
		Cfor0809		Cfor1415		Cfor1617		Cfor1819		Cfor2021		Cfor2627		Cfor2829		Cfor3031	
-	YY-M	191	191	211	213	217	225	188	188	233	233	162	162	219	221	235	239
D171523	-	191	191	211	215	217	225	184	186	233	233	170	172	219	221	233	239
D171524	-	191	191	211	227	217	217	188	188	233	233	0	0	219	219	235	241
D171525	-	191	191	211	213	217	217	186	186	233	233	0	0	219	219	239	239
D171526	-	191	191	211	213	217	217	184	186	233	233	0	0	221	221	233	233
D171527	-	191	203	211	221	217	217	184	186	233	233	170	172	219	219	233	239
D171528	-	191	203	211	227	217	217	184	184	233	233	0	0	219	221	239	239
D171531	-	187	191	211	213	217	217	184	188	233	233	0	0	219	221	239	239
D171532	-	191	191	211	213	217	217	184	184	233	233	162	172	219	219	233	239
D171533	-	191	191	211	221	217	217	176	176	233	233	0	0	219	221	233	239
D171534	-	191	191	211	213	217	217	176	188	233	233	162	162	219	219	235	235
D171535	-	187	191	213	221	217	217	184	186	233	233	0	0	219	221	233	239
D171536	-	191	203	211	211	217	217	176	176	233	233	0	0	219	219	233	239
D171537	-	191	191	211	211	217	217	184	188	233	233	170	172	219	221	235	239
D171538	-	191	191	215	221	217	217	184	186	233	233	0	0	219	221	233	237
D171539	-	191	191	211	213	217	217	176	176	233	233	0	0	219	219	233	239
D171540	-	191	191	211	213	217	225	184	184	233	233	0	0	219	221	233	237
D171541	-	191	203	211	227	217	217	188	188	233	233	170	172	219	219	233	233

D171542	-	191	191	213	213	217	217	176	188	233	233	0	0	219	219	239	239
D171583	-	191	203	211	211	217	217	184	184	233	233	0	0	219	219	235	239
D171584	-	191	191	211	213	217	217	184	186	233	233	0	0	219	219	233	239
D171585	-	187	191	213	215	217	217	176	186	233	233	0	0	219	219	237	239
D171586	-	191	191	221	221	217	217	184	186	233	233	0	0	219	221	239	239
D171587	-	191	203	221	227	217	225	186	186	233	233	0	0	219	221	239	239
D171588	-	187	191	211	211	217	217	184	184	233	233	0	0	219	221	239	239
D171589	-	191	191	211	211	217	217	184	184	233	233	162	162	219	219	239	241
D171590	-	191	191	211	213	217	217	188	188	233	233	0	0	219	221	235	235
D171591	-	191	203	211	213	217	217	184	184	233	233	0	0	219	219	237	239
D171592	-	191	203	221	227	217	217	184	184	233	233	170	172	219	221	239	239
D171593	-	191	203	221	221	217	217	176	176	233	233	0	0	219	221	233	233
D171594	-	191	191	213	221	217	217	184	186	233	233	162	172	219	219	233	239
D171595	-	191	191	213	221	217	217	188	188	233	233	0	0	219	219	235	239
D171596	-	203	203	211	211	217	225	188	188	233	233	170	172	219	221	239	239
D171597	-	191	191	211	215	217	225	176	184	233	233	0	0	219	219	233	233
D171598	-	191	191	213	227	217	217	188	188	233	233	0	0	219	219	239	239

Appendix 3.2 Assignment of Mangere and South-East Islands Parakeets Using Microsatellites, Mitochondrial DNA, and Morphological Markers.

Birds were assigned using microsatellite allele frequencies, mitochondrial control region haplotype, and crown plumage variation. P(FB), P(RC), P(F$_1$), and P(F$_2$) refer to the posterior probabilities of being a Forbes' parakeet, Chatham Island Red-crowned parakeet, F$_1$ hybrid, and F$_2$ hybrid respectively in the Bayesian assignment test. In the overall assignment, F, H, R and ? stand for Forbes' parakeet, Hybrids, Chatham Island Red-crowned parakeet, and assignment not made due to insufficient data respectively. H* denotes morphological Forbes' parakeets with Forbes' parakeet microsatellite assignment, but have an ancient hybridisation event detected only by mitochondrial DNA.

Mangere Island Population:

Metal Band	Colour Band	Microsatellites					mtDNA Assign	Morph. Assign	Overall Assignment
		P(FB)	P(RC)	P(F$_1$)	P(F$_2$)	Assign			
	BR-G	0.5752	0.1693	0.0141	0.2414	MS2	-	MP3	?
	BW-G	0.9841	0.0000	0.0001	0.0158	MS1	MT3	MP1	F
	G-BG	0.9705	0.0060	0.0008	0.0227	MS1	MT4	MP1	H*
	G-BO	0.9663	0.0081	0.0013	0.0243	MS1	MT3	MP1	F
	G-BR	0.9588	0.0103	0.0013	0.0296	MS1	MT3	MP1	F
	G-BW	0.7247	0.1101	0.0171	0.1481	MS2	MT1	MP1	H
	G-BY	0.9018	0.0140	0.0029	0.0813	MS2	MT3	MP1	H
	G-GW	0.8286	0.0540	0.0113	0.1061	MS2	MT2	MP1	H
	G-OW	0.0235	0.8580	0.0054	0.1131	MS2	-	-	?
	GO-W	0.9649	0.0078	0.0010	0.0264	MS1	-	MP1	?
	G-RB	0.9018	0.0140	0.0029	0.0813	MS2	MT3	MP1	H
	G-RG	0.8590	0.0359	0.0046	0.1006	MS2	MT3	MP1	H
	G-RO	0.9018	0.0140	0.0029	0.0813	MS2	MT3	MP1	H
	G-RW	0.0058	0.9687	0.0004	0.0251	MS3	MT1	MP1	H
	G-RY	0.9647	0.0088	0.0014	0.0251	MS1	MT3	MP1	F
	G-WB	0.8129	0.0460	0.0087	0.1324	MS2	MT3	MP1	H
	G-WG	0.8129	0.0460	0.0087	0.1324	MS2	MT3	MP1	H
	GW-G	0.0192	0.8163	0.0064	0.1582	MS2	MT3	MP1	H
	G-WO	0.0316	0.7513	0.0112	0.2059	MS2	MT3	MP1	H
	G-WR	0.9649	0.0078	0.0010	0.0264	MS1	-	MP1	?
	GW-W	0.9588	0.0103	0.0013	0.0296	MS1	MT3	MP1	F

	G-WY	0.0235	0.8580	0.0054	0.1131	MS2	MT1	MP1	H
	G-YR	0.0319	0.8126	0.0098	0.1457	MS2	MT3	MP1	H
	OW-G	0.6494	0.1747	0.0154	0.1606	MS2	MT4	MP1	H
	OY-G	0.9773	0.0063	0.0007	0.0158	MS1	MT3	MP1	F
	RB-G	0.0319	0.8126	0.0098	0.1457	MS2	MT2	MP1	H
	RG-G	0.9790	0.0063	0.0006	0.0141	MS1	MT3	MP1	F
	RO-G	0.8590	0.0359	0.0046	0.1006	MS2	MT3	MP1	H
	RW-G	0.9588	0.0103	0.0013	0.0296	MS1	-	-	?
	RW-W	0.9588	0.0103	0.0013	0.0296	MS1	MT2	MP1	H*
	RY-G	0.0199	0.7732	0.0218	0.1851	MS2	MT2	MP1	H
	WB-G	0.3381	0.4470	0.0072	0.2077	MS2	MT1	MP1	H
	WG-G	0.6858	0.1518	0.0145	0.1479	MS2	MT4	MP1	H
	WO-G	0.9841	0.0000	0.0001	0.0158	MS1	MT3	MP1	F
	WR-G	0.9563	0.0116	0.0014	0.0306	MS1	MT3	MP1	F
	W-RW	0.0302	0.8162	0.0052	0.1484	MS2	MT3	MP1	H
	W-WB	0.9590	0.0099	0.0016	0.0295	MS1	MT3	MP1	F
	W-WR	0.0077	0.9179	0.0023	0.0721	MS2	-	MP1	?
	WY-G	0.3381	0.4470	0.0072	0.2077	MS2	MT1	-	?
	YW-G	0.1174	0.6166	0.0251	0.2409	MS2	MT1	-	?
	Y-YG	0.0055	0.9892	0.0001	0.0052	MS3	MT4	-	?
D171508	GG-M	0.9588	0.0103	0.0013	0.0296	MS1	MT2	MP2	H
D171511	BW-M	0.2206	0.5455	0.0151	0.2187	MS2	MT1	MP1	H
D171514	M-RW	0.9702	0.0001	0.0001	0.0296	MS1	MT3	MP1	F
D171515	M-RB	0.4117	0.1852	0.0570	0.3461	MS2	MT2	MP2	H
D171516	M-RY	0.9781	0.0061	0.0006	0.0152	MS1	MT2	MP1	H*
D171517	M-RG	0.8062	0.0409	0.0032	0.1497	MS2	MT3	MP1	H
D171518	M-RO	0.8463	0.0285	0.0069	0.1183	MS2	MT4	MP1	H
D171519	M-WR	0.8591	0.0376	0.0055	0.0978	MS2	MT1	MP1	H
D171520	M-WB	0.4090	0.3450	0.0097	0.2363	MS2	MT3	MP1	H
D172001	M-WY	0.2668	0.4303	0.0313	0.2717	MS2	MT3	MP1	H
D172002	M-WG	0.9744	0.0000	0.0001	0.0255	MS1	-	MP1	?
D172003	M-WO	0.2738	0.4898	0.0102	0.2262	MS2	-	MP1	?
D172005	M-BW	0.8871	0.0267	0.0074	0.0788	MS2	MT1	MP1	H
D172006	M-BY	0.4294	0.3379	0.0209	0.2118	MS2	-	MP1	?
D172007	M-YR	0.0049	0.9517	0.0001	0.0432	MS3	MT3	MP3	H
D172008	M-YG	0.3955	0.2768	0.0114	0.3163	MS2	MT3	MP1	H
D172010	M-YB	0.8053	0.0544	0.0075	0.1327	MS2	MT3	MP1	H
D172011	M-GR	0.9647	0.0088	0.0014	0.0251	MS1	MT3	MP1	F
D172012	M-GW	0.4620	0.0567	0.0524	0.4289	MS2	-	MP1	?
D172013	M-GB	0.8871	0.0267	0.0074	0.0788	MS2	MT1	MP1	H
D172015	M-GO	0.1515	0.5966	0.0067	0.2452	MS2	MT3	MP1	H
D172016	RW-M	0.7565	0.0822	0.0158	0.1456	MS2	MT4	MP3	H
D172017	RB-M	0.9666	0.0070	0.0011	0.0253	MS1	-	MP1	?
D172018	RY-M	0.9801	0.0059	0.0004	0.0136	MS1	-	MP1	?
D172019	RG-M	0.9633	0.0084	0.0011	0.0273	MS1	-	MP1	?
D172020	RO-M	0.5941	0.1348	0.0163	0.2548	MS2	MT3	MP2	H
D172021	WR-M	0.9695	0.0071	0.0010	0.0224	MS1	MT4	MP1	H*
D172022	WB-M	0.3264	0.4178	0.0247	0.2312	MS2	MT3	MP1	F
D172023	WY-M	0.9379	0.0123	0.0022	0.0475	MS2	-	MP1	?
D172024	WG-M	0.8920	0.0147	0.0016	0.0918	MS2	MT3	MP1	H
D172025	BR-M	0.9773	0.0063	0.0007	0.0158	MS1	-	MP1	?

D172026	WO-M	0.9195	0.0141	0.0031	0.0633	MS2	MT3	MP1	H
D172027	BW-M	0.4706	0.2265	0.0160	0.2870	MS2	MT3	MP1	H
D172028	BY-M	0.9695	0.0071	0.0010	0.0224	MS1	MT1	MP1	H*
D172029	BG-M	0.9315	0.0142	0.0035	0.0508	MS2	MT3	MP1	H
D172030	BO-M	0.9797	0.0000	0.0001	0.0202	MS1	-	MP1	?
D172031	YR-M	0.2990	0.3306	0.0197	0.3508	MS2	-	MP1	?
D172032	YW-M	0.5370	0.2435	0.0081	0.2113	MS2	MT3	MP1	H
D172033	YB-M	0.8792	0.0318	0.0079	0.0811	MS2	-	MP1	?
D172034	YG-M	0.0891	0.7246	0.0067	0.1797	MS2	MT1	MP1	H
D172035	YO-M	0.6494	0.1747	0.0154	0.1606	MS2	MT3	MP1	H
D172036	GR-M	0.9704	0.0000	0.0002	0.0294	MS1	-	MP1	?
D172037	GW-M	0.6723	0.1526	0.0151	0.1600	MS2	MT3	MP1	H
D172038	GB-M	0.0395	0.8307	0.0062	0.1235	MS2	MT3	MP3	H
D172040	M-BO	0.8935	0.0005	0.0003	0.1058	MS2	-	MP1	?
D172042	M-YO	0.9588	0.0103	0.0013	0.0296	MS1	MT3	MP1	F
D172043	GO-M	0.7942	0.0764	0.0103	0.1191	MS2	MT2	MP1	H
D172044	M-OO	0.8602	0.0299	0.0115	0.0984	MS2	-	-	?
D172046	M-BB	0.9790	0.0063	0.0006	0.0141	MS1	-	-	?
D172047	M-	0.9246	0.0002	0.0004	0.0748	MS2	-	-	?
D172048	OW-M	0.0131	0.9095	0.0028	0.0745	MS2	-	-	?
D172049	OR-M	0.8062	0.0409	0.0032	0.1497	MS2	MT3	-	?
D172050	OY-M	0.9588	0.0103	0.0013	0.0296	MS1	-	-	?
D172051	OG-M	0.0068	0.9486	0.0008	0.0438	MS2	-	-	?
D172052	OB-M	0.7883	0.0433	0.0076	0.1608	MS2	MT1	-	?
D172053	M-OW	0.0088	0.8198	0.0110	0.1604	MS2	-	MP5	?
D172054	M-OR	0.9110	0.0001	0.0006	0.0884	MS2	-	-	?
D172055	M-OY	0.9695	0.0071	0.0010	0.0224	MS1	-	-	?
D172056	M-OG	0.7883	0.0433	0.0076	0.1608	MS2	MT2	-	?
D172057	M-OB	0.7429	0.0795	0.0094	0.1682	MS2	-	MP1	?
D172058	M-YY	0.0112	0.9180	0.0026	0.0681	MS2	-	MP5	?
D172059	M-GG	0.4389	0.1830	0.0227	0.3554	MS2	MT3	MP3	H
D172061	M-WW	0.5283	0.2723	0.0189	0.1804	MS2	MT3	MP1	H
D174701	R-GO	0.0630	0.7814	0.0039	0.1517	MS2	MT3	-	?
D174702	R-RW	0.3381	0.4470	0.0072	0.2077	MS2	-	-	?
D174703	R-BY	0.8871	0.0267	0.0074	0.0788	MS2	-	-	?
D174704	R-RB	0.5546	0.2268	0.0178	0.2009	MS2	-	-	?
D174705	R-OR	0.2005	0.5023	0.0277	0.2695	MS2	-	-	?
D174706	R-OY	0.0333	0.7325	0.0058	0.2284	MS2	-	-	?
D174707	R-GW	0.0694	0.8102	0.0044	0.1160	MS2	-	-	?
D174708	R-RY	0.0915	0.6325	0.0324	0.2436	MS2	-	-	?
D174709	R-BO	0.9685	0.0072	0.0006	0.0236	MS1	-	-	?
D174710	R-OG	0.6140	0.1090	0.0184	0.2586	MS2	-	-	?
D174711	R-GY	0.8871	0.0267	0.0074	0.0788	MS2	MT3	MP1	H
D174712	R-RG	0.3959	0.3347	0.0285	0.2409	MS2	MT3	MP1	H
D174713	R-WO	0.0851	0.6911	0.0180	0.2058	MS2	MT1	MP1	H
D174714	R-RO	0.9633	0.0084	0.0011	0.0273	MS1	MT3	MP1	F
D174715	R-YB	0.3959	0.3347	0.0285	0.2409	MS2	MT2	MP1	H
D174716	R-WY	0.9562	0.0093	0.0010	0.0334	MS1	-	-	?
D174717	R-YR	0.9649	0.0078	0.0010	0.0264	MS1	MT3	MP1	F
D174718	R-GB	0.9753	0.0068	0.0008	0.0171	MS1	MT3	MP1	F
D174719	R-OB	0.9649	0.0078	0.0010	0.0264	MS1	MT1	MP1	H

D174774	R-YG	0.1731	0.5534	0.0293	0.2442	MS2	MT3	MP1	H
D175001	BY-G	0.9505	0.0098	0.0011	0.0386	MS1	MT3	-	?
D175002	G-YW	0.8457	0.0296	0.0044	0.1204	MS2	MT2	MP1	H
D175003	G-YB	0.9732	0.0005	0.0008	0.0255	MS1	MT3	MP1	F
D175004	YB-G	0.9647	0.0088	0.0014	0.0251	MS1	MT3	MP3	H
D175005	G-YO	0.3139	0.3872	0.0323	0.2666	MS2	MT3	MP1	H
D175006	BG-G	0.7942	0.0764	0.0103	0.1191	MS2	MT3	MP1	H
D175007	G-YG	0.0916	0.6470	0.0153	0.2461	MS2	MT3	MP1	H
D175009	YG-G	0.9633	0.0084	0.0011	0.0273	MS1	MT3	MP2	H
D175010	BO-G	0.0656	0.7731	0.0070	0.1543	MS2	MT3	MP1	H
D175013	GR-G	0.9704	0.0000	0.0002	0.0294	MS1	MT3	MP1	F
D175014	G-GY	0.0055	0.9821	0.0002	0.0122	MS3	MT4	MP5	R
D175015	G-GO	0.2399	0.5949	0.0049	0.1603	MS2	MT3	MP2	H
D175016	G-OR	0.6191	0.1534	0.0135	0.2140	MS2	MT1	MP1	H
D175017	G-OB	0.0081	0.9271	0.0037	0.0611	MS2	MT4	MP4	H
D175018	GO-G	0.9685	0.0072	0.0006	0.0236	MS1	-	MP1	?
D175019	GB-G	0.5137	0.1787	0.0119	0.2956	MS2	MT2	MP5	H
D175020	GY-G	0.7181	0.0034	0.0005	0.2780	MS2	MT4	MP1	H
D175021	G-OG	0.7942	0.0764	0.0103	0.1191	MS2	MT3	MP1	H
D175022	G-OY	0.0060	0.9581	0.0006	0.0353	MS3	MT4	MP5	R
D175023	OR-G	0.0981	0.7065	0.0072	0.1882	MS2	MT1	MP1	H
D175101	RW-B	0.0262	0.8550	0.0055	0.1133	MS2	MT3	MP2	H
D175102	RB-B	0.0109	0.9118	0.0038	0.0735	MS2	MT2	MP1	H
D175103	RY-B	0.9845	0.0000	0.0000	0.0154	MS1	MT3	MP1	F
D175104	RG-B	0.9797	0.0001	0.0000	0.0202	MS1	MT3	MP1	F
D175105	B-RW	0.0549	0.6652	0.0213	0.2585	MS2	MT3	MP1	H
D175106	B-RB	0.0612	0.7482	0.0072	0.1833	MS2	MT3	MP1	H
D175107	B-RY	0.0342	0.8477	0.0031	0.1150	MS2	MT3	MP3	H
D175108	B-RG	0.1053	0.6002	0.0138	0.2807	MS2	MT3	MP1	H
D175109	B-RO	0.0165	0.7146	0.0044	0.2645	MS2	MT3	MP1	H
D175110	B-WR	0.7634	0.0774	0.0040	0.1552	MS2	-	MP3	?
D175111	B-WB	0.9563	0.0116	0.0014	0.0306	MS1	MT3	MP1	F
D175112	RO-B	0.9647	0.0088	0.0014	0.0251	MS1	-	MP1	?
D175113	B-WY	0.7783	0.0898	0.0108	0.1212	MS2	MT3	MP1	H
D175114	WR-B	0.9883	0.0000	0.0000	0.0116	MS1	MT3	MP1	F
D175115	WB-B	0.0161	0.8979	0.0034	0.0825	MS2	MT3	MP1	H
D175116	B-WG	0.0182	0.9058	0.0023	0.0738	MS2	MT3	MP1	H
D175117	WY-B	0.8792	0.0318	0.0079	0.0811	MS2	MT3	MP1	H
D175118	BW-B	0.8733	0.0026	0.0042	0.1199	MS2	MT3	MP1	H
D175119	B-WO	0.0096	0.9212	0.0024	0.0668	MS2	MT3	MP1	H
D175120	B-BR	0.2063	0.5930	0.0071	0.1936	MS2	MT3	MP1	H
D175121	B-BW	0.5908	0.1732	0.0142	0.2218	MS2	MT3	MP1	H
D175122	B-BY	0.0517	0.8150	0.0087	0.1246	MS2	MT3	MP1	H
D175123	WG-B	0.5610	0.2184	0.0081	0.2126	MS2	MT3	MP1	H
D175124	B-BG	0.9010	0.0301	0.0035	0.0654	MS2	MT3	MP2	H
D175125	B-BO	0.9745	0.0062	0.0005	0.0187	MS1	MT3	MP3	H
D175126	WO-B	0.0131	0.8850	0.0053	0.0965	MS2	MT3	MP1	H
D175127	BR-B	0.0252	0.8475	0.0024	0.1249	MS2	MT3	MP2	H
D175128	B-YR	0.9741	0.0072	0.0009	0.0178	MS1	MT3	MP1	F
D175129	BY-B	0.9130	0.0189	0.0033	0.0648	MS2	MT3	MP1	H
D175130	B-YW	0.9321	0.0178	0.0023	0.0478	MS2	MT1	MP1	H

D175131	B-YB	0.2387	0.2483	0.0622	0.4508	MS2	MT4	MP1	H
D175132	BG-B	0.9674	0.0001	0.0001	0.0325	MS1	MT1	MP1	H*
D175133	B-YG	0.0992	0.7027	0.0105	0.1876	MS2	MT2	MP1	H
D175134	BO-B	0.0189	0.8378	0.0132	0.1301	MS2	MT4	MP4	H
D175135	YR-B	0.9647	0.0088	0.0014	0.0251	MS1	MT3	MP1	F
D175136	YW-B	0.9753	0.0068	0.0008	0.0171	MS1	-	MP1	?
D175139	B-GR	0.8792	0.0318	0.0079	0.0811	MS2	MT3	MP1	H
D175140	B-GW	0.0060	0.9579	0.0006	0.0355	MS3	-	MP4	?
D175141	YG-B	0.3631	0.2892	0.0113	0.3364	MS2	MT3	MP1	H
D175142	B-GB	0.8939	0.0352	0.0037	0.0672	MS2	MT1	MP1	H
D175143	B-GY	0.0086	0.9223	0.0010	0.0680	MS2	MT4	MP4	H
D175144	YO-B	0.1287	0.3877	0.0830	0.4005	MS2	MT4	MP4	H
D175145	B-GO	0.0054	0.9641	0.0003	0.0302	MS3	MT2	MP4	H
D175146	B-BkW	0.3222	0.4241	0.0146	0.2391	MS2	MT3	MP1	H
D175173	GR-B	0.0055	0.9629	0.0013	0.0303	MS3	MT3	MP4	H
D175174	GG-B	0.0162	0.8840	0.0047	0.0950	MS2	MT3	MP1	H
D175175	GW-B	0.9705	0.0060	0.0008	0.0227	MS1	MT4	MP1	H*
D175176	GY-B	0.0173	0.8742	0.0050	0.1034	MS2	MT3	MP1	H
D175177	OO-B	0.4073	0.2585	0.0187	0.3155	MS2	MT1	MP1	H
D175178	YY-B	0.7783	0.0898	0.0108	0.1212	MS2	MT3	MP1	H
D175179	RR-B	0.9649	0.0078	0.0010	0.0264	MS1	MT4	MP1	H*
D175180	B-OR	0.3381	0.4470	0.0072	0.2077	MS2	MT3	MP1	H
D175181	B-	0.9802	0.0001	0.0000	0.0197	MS1	MT3	MP1	F
D175182	B-OW	0.0099	0.9367	0.0011	0.0522	MS2	MT3	MP1	H
D175183	B-OB	0.8162	0.0288	0.0101	0.1449	MS2	MT2	MP1	H
D175184	B-OG	0.1982	0.5586	0.0071	0.2360	MS2	MT3	MP1	H
D175186	OR-B	0.9026	0.0220	0.0028	0.0726	MS2	MT3	MP1	H
D175187	GO-B	0.9563	0.0116	0.0014	0.0306	MS1	MT3	MP1	F
D175190	OY-B	0.9588	0.0103	0.0013	0.0296	MS1	MT3	MP1	F
D175191	OY-B	0.2697	0.4204	0.0171	0.2928	MS2	MT2	MP3	H
D175192	OG-B	0.7942	0.0764	0.0103	0.1191	MS2	MT1	MP1	H
D175193	WW-B	0.9413	0.0108	0.0020	0.0459	MS2	MT1	MP1	H
D175194	BkR-B	0.1127	0.6411	0.0127	0.2335	MS2	MT3	MP1	H
D175197	B-BkR	0.8590	0.0359	0.0046	0.1006	MS2	MT3	MP1	H
D175198	B-BkO	0.0086	0.9302	0.0024	0.0588	MS2	MT4	MP4	H
D175199	B-BkG	0.0096	0.8590	0.0116	0.1199	MS2	MT2	MP4	H
D175200	B-BkY	0.6858	0.1518	0.0145	0.1479	MS2	MT3	MP1	H
D175201		0.0290	0.8622	0.0034	0.1054	MS2	MT3	-	?
D175202		0.5610	0.2184	0.0081	0.2126	MS2	MT3	-	?
D175203		0.8496	0.0425	0.0049	0.1030	MS2	MT3	-	?
D175204		0.1223	0.7090	0.0050	0.1637	MS2	MT3	-	?
D175205		0.9839	0.0059	0.0004	0.0099	MS1	MT3	-	?
D175206		0.3381	0.4470	0.0072	0.2077	MS2	MT1	-	?
D175207		0.9563	0.0116	0.0014	0.0306	MS1	MT3	MP1	F
D175208		0.0981	0.7065	0.0072	0.1882	MS2	MT3	MP2	H
D175209		0.3786	0.3669	0.0095	0.2450	MS2	MT3	MP1	H
D175210		0.7783	0.0898	0.0108	0.1212	MS2	MT3	MP1	H
D175211		0.9753	0.0068	0.0008	0.0171	MS1	MT2	MP3	H
D175212		0.6494	0.1747	0.0154	0.1606	MS2	MT3	-	?
D175213		0.9695	0.0071	0.0010	0.0224	MS1	MT3	-	?
D175214		0.9563	0.0116	0.0014	0.0306	MS1	MT3	MP1	F

D175215		0.3381	0.4470	0.0072	0.2077	MS2	MT4	MP3	H
D175216		0.8479	0.0011	0.0005	0.1505	MS2	MT3	MP3	H
D175218		0.6494	0.1747	0.0154	0.1606	MS2	MT3	-	?
D175219		0.1496	0.6475	0.0071	0.1959	MS2	MT3	-	?
D175223		0.8652	0.0322	0.0043	0.0983	MS2	MT3	MP1	H
D175225		0.0981	0.7065	0.0072	0.1882	MS2	MT3	-	?
D175226		0.9376	0.0176	0.0021	0.0427	MS2	MT3	MP2	H
D175227		0.1127	0.6411	0.0127	0.2335	MS2	MT3	-	?
D175228		0.9590	0.0099	0.0016	0.0295	MS1	MT3	-	?
D175230		0.0116	0.9059	0.0043	0.0781	MS2	MT3	-	?
D175231		0.0068	0.9459	0.0007	0.0466	MS2	MT1	-	?
D175232		0.0162	0.8840	0.0047	0.0950	MS2	MT3	-	?
D175234		0.0696	0.6907	0.0119	0.2278	MS2	MT3	MP1	H
D175237		0.0260	0.8479	0.0062	0.1198	MS2	MT3	-	?
D175243		0.2571	0.4761	0.0296	0.2372	MS2	MT3	-	?
D175248		0.0099	0.9367	0.0011	0.0522	MS2	MT3	-	?
D175250		0.2918	0.3923	0.0275	0.2884	MS2	MT3	-	?
D175255		0.9802	0.0001	0.0000	0.0197	MS1	MT4	-	?
D175267		0.3101	0.3664	0.0283	0.2952	MS2	MT4	-	?
D175271		0.9633	0.0084	0.0011	0.0273	MS1	MT3	-	?
D175272	Y-GR	0.0053	0.9535	0.0009	0.0404	MS3	MT1	MP4	H
D175273	Y-OR	0.0056	0.9503	0.0021	0.0420	MS3	MT3	-	?
D175275	YB-Y	0.0055	0.9687	0.0006	0.0252	MS3	MT3	-	?
D175276	YO-Y	0.0059	0.9231	0.0044	0.0666	MS2	MT4	-	?
D175278	YW-Y	0.0055	0.9853	0.0001	0.0091	MS3	-	-	?

South-East Island Population:

Metal Band	Colour Band	Microsatellites					mtDNA Assign	Morph. Assign	Overall Assignment
		P(FB)	P(RC)	P(F$_1$)	P(F$_2$)	Assign			
	YY-M	0.0405	0.6491	0.0312	0.2793	MS2	-	-	?
D171523		0.0054	0.9766	0.0002	0.0178	MS3	MT2	MP5	H
D171524		0.3825	0.2422	0.0445	0.3308	MS2	MT4	MP5	H
D171525		0.0247	0.8649	0.0038	0.1066	MS2	MT4	MP5	H
D171526		0.0050	0.9728	0.0001	0.0222	MS3	MT4	MP5	R
D171527		0.0054	0.9606	0.0004	0.0336	MS3	MT4	MP5	R
D171528		0.0061	0.9360	0.0031	0.0548	MS2	MT4	MP5	H
D171531		0.0055	0.9629	0.0013	0.0303	MS3	MT4	MP5	R
D171532		0.0062	0.8967	0.0022	0.0949	MS2	MT2	MP5	H
D171533		0.0054	0.9622	0.0004	0.0320	MS3	MT4	MP5	R
D171534		0.7507	0.0590	0.0048	0.1855	MS2	MT2	MP5	H
D171535		0.0051	0.9667	0.0008	0.0274	MS3	MT4	MP5	R
D171536		0.0054	0.9626	0.0003	0.0317	MS3	MT4	MP5	R
D171537		0.0272	0.6694	0.0140	0.2894	MS2	MT4	MP5	H
D171538		0.0051	0.8658	0.0066	0.1225	MS2	MT4	MP5	H
D171539		0.0062	0.8967	0.0022	0.0949	MS2	MT4	MP5	H
D171540		0.0057	0.8647	0.0131	0.1164	MS2	MT4	MP5	H
D171541		0.0051	0.9174	0.0004	0.0771	MS2	MT4	MP5	H
D171542		0.1240	0.7257	0.0054	0.1448	MS2	MT4	MP5	H
D171583		0.0169	0.6735	0.0135	0.2960	MS2	MT4	MP5	H
D171584		0.0062	0.8967	0.0022	0.0949	MS2	MT4	MP5	H
D171585		0.0134	0.7810	0.0170	0.1886	MS2	MT4	MP5	H
D171586		0.0070	0.9523	0.0009	0.0398	MS3	MT4	MP5	R
D171587		0.0058	0.9571	0.0021	0.0350	MS3	MT4	MP5	R
D171588		0.0054	0.9766	0.0003	0.0177	MS3	MT4	MP5	R
D171589		0.0182	0.8922	0.0023	0.0873	MS2	MT4	MP5	H
D171590		0.4878	0.1703	0.0102	0.3317	MS2	MT4	MP5	H
D171591		0.0288	0.7626	0.0171	0.1915	MS2	MT2	MP5	H
D171592		0.0066	0.9323	0.0032	0.0579	MS2	MT4	MP5	H
D171593		0.0053	0.9794	0.0000	0.0152	MS3	MT4	MP5	R
D171594		0.0069	0.8911	0.0023	0.0996	MS2	MT4	MP5	H
D171595		0.3706	0.3132	0.0294	0.2867	MS2	MT4	MP5	H
D171596		0.0055	0.9890	0.0000	0.0055	MS3	MT4	MP5	R
D171597		0.0049	0.9645	0.0001	0.0305	MS3	MT4	MP5	R
D171598		0.2228	0.5827	0.0075	0.1871	MS2	MT4	MP5	H

Appendix 3.3   Genetic Analysis of Interspecific Hybridisation in the World's Only Forbes' Parakeet (*Cyanoramphus forbesi*) Natural Population

Chi-hang Chan[1], Kaye N. Ballantyne[1], Hilary Aikman[2], Denise Fastier[3], Charles H. Daugherty[1], and Geoffrey K. Chambers[1]

[1]*Institute for Molecular Systematics, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington, New Zealand.*

[2]*Wellington Conservancy, Department of Conservation, PO Box 5086, Wellington, New Zealand.*

[3]*Chatham Islands Area Office, Department of Conservation, PO Box 114, Chatham Islands, New Zealand.*

Abstract

Genetic introgression from Chatham Island Red-crowned parakeet (*Cyanoramphus novaezelandiae chathamensis*) is a long-standing problem in the conservation of the rare Forbes' parakeet (*C. forbesi*) on Mangere Island, New Zealand. Microsatellite genotypes, mitochondrial DNA sequences, and morphological markers have been applied to study the situation. An extensive history of hybridisation was detected in the single remaining Forbes' parakeet population, where no less than 81% of all birds screened are considered hybrids. Genetic markers showed that a large proportion of birds identified as Forbes' parakeets by crown plumage are cryptic hybrids. Only a small proportion of birds with Forbes' parakeet morphotype were found to be genetically distinct from Chatham Island

Red-crowned parakeets using microsatellite and mitochondrial DNA assignment tests, but these still represent an Evolutionarily Significant Unit (ESU) for conservation.

Introduction

Forbes' parakeet (*Cyanoramphus forbesi*) is a highly threatened parrot species found on Mangere Island and the adjacent Little Mangere Island in the Chatham Islands group of New Zealand (Figure 1). Increased urgent attention towards its conservation was prompted after an allozyme electrophoresis study (Triggs & Daugherty 1996) suggested elevation of Forbes' parakeet from a subspecies of Yellow-crowned parakeet (*C. auriceps forbesi*) to its present full species status. Further studies using mitochondrial control region DNA sequences vindicated this view and convincingly placed Forbes' parakeet phylogenetically basal to all other New Zealand *Cyanoramphus* parakeets (Boon et al. 2000). Forbes' parakeet is classified by the IUCN as an endangered species.

The Chatham Island Red-crowned parakeet (*C. novaezelandiae chathamensis*) has not been ranked into IUCN threat categories as a subspecies, but the New Zealand Red-crowned parakeet (*C. novaezelandiae*), in general, is ranked as vulnerable. The Chatham Island Red-crowned parakeet can be found on the main Chatham Island, Pitt Island, Rangatira (also known as South-East Island), but also visits and breeds on Mangere and Little Mangere Islands (Figure 1). Morphogically, Forbes' parakeets are distinguished from Chatham Island Red-crowned parakeets by a yellow crown with red frontal band that does not extend beyond the eyes. They differ from mainland Yellow-crowned parakeets (*C. auriceps*) by having yellowish cheeks and are generally larger in size. In contrast, Chatham Island Red-crowned parakeets, a subspecies of New Zealand Red-crowned parakeets, are very similar to the mainland

subspecies (*C. n. novaezelandiae*), in having only red crown plumage. Hybrids between Forbes' and Chatham Island Red-crowned parakeets can show a range of crown colourations intermediate between the two parent species (Nixon 1982). Forbes' parakeets seem to prefer native forest habitats to open vegetation, while Chatham Island Red-crowned parakeets are more commonly seen on scattered patches of grass (Taylor 1975). Forest clearance for farming on Mangere Island is believed to have promoted interspecific hybridisation between the two taxa in the past (Taylor 1975).

Examination of mitochondrial control region DNA sequences showed four distinct haplotypes from Mangere Island parakeets, and suggested past hybridisations with Chatham Island Red-crowned parakeets may have modified the Forbes' parakeet gene pool (Boon et al. 2001). Haplogroups 1 and 2 (denoted by MT1 and MT2 hereafter) sit respectively within, and basal to, a Red-crowned parakeet clade, and were suggested to represent lineages derived from Red-crowned parakeets hybridising with Forbes' parakeets in the past. Haplogroup 3 (MT3) is basal to all other New Zealand parakeets, and is thought to be the true ancestral Forbes' parakeet haplotype. Birds with the Chatham Island Red-crowned parakeet haplotype (MT4) are also found on Mangere Island.

Interspecific hybridisation can potentially assimilate and displace native genotypes (Huxel 1999), or threaten the existence of rare species (Rhymer & Simberloff 1996; Wolf et al. 2001). In the case of Forbes' parakeet, where introgression is from the generally more abundant Chatham Island Red-crowned parakeet, there is a high potential risk of complete extinction of the rare ancestral Forbes' parakeet genotypes. Conversely, hybridisation can also be a valuable source of genetic diversity capable of simulating the diversification of populations (Anderson & Stebbins 1954; Dowling & Secor 1997), and leading to rapid adaptive

evolution (Lewontin & Birch 1966). Through the use of molecular markers, studies in hybrid zones have greatly enhanced opportunities in understanding hybridisation patterns and the role of hybridisation in evolution (reviewed in Barton 2001).

Aimed at identifying 'populations possessing genetic attributes significant for present and future generations of the species in question', the Evolutionarily Significant Unit (ESU) concept was first proposed by Ryder (1986) in order to provide a rational basis for setting priorities in conservation that reflects the underlying genetic diversity. Various different criteria have been suggested to define ESUs (reviewed in Fraser & Bernatchez 2001). For example, Moritz (1994) defined ESUs as 'reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci', whereas Crandall et al. (2000) recommended ecological data and genetic variation of adaptive significance be taken into account in establishing ESUs.

To further understand the role that hybridisation plays in Forbes' parakeet and to help guide future management strategies, we first need to accurately identify hybrids and parental types. A scoring scheme based on crown morphology has been developed to identify Forbes' parakeets and their hybrids (Nixon 1982; Greene 2000). However, the correlation between crown plumage patterns and their underlying genetics had not yet been tested. Identification of "pure" Forbes' parakeets in the Mangere Island population is difficult, as it is not easy to find reliable diagnostic markers that are capable of performing this task. This matter is further complicated by the possibility that biological samples obtained in the field may be from birds nearest to the original Forbes' parakeets rather than "pure" Forbes' parakeets. We have previously isolated microsatellite loci from Forbes' parakeet (Chan et al. 2005), and along with mitochondrial control region markers (Boon et al. 2001), it is now possible to investigate

the relationship between morphology and genetics in Forbes' parakeet, and to examine the extent of hybridisation at the molecular level.

Materials and Methods

Sample Collection, Morphological Identification, and DNA Extraction

A total of 250 blood and feather samples were collected from the mixed parakeet population on Mangere Island, and a further 35 Chatham Island Red-crowned parakeet blood samples were collected from Rangatira. Most birds were caught in mist-nets. Three breast feathers were taken from all birds caught and were kept in labelled paper envelopes before being sent off the islands. Blood samples were also taken from the wing when liquid nitrogen was available for their storage.

Parakeets were assigned to morphological categories based on crown plumage. A simple five-category scoring scheme (developed by Greene 2000, adapted from Nixon 1982) was used to enable easy field application. MP1 is a bird with Forbes' parakeet morphology: a pure yellow crown and a red frontal band that does not extend to the eye. MP5 has Chatham Island Red-crowned parakeet morphology, with no yellow in the crown. MP2-MP4 cover the plumage variations found between these two. Categories were assigned using a combination of direct field observation and photographic records of the parakeets. A number of parakeets had feather samples collected but could not be assigned morphological categories because they were chicks when sampled and their plumage was not yet fully developed.

DNA was extracted from 3μl aliquots of blood samples using a phenol / chloroform method (Sambrook et al. 1989), or from feather tips with DNeasy Tissue Kit (Qiagen).

Screening of Mitochondrial Control Region Haplotypes

A convenient PCR-RFLP screening system has been designed to score the samples for mitochondrial control region DNA haplotypes (Ballantyne et al. 2004). Briefly, the mitochondrial control region was PCR amplified using primers designed by Boon et al. (2001), and the final 1.6kb amplified products were digested with various combinations of restriction enzymes, *Cla*I, *Hae*III, *Hind*III, and *Rca*I (Roche). Agarose gel electrophoresis was then used to analyse the digest fragments and produce characteristic patterns that could be used to assign individuals to membership of haplogroup. The reliability of this scoring method was confirmed by DNA sequencing of PCR products from randomly selected samples. DNA sequencing was performed on an ABI Prism 377 sequencer (Applied Biosystems).

Microsatellite Genotyping

Six loci, *Cfor0809*, *Cfor1415*, *Cfor1617*, *Cfor2021*, *Cfor2829*, and *Cfor3031* were selected for this population study. Two other previously isolated polymorphic loci were not used. These were *Cfor1819*, which showed size homoplasy at the 184bp allele by DNA sequencing, and *Cfor2627*, where some individuals are homozygous for null alleles (Chan et al. 2005).

PCR amplifications were carried out in 1x PCR buffer (Qiagen), 2mM MgCl$_2$, 50mM KCl, 0.1mM dNTPs, 1μM fluorescein-12-dUTP (Roche), 0.4μM of each primer (0.8μM for *Cfor2021*), and 1U *Taq* DNA polymerase (Qiagen). The reactions were run on a Perkin Elmer 480 thermal cycler under conditions described in Chan et al. (2005). The products were analysed using an ABI Prism 377 sequencer and GeneScan Analysis 3.7 software (Applied Biosystems).

Evaluation of the Stepwise Mutation Model in Forbes' Parakeet Microsatellite Loci

A likelihood test was performed using the software MISAT (Nielsen 1997) to assess the goodness-of-fit of the stepwise mutation model (SMM; Ohta & Kimura 1973) to the allele frequency spectra of the loci used in this study. The estimator of variance in allele size θ (Valdes et al. 1993), the estimated proportion of multi-step mutations p, and the likelihood value $L(\theta)$ were estimated using a Markov chain recursion method set for 10^5 runs. The null hypothesis that a locus evolves via SMM was tested by calculation of the likelihood ratio $-2\log\lambda$, following Nielsen & Palsbøll (1999), where $\lambda=\max[L(\theta, p=0)]/\max[L(\theta, p)]$. At present, the design of this software does not cater for loci with more than one repetitive theme. Here, the statistics for the locus *Cfor2829*, which contains both dinucleotide and tetranucleotide repeats (Chan et al. 2005) were not calculated.

Genetic Differentiation Between Mangere Island and Rangatira Populations at Microsatellite Loci

The estimator of F_{ST} (Weir & Cockerham 1984), and the inbreeding coefficient F_{IS} were calculated using the Microsatellite Analyser software (version M3.15; Dieringer & Schlötterer 2002). The alternate statistic R_{ST} (Slatkin 1995), which is analogous to F_{ST} but also allows for allele size differences under the SMM (Ohta & Kimura 1973), was estimated by the GENEPOP software (version 3.4; Raymond & Rousset 1995). Differentiation between the Mangere Island and Rangatira populations was also assessed by Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) as implemented in the software GeneticStudio (version 2.01; Dyer & Sork 2001).

Testing for Genetic Bottleneck Effects in the Parakeet Populations

In a bottlenecked population, reduction in allelic diversity (allele numbers) and heterozygosity can be observed, and allelic diversity is reduced faster than heterozygosity. Therefore, recently bottlenecked populations are expected to show heterozygosity values larger than those expected from the observed number of alleles in the population (Cornuet & Luikart 1996). The possibility of recent bottlenecks in the Mangere Island and Rangatira parakeet populations was tested with microsatellite loci using the software Bottleneck (version 1.2.02; Cornuet & Luikart 1996). Three tests for heterozygosity excess, each one under the infinite allele model (IAM; Kimura & Crow 1964), stepwise mutation model (SMM; Ohta & Kimura 1973), and the two-phase mutation model (TPM; Di Rienzo et al. 1994) are implemented by Bottleneck. All bottleneck tests were run for 10^6 replications, and the TPM tests were run assuming 95% single-step mutations and 5% multi-step mutations.

Bayesian Clustering and Population Assignment

Assignment tests were performed with the software NewHybrids (version 1.1b3; Anderson & Thompson 2002) using the microsatellite dataset. NewHybrids is similar to the Bayesian method of Pritchard et al. (2000), but given some basic information about the population structure, NewHybrids provides a more detailed picture using an inheritance model defined by genotype. The accuracy of the software in assigning an individual to the correct population was tested with a simulated genotypic dataset of two populations containing 285 individuals using the Easypop software (version 1.8; Balloux 2001). The results (not included) showed that 284 individuals were assigned to their correct population with a posterior probability ≥0.95. To determine the best strategy for the assignment runs, a test run was performed with a simple hypothetical hybridising population consisting of parental species, F_1 and F_2 hybrids,

and various first generation backcrosses. The test was performed using genotypic class and allele frequency assumptions described in Anderson & Thompson (2002) for the two parental classes, F_1 and F_2 hybrids. Under these settings, the assignment results showed that all parental individuals were assigned with a posterior probability ≥0.95 to their respective species, but hybrids and backcrosses showed variable success with respect to assignment scores, e.g. some backcross genotypes had posterior probabilities as high as 0.91 for assignment to a parent species. The parakeet microsatellite genotypic dataset was run for 10^6 sweeps after a burn-in period of 10^5 sweeps. The run was repeated 30 times and each run required 3.75 hours on a Macintosh computer with a PowerPC G3 900Mhz processor.

Ordination and Clustering of Individual Microsatellite Genotypes

Genetic distances between individuals, including the proportion of shared alleles (Bowcock et al. 1994) and deltamu $(\delta\mu)^2$ (Goldstein et al. 1995) distances were estimated with the Microsatellite Analyser software (version M3.15; Dieringer & Schlötterer 2002). The proportion of shared alleles distance matrix was analysed through Principal Coordinates Analysis implemented in the software PCO (Anderson 2003). Neighbor-joining trees (Saitou & Nei 1987) were constructed from 100 bootstrap replica $(\delta\mu)^2$ matrices with the Neighbor module, in the Phylip software package (version 3.61; Felsenstein 1989).

Correlation Between Genetic and Morphological Characters

The relationship between microsatellite assignments, mitochondrial control region DNA haplogroups, and crown plumage variations was analysed by Multiple Correspondence Analysis using the MCA module of the ADE-4 software package (Thioulouse et al. 1997). This analysis was performed

with 169 Mangere Island and the 34 Rangatira parakeet samples from which all three types of data (morphological, microsatellite and mitochondrial DNA) were collected.

Results

Morphological and Mitochondrial DNA Control Region Haplotype Scorings

Crown plumage morphotype data were collected for 169 Mangere Island parakeets and 34 Rangatira parakeets (full dataset can be obtained from authors). In the mixed population on Mangere Island, 136 (80% of total) of the 169 birds showed Forbes' parakeet crown morphology (MP1), 3 (2% of total) showed Chatham Island Red-crowned parakeet crowns (MP5), and 30 (18% of total) showed intermediate hybrid crown morphotypes (10 MP2, 11 MP3, and 9 MP4). On Rangatira, all 34 parakeets scored have typical Chatham Island Red-crowned parakeet morphology (MP5). Thus the preliminary morphological survey showed that the population on Mangere Island is predominantly made up of Forbes' parakeets, while all Rangatira parakeets are Chatham Island Red-crowned parakeets, in line with general expectations.

Screening of 203 of the Mangere Island parakeets and 34 of the Rangatira parakeets for their mitochondrial DNA inheritance patterns showed that, on Mangere Island, 136 (67% of total) belong to the Forbes' parakeet MT3 maternal lineage, 22 (11% of total) birds are maternally linked to Chatham Island Red-crowned parakeets (MT4), and respectively 26 (13% of total) and 19 (9% of total) parakeets have the MT1 and MT2 maternal lineages. This showed at least some of the Forbes' parakeet morphotypes (MP1) must have Forbes' parakeet mitochondrial DNA control region haplotype MT3, supporting the observation by Boon et al. (2001). The data also suggested the presence of cryptic hybrids on Mangere Island that resemble Forbes' parakeet very closely morphologically. In the Rangatira

population, the majority (30; 88% of total) of birds have Chatham Island Red-crowned parakeets MT4 maternal haplotype. A further 4 individuals (12% of total) with the MT2 haplotype are also present.

Mutation Pattern at Microsatellite Loci in Forbes' Parakeet

Allele frequency distributions varied across the microsatellite loci screened. For each of the loci *Cfor0809*, *Cfor1617*, *Cfor2021*, and *Cfor2829*, the recorded patterns are fairly similar between the two populations, but for *Cfor1415* and *Cfor3031*, some differences between the populations are apparent (Figure 2). Using the method described in Nielsen (1997), all five of the loci tested conformed to SMM expectations in the Rangatira population. However, in the Mangere Island population, only *Cfor1617* and *Cfor3031* conformed to strict SMM predictions, and the TPM is a better descriptor for *Cfor0809*, *Cfor1415*, and *Cfor2021* (Table 1). It is worth noting that stochasticity and sampling effects to some unknown extent may possibly account for some of the differences, and for loci *Cfor1617* and *Cfor2021*, distinguishing between the mutation models is difficult since there are only two observed alleles at each locus.

Sequencing of alleles from these six loci showed that the structure of the repeat motifs are identical between the two populations studied, and that size homoplasy was not detected.

Genetic Differentiation Between Populations and Estimation of Bottleneck Effects

Microsatellite variability was estimated between the Mangere Island and Rangatira parakeet populations at $F_{ST} = 0.12$ and $R_{ST} = 0.20$. The higher R_{ST} value suggested the two populations differed more in terms of allele sizes than allele frequencies. Significant differences between the two

populations was also suggested by AMOVA (0.18, $P = 0.01$). A moderate level of inbreeding in the populations was apparent as suggested by an inbreeding coefficient F_{IS} of 0.15.

Bottleneck testing using a one tailed Wilcoxon sign-rank test for heterozygosity excess in the Mangere Island population suggested against a significant bottleneck under the IAM ($P = 0.22$), SMM ($P = 0.78$), and TPM ($P = 0.58$). In the Rangatira population, tests for bottleneck were insignificant under either the IAM ($P = 0.05$), or SMM ($P = 0.50$), or TPM ($P = 0.41$).

Bayesian Assignment of Individuals Using Microsatellite Genotypes
Based on the posterior probabilities in the parental classes P(Forbes') and P(Red-crowned), parakeets from the Mangere Island and Rangatira were scored into three classes. Birds with P(Forbes') ≥ 0.95 were scored MS1 (Forbes' parakeet), those with P(Red-crowned) ≥ 0.95 were assigned as MS3 (Chatham Island Red-crowned parakeet), and all other birds which did not fit into either of these two classes were scored MS2 (hybrids or unable to assigned). Under this classification, the sampled population on Mangere Island was made up of 67 Forbes' parakeets (27% of total), 171 hybrids or unassigned (68% of total), and 12 Chatham Island Red-crowned parakeets (5% of total). On Rangatira, the sampled population was made up of 13 Chatham Island Red-crowned parakeets (37% of total) and 22 hybrids or unassigned individuals (63% of total).

A "pure" Forbes' parakeet, therefore, is required to pass three tests. It must be classified MS1 by microsatellites, MT3 by mitochondrial DNA haplotypes, and MP1 by morphology. A "pure" Chatham Island Red-crowned parakeet would have an MS3 microsatellite assignment, mitochondrial DNA haplogroup MT4, and MP5 class morphology. Birds

which do not match either or the other of these two strict criteria were put in a hybrids category, which also include birds unable to be assigned in the microsatellite assignment test. Applying this classification to the 169 Mangere Island and 34 Rangatira samples which have available microsatellite, mitochondrial DNA and morphological data, the sampled Mangere Island population appears to be made up of 30 (18%) of Forbes' parakeets, 137 (81%) of hybrids, and 2 (1%) of Chatham Island Red-crowned parakeets. On Rangatira, the samples consist of 12 (35%) Chatham Island Red-crowned parakeets and 22 (65%) hybrids.

Microsatellite Genetic Distances Between Individuals

A cumulative 52.6% of the total variability was represented by the two PCO axes in a Principal Coordinate Analysis of a distance matrix calculated using the Bowcock et al. (1994) distance based on proportion of shared alleles (Figure 3). This genetic distance data was compared to the assignment method described in the previous section, using mitochondrial DNA, morphological, and Bayesian analysis of microsatellite data. The Chatham Island Red-crowned parakeets assigned using the three criteria appeared to cluster fairly closely as a group towards the right hand side in the PCO plot (indicated by triangles), while Forbes' parakeets assigned by the three criteria (indicated by circles) are spread out more and lie towards the left in Figure 3. A large cloud of hybrids or unassigned birds are scattered widely in the centre of the plot but also lie more towards the Forbes' parakeet cluster.

Neighbor-joining trees constructed using $(\delta\mu)^2$ distances (Figure 4) seem to be of little value in identifying hybrids in the populations. The performance of the test was even worse when other genetic distance measures were used (data not shown). Bootstrap support values tend to be quite low, the highest being only 50%. The tree shows that the majority

of birds, regardless of their assigned identities, formed a large unresolved cluster. However, one clear extended branch is apparent and is largely made up of Mangere Island parakeets. Among the individuals found along this branch, eight were assigned as "pure" Forbes' parakeets and three were assigned as hybrids by the three criteria test.

Relationships Between Genetic and Morphological Variables

Association between parameters for "Red-crownedness" (microsatellite class MS3, mitochondrial haplogroup MT4, and morphological scales MP5) is clearly shown in the MCA plot of the variables (Figure 5). There seemed to be a somewhat weaker correlation between Forbes' parakeet parameters (MS1, MT3, and MP1), which are represented in less than 50% of the screened samples. Other "hybrid" factors (MS2, MT1, MT2, MP2-4) appeared as random features, except that MP4 was closer to the Red-crowned parameters and MP2 fell close to MP3. The plot can be interpreted to suggest that Forbes' parakeet phenotype and genotypes are not as clearly defined as the Chatham Island Red-crowned parakeet.

A more detailed analysis of the correlations was made by pairwise comparison of the three scoring variables in the Mangere Island population (Table 2). Comparing mitochondrial haplotypes with morphology, a total of 40 cryptic hybrids could be identified, that is, those which showed Forbes' parakeet morphotype, but have Red-crowned related mitochondrial haplotypes (Table 2a). The presence of these birds shows that individuals which look like Forbes' parakeets can have a hybridisation history undetectable by plumage phenotype. The microsatellite data also confirm that morphological Forbes' parakeets are actually made up of both "pure" Forbes' parakeets and hybrids (Table 2b). Most birds (74%) that were assigned by microsatellites as Forbes' parakeets (MS1) were birds that have a Forbes' parakeet mitochondrial

lineage (MT3; Table 2c), indicating a fairly high level of agreement between the two genetic tests.

Discussion

The Present Status of the Forbes' Parakeet Population

The results obtained in this study clearly show that Forbes' parakeets have already hybridised fairly extensively with Chatham Island Red-crowned parakeets. Despite this historical record of interspecific hybridisation written in the genes of their descendants, there is still a group of "pure" Forbes' parakeets that remains entirely genetically distinct from Chatham Island Red-crowned parakeets. These parakeets may not be genuine versions of the original Forbes' parakeet types that once inhabited Mangere Island before habitat modification and large-scale hybridisation. Nonetheless, they are likely to represent the nearest "pure" Forbes' parakeet types that still exist anywhere at present.

The original Forbes' parakeet population is believed to have experienced a reduction in population size when Mangere Island was cleared for farming (Taylor 1975). A lack of documentation regarding the parakeet population size before or after forest clearance on Mangere Island makes it quite difficult to determine whether the population is likely to have experienced a genetic bottleneck. Our microsatellite evidence does not support a significant recent genetic bottleneck in the Mangere Island population, but the low numbers of individuals observed by Taylor (1975) suggest there was a demographic bottleneck of uncertain duration in the history of this population.

Hybridisation can introduce adaptive genetic variability into a population (Seehausen 2004), and may even contribute positively to the evolution of a species (see Barton 2001). Further hybridisation in the Mangere Island

parakeet population, however, would only result in more complete mixing of the gene pool between the two species. The low numbers of "pure" or near-"pure" Forbes' parakeets also suggest that hybridisation has been slowly displacing the Forbes' parakeet parental genotypes, and may constitute a threat to the long-term survival of the integrity of the species, not withstanding the hypothetical benefits.

In any recently derived group of parrots such as the *Cyanoramphus* parakeets (Boon et al. 2001), a potential to hybridise persists due to the relatively slow development of postzygotic isolating mechanisms (Bigelow 1965; Prager & Wilson 1975; Grant & Grant 1992). Despite field observations showed Mangere Island parakeets preferentially mate with morphologically similar parakeets (unpublished data), knowledge about pre-mating barriers between these two species of parakeets is still very limited. The abundance of hybrids on Mangere Island suggests there may be very little pre-mating isolation between Forbes' parakeets, Chatham Island Red-crowned parakeets and their hybrids under the ecological conditions now present.

A major obstacle for the conservation of Forbes' parakeets is arriving at a correct and practical definition of the "pure" Forbes' parakeet. The previous system based solely on morphology suffers from a serious failing in its ability to identify cryptic hybrids. Resolution based on molecular genetics alone is also limited due to failure to resolve some hybrid individuals in the microsatellite assignment test. Also, our PCO plot of genetic distances (Figure 3) indicated that the birds classified as Forbes' parakeet can have very variable microsatellite genotypes which some hybrids or unresolved samples shared, and it suggested a number of birds having similar microsatellite genotypes as Forbes' parakeets had a hybridisation history only detectable by mitochondrial haplotypes or crown plumage. Therefore, the three criteria identification system based on both

genetic and morphological factors is suggested as the best available present solution.

All the birds that we classified as "pure" Forbes' parakeets undoubtedly form an ESU. These birds are important regardless of whether or not they represent the original Forbes' parakeet types. Their significance derives from the fact that they all have the MT3 mitochondrial haplogroup that is phylogenetically basal to all other New Zealand *Cyanoramphus* species (Boon et al. 2001), they still show significant divergence from Chatham Island Red-crowned parakeets with respect to allele frequencies at nuclear microsatellite loci, and they are morphologically unique.

Number of Loci and Power of Assignment Using Microsatellite Genotypes
The resolution and power of assignment tests are dependent on the number of loci included in the analysis and the methods used. Earlier studies using restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), and protein electrophoresis have suggested that four or five markers would be sufficient to classify parental, F_1 hybrids and simple backcrosses, but that these may not be able to distinguish more complex backcrosses (Boecklen & Howard 1997). Removal of loci *Cfor1415*, *Cfor1617*, and *Cfor3031* in our assignment test changed the outcome more than removal of other loci (data not shown), suggesting that more robust assignments might be achieved by using more informative loci. This very desirable objective is limited in parrots by the technical difficulties inherent in isolating a large number of microsatellite loci from the taxa (Hughes et al. 1998; Robertson et al. 2000; Russello et al. 2001; Caparroz et al. 2003; Sainsbury et al. 2004). This may due to a generally low abundance of microsatellites in birds (Primmer et al. 1997).

General Outlook for the Conservation of Forbes' Parakeet

Our results have shown that reasonable numbers of Forbes' parakeets that are both morphologically and genetically distinct from Chatham Island Red-crowned parakeets still exist in the Mangere Island population. Although these individual parakeets do have a greater or lesser history of genetic introgression, and none may represent the original Forbes' parakeets present before the modification of the habitat, they are distinct, and hence deserve special conservation attention. Acceptance of some genetic introgression and hybridisation as part of the natural history of Forbes' parakeet does not reduce the survival chances of the species as a whole or diminish its importance.

Hybrids now present on the island were likely to have arisen in part by natural process and in part from anthropogenic causes, since the two species probably co-existed (and hybridised) before human arrival on Mangere Island. Subsequent human activities have certainly promoted interspecific hybridisation. Natural hybrids themselves may deserve some form of special protection (Allendorf et al. 2001) to preserve genetic diversity, but in this case, they should only be protected if this can be achieved without threatening the "pure" or near-"pure" Forbes' parakeets via further backcross hybridisation. The ongoing survival of Forbes' parakeets will depend on careful monitoring and genetic control of the population.

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Figure 1.   Map of the Chatham Islands group of New Zealand, showing Mangere Island and Rangatira where field samples were collected.
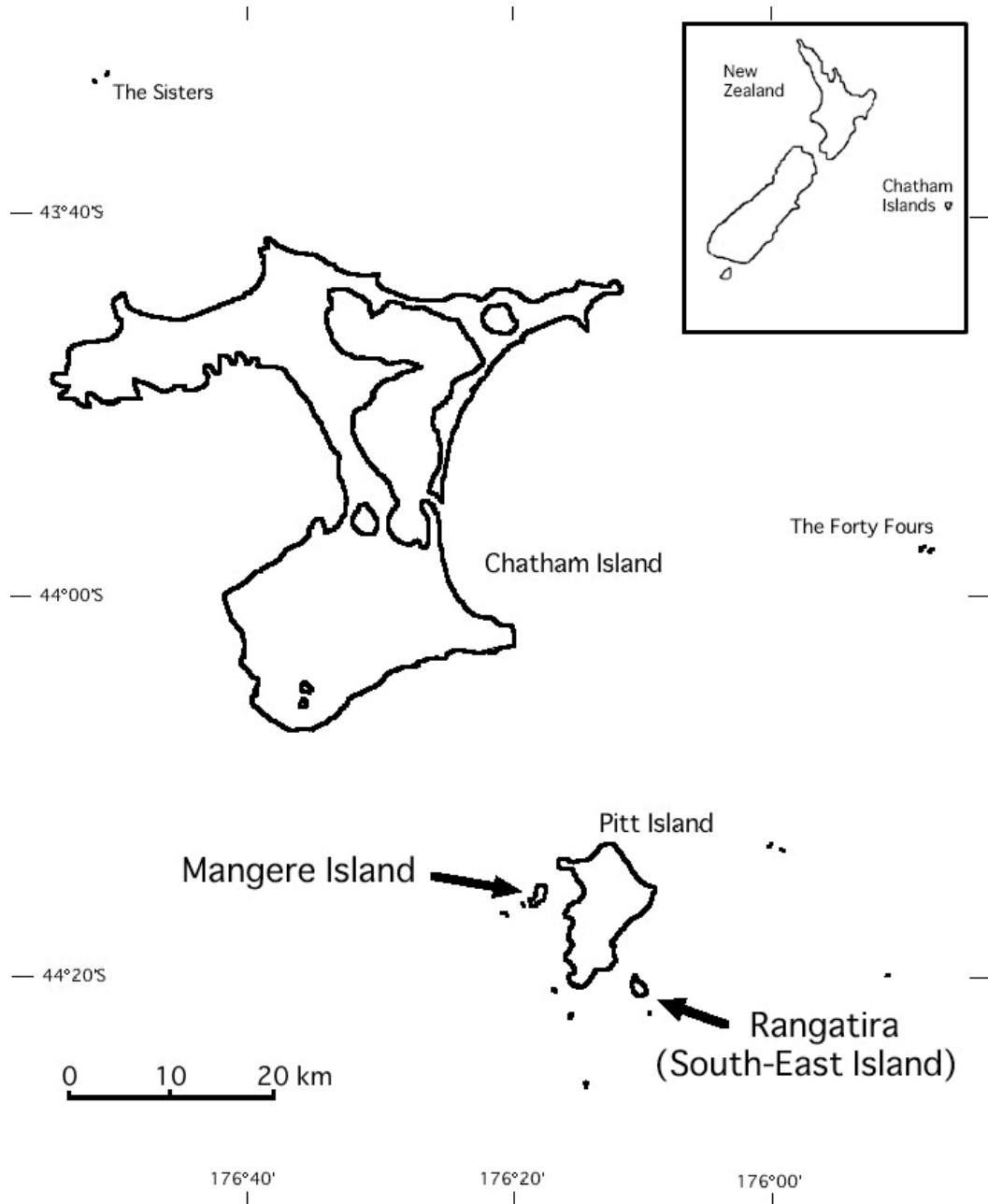
Figure 2.   Allele frequency distributions of the six microsatellite loci in two populations of parakeets.   The Mangere Island parakeets and Rangatira parakeets are respectively represented by black and grey bars. The x-axis in each plot shows the allele sizes, and the y-axis shows the allele frequencies.
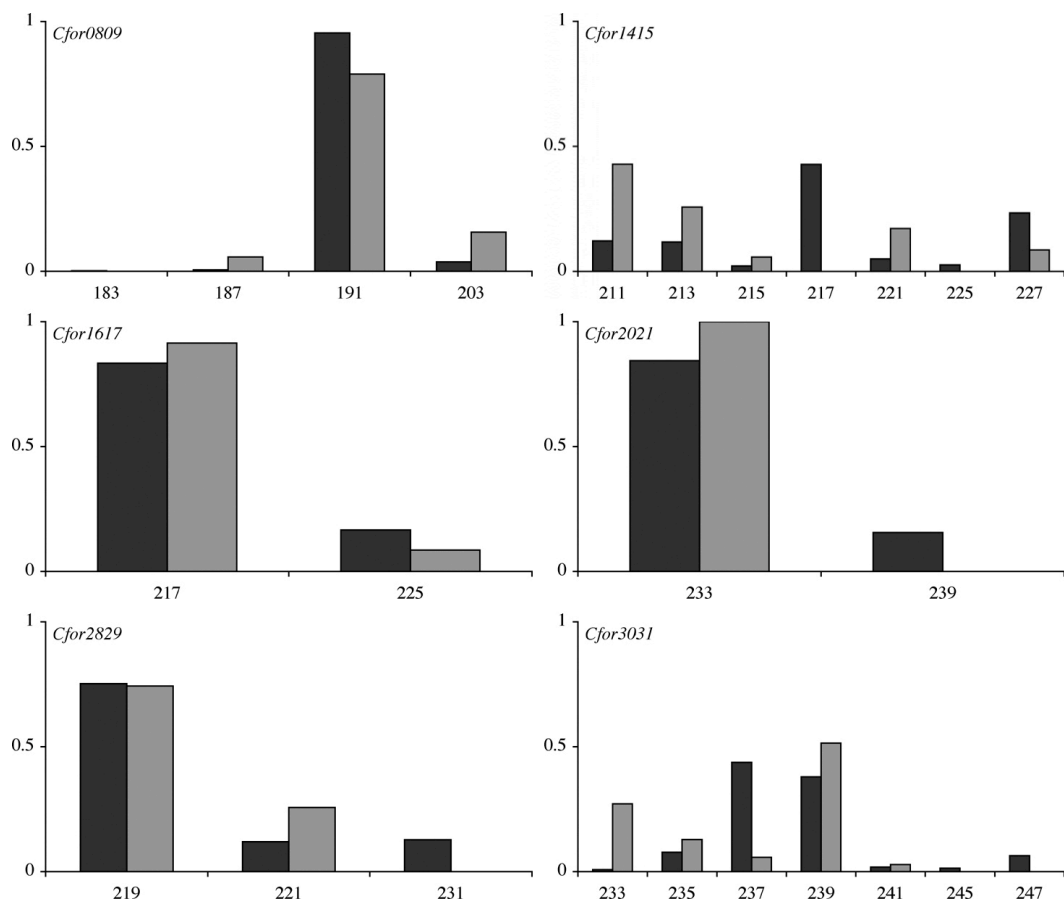
Figure 3.   Principal Coordinate Analysis of distance matrix constructed using proportion of shared microsatellite alleles distances (Bowcock et al. 1994). PCO axis 1 and 2 respectively represent 31.5% and 21.1% of the total variability. Birds were assigned as Forbes' parakeet, hybrid, or Chatham Island Red-crowned parakeet using mitochondrial DNA control region, morphological data, and microsatellites data by Bayesian method. The symbol ● represents Forbes' parakeets, ✳ represents hybrids, and ▲ represents Chatham Island Red-crowned parakeets classified using the three criteria test.
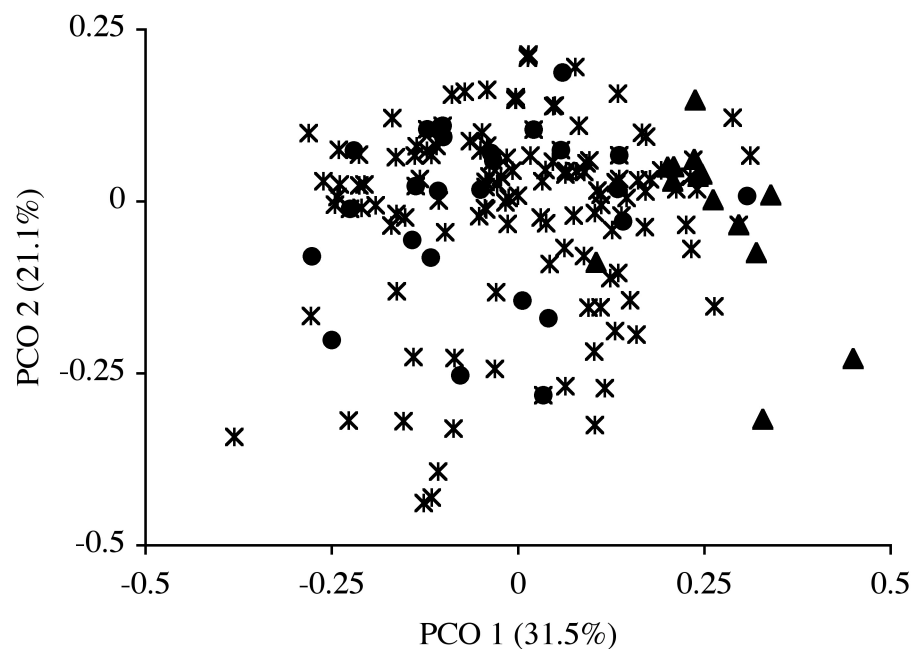
Figure 4. Neighbor-joining tree built from $(\delta\mu)^2$ genetic distances with 100 bootstrap pseudo replicates. Forbes' parakeets, hybrids, and Chatham Island Red-crowned parakeets were represented by letters F, H, and R respectively. Parakeets with no available mitochondrial DNA and/or morphological data were labelled M (Mangere Island) or S (Rangatira) dependent on the site of sample collection.
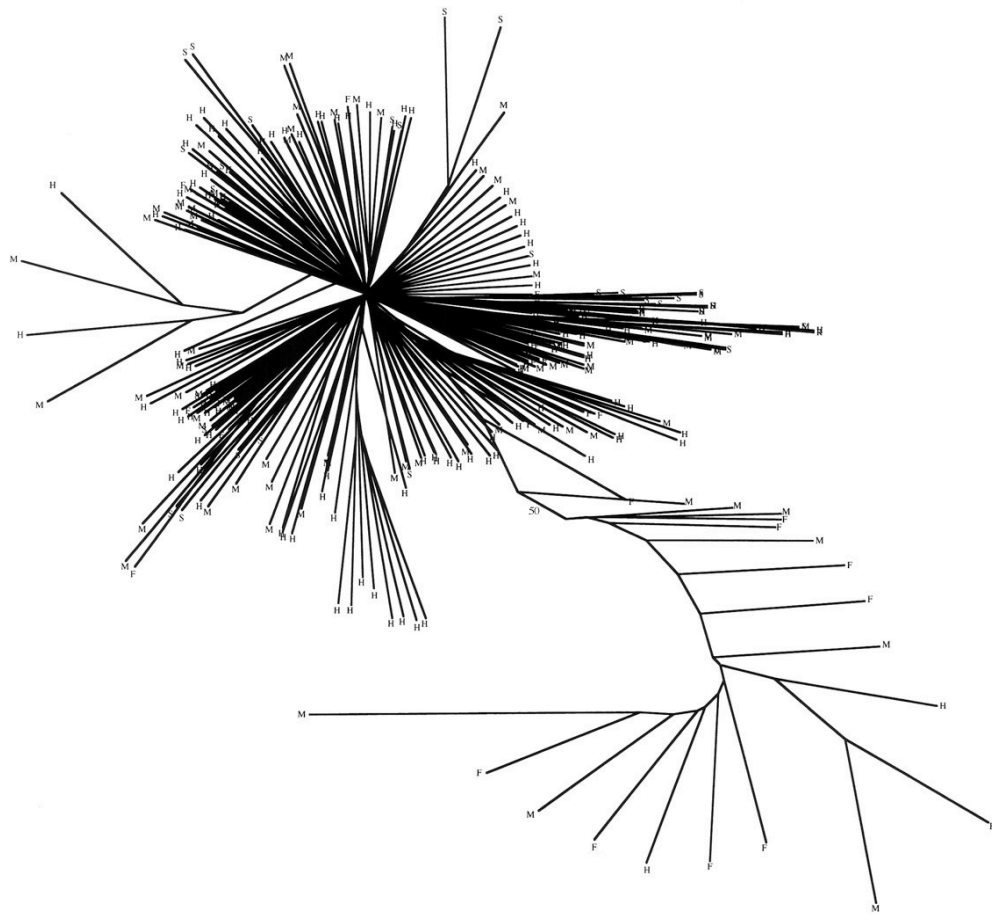
Figure 5.   Relationships between genetic and morphological variables analysed by Multiple Correspondence Analysis. Ellipses from centre show correlations fit 50, 90, and 99% of samples.   The x- and y-axes respectively represent 24.1% and 13.0% of the total variation between the variables.   Forbes' parakeet characters are symbolised by ●, hybrid characters are symbolised by ✱, and Chatham Island Red-crowned parakeet characters are symbolised by ▲.
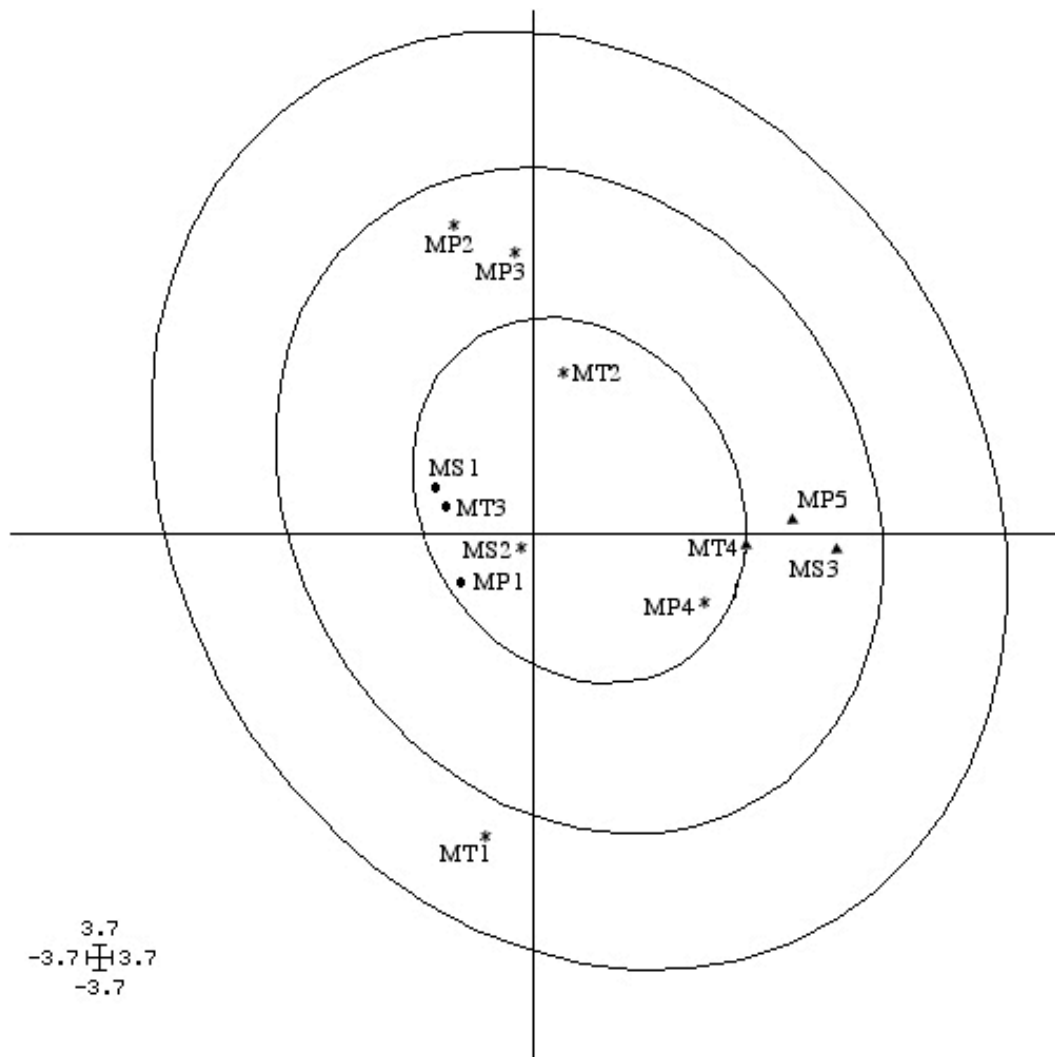
Table 1.　　Mutation behaviour of *Cfor* loci.　*p* indicates the proportion of multi-step mutations at max $L(\theta)$.

Locus	Population	*p*	-2logλ	Mutation Model
Cfor0809	Mangere	0.475	7.50	TPM
	Rangatira	0.400	1.26	SMM
Cfor1415	Mangere	0.025	14.68	TPM
	Rangatira	0.025	0.21	SMM
Cfor1617	Mangere	0.475	0.99	SMM
	Rangatira	0.475	0.29	SMM
Cfor2021	Mangere	0.475	3.84	TPM
	Rangatira	-	-	-
Cfor3031	Mangere	0.000	0.00	SMM
	Rangatira	0.000	0.00	SMM

Table 2.　　Relationship between variables among 169 Mangere Island samples.　a) mitochondrial haplotypes versus morphology; b) microsatellites versus morphology; c) microsatellites versus mitochondrial haplotypes.　Number of individuals in each category is in brackets.

a)　Mitochondrial haplotypes	Morphology scores				
	MP1 (136)	MP2 (10)	MP3 (11)	MP4 (9)	MP5 (3)
MT1 (21)	20	0	0	1	0
MT2 (18)	11	2	2	2	1
MT3 (112)	96	8	7	1	0
MT4 (18)	9	0	2	5	2

b)　Microsatellites	Morphology scores				
	MP1 (136)	MP2 (10)	MP3 (11)	MP4 (9)	MP5 (3)
MS1 (43)	38	2	3	0	0
MS2 (119)	97	8	7	6	1
MS3 (7)	1	0	1	3	2

c)　Microsatellites	Mitochondrial haplotypes			
	MT1 (21)	MT2 (18)	MT3 (112)	MT4 (18)
MS1 (43)	3	4	32	4
MS2 (119)	16	13	78	12
MS3 (7)	2	1	2	2

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Chapter 4:   Microsatellite Evolution in Parrots

4.1   Introduction

Although isolating microsatellite loci can be tedious (see Zane et al. 2002), once developed, microsatellite primers can often be used productively across a range of more or less closely related species (Moore et al. 1991; Primmer et al. 1996) because the priming sites of some microsatellites can be conserved over millions of years, e.g. in turtles (FitzSimmons et al. 1995), birds (Primmer et al. 1996), fish (Rico et al. 1996), and wasps (Ezenwa et al. 1998).

Microsatellites are often highly polymorphic with respect to repeat number variations (Weber & May 1989; Weber 1990). However, diversity in microsatellite alleles can also arise from indels and / or single base substitutions within repeats (Estoup et al. 1995; Angers & Bernatchez 1997; Viard et al. 1998; Taylor et al. 1999) and from variations in the flanking sequences (FitzSimmons et al. 1995; Grimaldi & Crouau-Roy 1997).

Variations observed in microsatellite loci are often caused by repeat number changes, and often conform to the infinite allele model (IAM; Kimura & Crow 1964), the stepwise mutation model (SMM; Ohta & Kimura 1973), or the two-phase model (TPM; Di Rienzo et al. 1994) at the population level. However, insertion or deletion events, and single nucleotide polymorphisms also occur frequently within some microsatellite loci, suggesting the existence of a more complex, and presently unknown pattern of evolution (reviewed by Chambers & MacAvoy 2000).

The evolution of parrots has been studied fairly extensively. However, no consensus has yet been reached regarding their current global phylogeny.

A wide range of characters has been employed in constructing parrot phylogenies, including morphological and anatomical features (Burton 1974; Smith 1975), karyotypes (Schmutz & Prus 1987), protein electrophoresis (Christidis et al. 1991; Triggs & Daugherty 1996), and mitochondrial DNA variation (Ovenden et al. 1987; Birt et al. 1992; Leeton et al. 1994; Miyaki et al. 1998; Boon et al. 2000; Groombridge et al. 2004; Ribas & Miyaki 2004). One of the factors that has created practical difficulties in constructing a reliable global phylogeny for parrots is the rearrangement of the mitochondrial genome in some species (Eberhard et al. 2001). So far, it has been recognised that the global parrot biota is made up of three main vicariant lineages: African (AF), Australasian (AU), and Neotropical (NT), with subdivisions within each lineage (see Boon 2000). Amplification of conserved microsatellite loci across related species presents a possibility of constructing phylogenies based on these loci, either using distance methods (Bowcock et al. 1994), or using standard phylogenetic methods based on flanking region sequence changes between taxa (Zardoya et al. 1996; Sainsbury 2004).

With the aims of enhancing the understanding of parrot evolution and the evolutionary process of microsatellite loci, this chapter evaluates the use of flanking sequences of loci developed for Forbes' parakeet (see Chapter 2) for phylogenetic construction. Microsatellite repeat motif evolution is investigated through mapping changes on to a phylogeny derived from Boon (2000).

4.2   Methods

4.2.1 Cross-species Amplification of Microsatellite Loci

Genomic DNA from a range of parrot tissue samples (Table 4.1) was available in the IMS collection. These were extracted by Boon (2000) and Sainsbury (2004). For this study, DNA from *Coracopsis nigra barkyli* and

Poicephalus senegalus (see Boon 2000) was extracted from feather samples available in the IMS collection with DNeasy Tissue Kit (Qiagen), following the manufacturer's protocol.

PCR amplification conditions were as described for Forbes' parakeet in Chapter 2, section 2.2.16, except that the primer annealing temperature was lowered from the optimal temperature (see Chapter 2, Table 2.1) for Forbes' parakeet to 50ºC in some non-*Cyanoramphus* species (see Table 4.1). The criterion for successful amplification were as described by Ezenwa et al. (1998), namely, the detection by agarose gel electrophoresis of a clear band within 200 bp of the expected length. PCR products judged to have been successfully amplified were characterised by DNA sequencing of both strands as described in Chapter 3, section 3.2.1. The DNA sequences were aligned manually with the aid of the MacClade software (version 4.06; Maddison & Maddison 2003).

4.2.2 Species Examined in this Study

Parrot taxa are classified following Boon (2000; see Table 4.1), recognising three major geographical groupings: African (AF), Australasian (AU), and Neotropical (NT). African parrot samples used in this study were: *Coracopsis nigra barkyli* (Seychelles black parrot), *Poicephalus senegalus* (Senegal parrot), *Poicephalus meyeri* (Meyer's parrot), and *Psittacus erithacus* (African grey parrot). Australasian parrots can be classified into four main lineages, AU-NZ (New Zealand *Nestor* and *Strigops*), AU-C (Cockatoos, Cockatiels and Galah), AU-L (Lorikeets), and AU-1 (all other Australasian parrots). AU-NZ parrots studied were: *Strigops habroptilus* (Kakapo), *Nestor notabilis* (Kea), and *Nestor meridionalis* (Kaka). AU-C species studied were: *Nymphicus hollandicus* (Cockatiel), *Eolophus roseicapillus* (Galah), and *Cacatua haematuropygia* (Red-vented cockatoo). AU-L species studied were: *Glossopsitta pusilla*

(Little lorikeet), and *Trichoglossus haematodus* (Rainbow lorikeet). AU-1 parrots studied included: *Cyanoramphus forbesi* (Forbes' parakeet), *Cyanoramphus novaezelandiae novaezelandiae* (Red-crowned parakeet), and the Chatham Island subspecies *Cyanoramphus novaezelandiae chathamensis* (Chatham Island Red-crowned parakeet), *Cyanoramphus auriceps* (Yellow-crowned parakeet), *Cyanoramphus malherbi* (Orange-fronted parakeet), *Cyanoramphus hochstetteri* (Reischek's parakeet), *Cyanoramphus unicolor* (Antipodes Island green parakeet), *Cyanoramphus saisetti* (New Caledonian Red-crowned parakeet), *Eunymphicus cornutus* (Horned parakeet), *Eunymphicus uvaeensis* (Ouvea parakeet), *Barnardius barnardi* (Mallee ringneck parrot), *Micropsitta pusio* (Buff-faced pygmy parrot), *Platycercus eximius* (Eastern rosella), *Psittacula alexandri* (Red-breasted parakeet), *Psephotus varius* (Mulga parrot), and *Melopsittacus undulatus* (Budgerigar). The Neotropical (NT) parrot studied was *Ara ararauna* (Blue-and-gold macaw).

4.2.3 *Characterization of Flanking Region Sequences*

The sequences obtained were searched for homology to published sequences at the Genbank/EMBL/DDBJ databases (http://www.ncbi.nlm.nih.gov) using the nucleotide BLAST tool. To determine whether the microsatellite loci contained coding regions, the entire *C. forbesi* loci sequences were checked for stop codons in all 6 possible reading frames using the MAPDRAW software (Lasergene version 4.0; DNASTAR Inc.). Non-triplet insertions or deletions that would result in frameshifts were checked in interspecies sequence alignments constructed with the MacClade software (version 4.06; Maddison & Maddison 2003).

4.2.4 Phylogenetic Trees Construction Within Cyanoramphus

Using the flanking sequences of microsatellite loci, phylogenetic trees were constructed between *Cyanoramphus* species. The microsatellite repeat arrays were excluded in the analysis to avoid confusion caused by repeat number mutations. Choice of loci was determined by the proportion of variable and informative sites in the flanking regions between the species of interest. The two loci with the largest number of variable sites, *Cfor1415* and *Cfor3031* (with respectively 3.87% and 2.33% of variable sites), were used.

Using the software PAUP (version 4.0b10PPC; Swofford 1998), phylogenies were constructed using the maximum parsimony (Camin & Sokal 1965) and maximum likelihood (Felsenstein 1981) algorithms. All trees were unrooted. Confidence intervals of phylogenies were determined using the bootstrap method (Efron 1979; Felsenstein 1985) with 1000 replicates.

4.2.5 Construction of Phylogeny Between Parrot Taxa

The flanking sequences at most microsatellites do not show sufficient variation to produce well supported phylogenies for parrot taxa (see section 4.3.3). The matter is further complicated by the limited availability of other DNA sequences characterized across taxa (see Boon 2000 for instance). A neighbour-joining phylogeny (Saitou & Nei 1987) can be constructed using the PAUP software (version 4.0b10PPC; Swofford 1998) from only 267 nt available common mitochondrial cytochrome *b* sequences (Boon 2000) between taxa in this study. Since mitochondrial cytochrome *b* sequences are not available for all taxa examined in this microsatellite study, the original neighbour-joining tree has been expanded by incorporating some of the placements from mitochondrial 12S rRNA trees in Boon (2000) to allow mapping of microsatellite arrays in a wider

range of taxa. Two loci, *Cfor1819* and *Cfor3031*, were selected for the mapping exercise since these loci contain simple, comprehensible and variable repeat structures between parrot taxa.

4.3   Results

4.3.1 Cross-species Microsatellite Amplifications

All nine loci were amplified successfully from all *Cyanoramphus* species tested, but the results of amplification reactions varied in other parrots (Table 4.1).   Sequencing of the PCR products confirmed that all successful cross-species PCRs amplified the same loci as in *Cyanoramphus forbesi*.

4.3.2 Search for Coding Regions within Flanking Sequences

Alignment searches of *Cfor* loci on GenBank/EMBL/DDBJ online databases showed no matches with non-*Cfor* entries (as at December 2004) with scores > 50 bits or E-values < 0.001. Translation of the *Cfor* sequences showed that all loci have stop codons in one or more reading frames, indicating the possibility of the presence of open reading frames. However, since all the loci have either di- or tetranucleotide repeat units, any repeat unit number mutations would disrupt open reading frames. The possibility is low that any of these loci contain an open reading frame or substantial part of one.

Table 4.1   Amplification of *Cfor* loci in other parrot and cockatoo species. Symbols + = amplification of a microsatellite, - = no amplification. Δ indicates a reduction of annealing temperature to 50°C is required to achieve amplification.

	Species	Locus								
		Cfor0809	Cfor1415	Cfor1617	Cfor1819	Cfor2021	Cfor2223	Cfor2627	Cfor2829	Cfor3031
Australasian	*Cyanoramphus novaezelandiae chathamensis*	+	+	+	+	+	+	+	+	+
	Cyanoramphus novaezelandiae novaezelandiae	+	+	+	+	+	+	+	+	+
	Cyanoramphus auriceps	+	+	+	+	+	+	+	+	+
	Cyanoramphus malherbi	+	+	+	+	+	+	+	+	+
	Cyanoramphus hochstetteri	+	+	+	+	+	+	+	+	+
	Cyanoramphus unicolor	+	+	+	+	+	+	+	+	+
	Cyanoramphus saisetti	+	+	+	+	+	+	+	+	+
	Eunymphicus cornutus	+	+	+	+	+	+	+	+	+
	Eunymphicus uvaeensis	+	+	+	+	+	+	+	+	+
	Platycercus eximius	Δ	+	+	+	-	Δ	-	-	+
	Psephotus varius	-	+	Δ	-	-	-	+	-	+
	Barnardius barnardi	+	+	+	+	-	Δ	+	+	-
	Melopsittacus undulatus	-	-	-	Δ	-	-	+	-	-
	Micropsitta pusio	+	-	+	+	+	-	+	-	+
	Psittacula alexandri	+	+	+	+	-	-	+	-	+
	Trichoglossus haematodus	+	-	+	-	+	-	+	-	+
	Glossopsitta pusilla	Δ	-	+	+	+	-	+	-	+
	Strigops habroptilus	-	-	-	Δ	-	Δ	-	-	-
	Nestor notabilis	+	-	Δ	-	-	-	-	-	-
	Nestor meridionalis	+	-	Δ	-	-	-	-	-	-
African	*Coracopsis nigra barkyli*	+	-	-	+	+	-	+	-	+
	Poicephalus senegalus	+	-	+	+	Δ	-	+	+	+
	Poicephalus meyeri	+	-	+	+	-	-	+	-	-
	Psittacus erithacus	+	-	+	+	Δ	-	+	-	+
Neotropical	*Ara ararauna*	+	+	+	Δ	-	-	+	-	-
Cacatuidae	*Nymphicus hollandicus*	+	+	+	+	+	+	+	+	-
	Eolophus roseicapillus	Δ	-	+	-	-	-	+	-	-
	Cacatua haematuropygia	Δ	-	-	-	-	-	+	-	-

4.3.3 Within-Cyanoramphus Phylogenetic Relationships

Phylogenetic trees for *Cyanoramphus* parakeets were constructed with the flanking sequences of *Cfor1415* (181 bp) and *Cfor3031* (215 bp) loci (Figure 4.1). Both trees showed a closer relationship between *C. unicolor* and *C. hochstetteri* than between other species. In the tree constructed based on flanking sequences of *Cfor3031*, the two subspecies of Red-

crowned parakeets (*C. novaezelandiae chathamensis*, and *C. n. novaezelandiae*) did not cluster together as expected (Figure 4.1b). This reflects the fact that the use of short sequences for phylogenetic constructions may lead to errors in the resulting tree topologies. Resolution is generally lower than for phylogenies previously constructed by Triggs & Daugherty (1996) and by Boon (2000) due to a lack of signal in the microsatellite flanking regions. Resolution did not improve when the flanking sequences from *Cfor1415* and *Cfor3031* were concatenated (data not shown). The trees obtained here do not have high bootstrap support for most branches. In *Cfor1415* interruptions to the core repeat tract were observed in *C. novaezelandiae chathamensis*, *C. auriceps*, and *C. malherbi* (Figure 4.1a). A G to A substitution in the middle of the *Cfor1415* repeats divides the TG repeats into two sections in *C. novaezelandiae chathamensis*, whereas in *C. auriceps* and *C. malherbi*, G to T substitutions were observed at the beginning of the tracts. These interruption patterns, however, may only be variation between individuals as the sample size screened is small. There is no major change of the microsatellite motif in *Cfor3031* (Figure 4.1b). In other loci, repeat structure changes are minor across *Cyanoramphus* (see Table 4.2).

The poor resolution and low bootstrap support of the trees suggest that the flanking regions do not show high enough differentiation between *Cyanoramphus* parakeets to allow a detailed and reliable phylogeny to be constructed.

4.3.4   Evolution of Cfor1819 in Parrots

Polymorphism in this locus was in the form of variation in $(CA)_n$ repeats. A change of the theme is observed in the two *Eunymphicus* species, where a $(CCA)_n$ motif has replaced the $(CA)_n$ motif from the second repeat unit (Figure 4.2a; Table 4.2). Interruptions in the repeat tracts were observed

in *C. n. chathamensis* and *B. barnardi*. In both species the interruptions
were made up of C mononucleotide repeats.

There is a clear pattern of microsatellite evolution in species related to
Cyanoramphus (Figure 4.2a). Parrots of the AF, NT, AU-NZ, AU-L
lineages and some AU-1 (*Melopsittacus*, *Micropsitta*, and *Psittacula*)
species do not show dinucleotide $(CA)_n$ repeats at this locus.

Figure 4.1   Phylogeny of *Cyanoramphus* parakeets constructed using
microsatellite flanking regions of (a) *Cfor1415* and (b) *Cfor3031*.
Maximum likelihood (ML) trees are positioned on the left, and maximum
parsimony (MP) trees are positioned on the right. The core repeat motifs
are mapped on the trees.

ML   MP

Cyanoramphus auriceps
(GT)$_8$

62   65

Cyanoramphus malherbi
(GT)$_{10}$

Cyanoramphus novaezelandiae novaezelandiae
(GT)$_{12}$

64   59

Cyanoramphus forbesi
(GT)$_{10}$

Cyanoramphus novaezelandiae chathamensis
(GT)$_{10}$

Cyanoramphus hochstetteri
(GT)$_9$

87   87

Cyanoramphus unicolor
(GT)$_9$

Cyanoramphus saisetti
(GT)$_8$
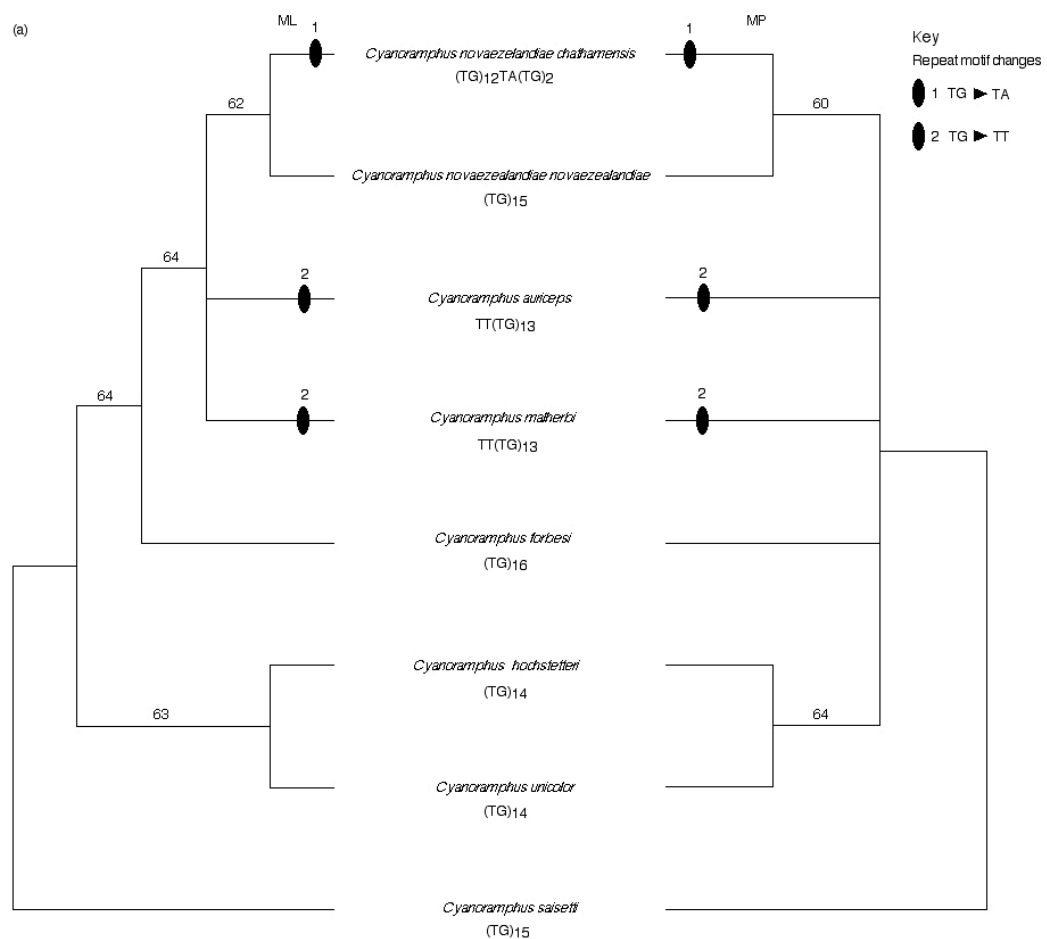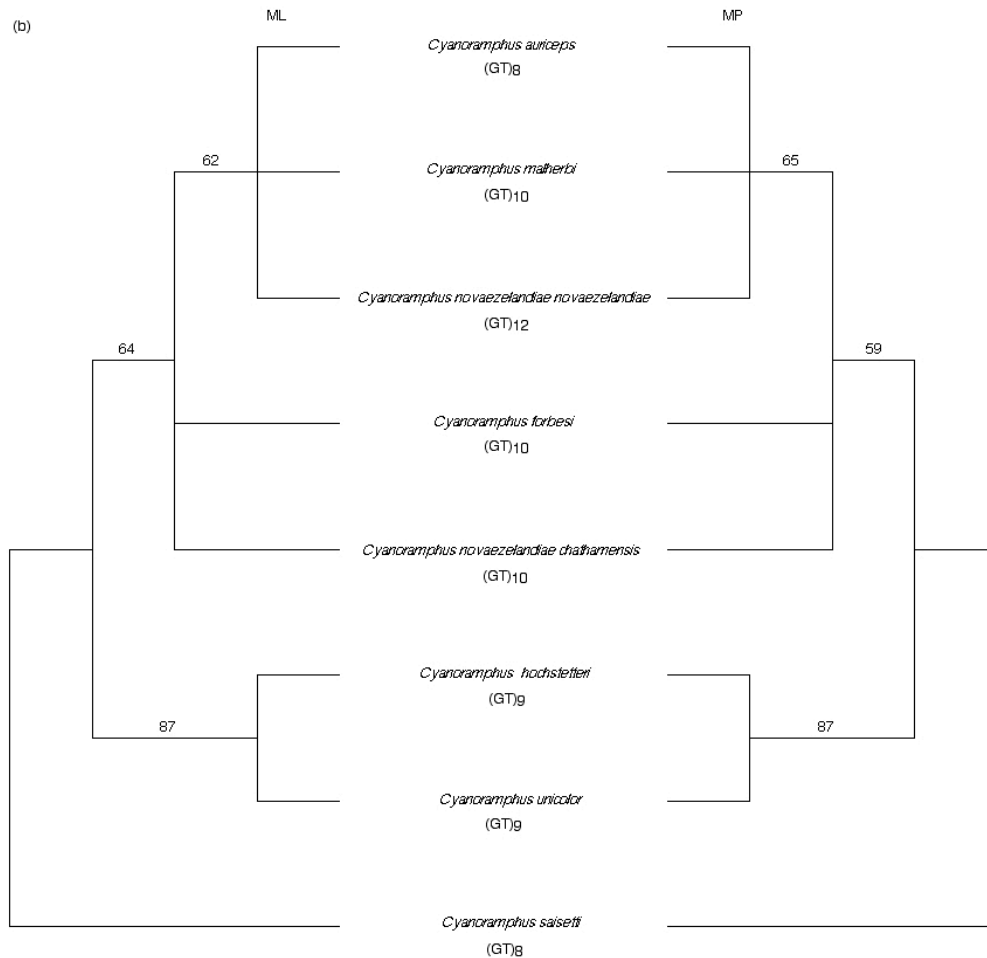
4.3.5 Evolution of Cfor3031 in Parrots

The repeat unit at this locus is (GT)$_n$. This locus is not very variable across species (Figure 4.2b; Table 4.2). Most groups have (GT)$_3$ repeat at this locus, except *Poicephalus senegalus* and *Psittacula erithacus* which had a (GT)$_6$ repeat, and *Cyanoramphus* and *Eunymphicus* species where larger number of repeats (n > 7) were observed. Groups with 3 or 6 GT repeats showed no within-species polymorphism in the individuals examined, while polymorphism was observed in *Cyanoramphus* and *Eunymphiucs* species.

Figure 4.2   Diagrammatic representation of phylogeny of parrot species. Neighbour-joining (Saitou & Nei 1987) phylogenetic constructions from 267 nt of cytochrome *b* sequences data (Boon 2000) are shown in bold lines.   Taxa from which cytochrome *b* data are unavailable have been added onto the phylogeny and represented by finer lines based on phylogenetic analysis of 12S rRNA data in Boon (2000).   Repeat motif changes for (a) *Cfor1819*, and (b) *Cfor3031* are mapped on the trees.   N/A indicates non-amplification of the microsatellite locus in a taxon.
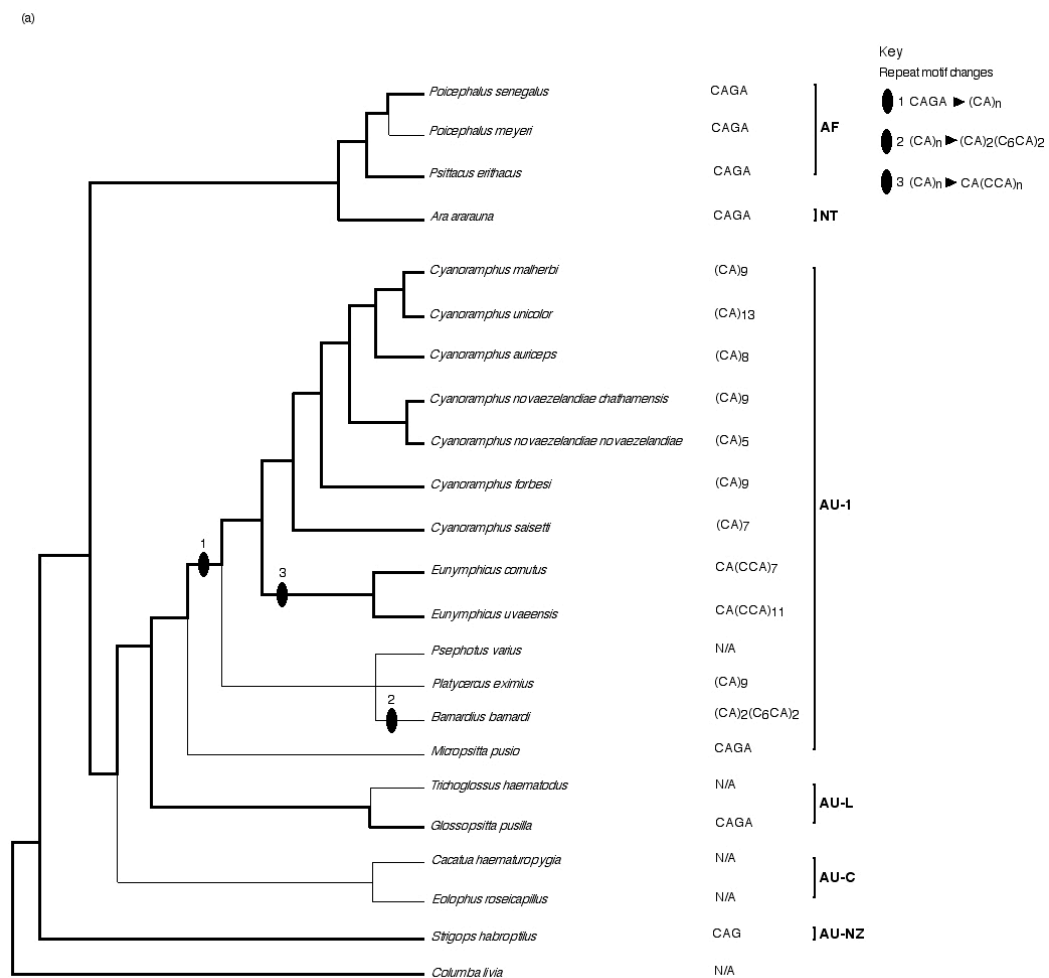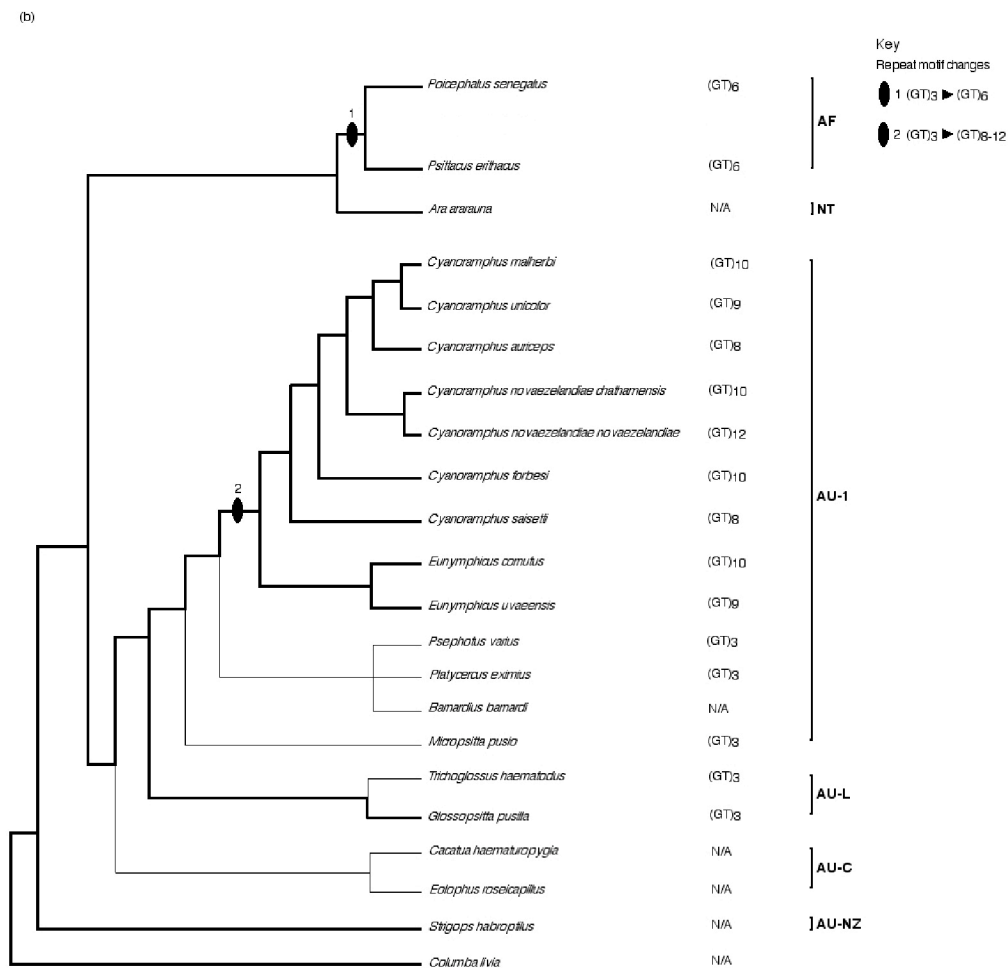
4.3.6 General Pattern of Microsatellite Evolution between Parrots

In loci *Cfor1819*, *Cfor2021*, *Cfor2627* and *Cfor3031*, the dinucleotide repeat tracts are bordered at one end by tracts of C_n/G_n mononucleotides (Table 4.2). The length of these mononucleotide tracts varied between species, and substitution or insertion mutations within these mononucleotide tracts can be observed in some species. Although in *Cfor3031*, species with longer dinucleotide repeats have less G mononucleotide repeats in the immediate bordering sequences, such pattern was not observed in other loci. In some species, the dinucleotide repeat tracts were interrupted with runs of mononucleotides of the same bases as the bordering mononucleotide tracts. This suggests the

bordering mononucleotides may play a role in the origin of the dinucleotide repeats.

Table 4.2 Association of dinucleotide microsatellite repeats with bordering mononucleotide repeat tracts.

Locus / Species / Lineage		Repeat Structure of Microsatellite and Bordering Sequences
Cfor1819		
C. nigra barkyli	AF	$CAGAC_4$
P. senegalus	AF	$CAGAC_5$
P. meyeri	AF	$CAGAC_5$
P. erithacus	AF	$CAGAC_4$
A. ararauna	NT	$CAGAC_5$
C. n. chathamensis	AU-1	$(CA)_9C_6$
C. n. novaezelandiae	AU-1	$(CA)_5C_4$
C. auriceps	AU-1	$(CA)_8C_4$
C. malherbi	AU-1	$(CA)_9C_7$
C. hochstetteri	AU-1	$(CA)_{12}C_4$
C. unicolor	AU-1	$(CA)_{13}C_4$
C. forbesi	AU-1	$(CA)_9C_4$
C. saisetti	AU-1	$(CA)_7C_6$
E. cornutus	AU-1	$CA(CCA)_7C_6$
E. uvaeensis	AU-1	$CA(CCA)_{11}C_3$
P. eximius	AU-1	$(CA)_9C_6$
B. barnardi	AU-1	$(CA)_2(C_6CA)_2CC$
P. alexandri	AU-1	$CAGAC_5$
M. pusio	AU-1	$CAGAC_5$
M. undulatus	AU-1	$CAGAC_4$
G. pusilla	AU-L	$CAGAC_5$
N. hollandicus	AU-C	$(CA)_{10}C_6$
S. habroptilus	AU-NZ	$CAGC_4TC$
Cfor2021		
C. nigra barkyli	AF	$G_6(TG)_2G(TG)_2G(TG)_2G(TG)_4$
P. senegalus	AF	$G_6TGTAG(TG)_2G(TG)_5$
P. erithacus	AF	$G_6TGTAG(TG)_2G(TG)_3CGTG$
C. n. chathamensis	AU-1	$G_4(TG)_2CGG(TG)_2G(TG)_6$
C. n. novaezelandiae	AU-1	$G_4(TG)_2CGG(TG)_2G(TG)_6$
C. auriceps	AU-1	$G_4(TG)_2CGG(TG)_2G(TG)_6$
C. malherbi	AU-1	$G_4(TG)_2CGG(TG)_2G(TG)_6$
C. hochstetteri	AU-1	$G_4(TG)_2CGG(TG)_2GG(TG)_3$
C. unicolor	AU-1	$G_4(TG)_2CGG(TG)_2GG(TG)_3$
C. forbesi	AU-1	$GAGG(TG)_2CGG(TG)_2G(TG)_9$
C. saisetti	AU-1	$G_4(TG)_2CGG(TG)_2G(TG)_6$
E. cornutus	AU-1	$G_4(TG)_2CGG(TG)_2G(TG)_4$
E. uvaeensis	AU-1	$G_4(TG)_2CGG(TG)_2G(TG)_7$
M. pusio	AU-1	$G_4(TG)_3G(TG)_5$
G. pusilla	AU-L	$G_6(TG)_2G(TG)_4TT$
T. haematodus	AU-L	$G_6(TG)_2G(TG)_2G(TG)_2G(TG)_4$
N. hollandicus	AU-C	$G_4(TG)_2CGG(TG)_2G(TG)_6$

Cfor2627

C. nigra barkyli	AF	$G_3TG_4(AG)_2(TGAG)_2(TG)_6T_2(TG)_7T_2(TG)_3AG(TG)_4T_2(TG)_7$
P. senegalus	AF	$G_3ATGAGG(AG)_2TGAGTTCA(TG)_4CG(TG)_6$
P. meyeri	AF	$GAGATGAGG(AG)_2TGAGTTCA(TG)_4CA(TG)_6$
P. erithacus	AF	$G_3ATGAGG(AG)_2TGAGTTCG(TG)_3C(TG)_5$
A. ararauna	NT	$G_3TGAGA(AG)_2TG(AG)_2(TG)_3CAT$
C. n. chathamensis	AU-1	$G_4TG_4(AG)_2(TG)_{14}(CG)_2(CA)_2TG$
C. n. novaezelandiae	AU-1	$G_4TG_4(AG)_3(TG)_7(CA)_2TG$
C. auriceps	AU-1	$GCGGTG_4(AG)_3(TG)_{12}CG(TG)_6(CA)_2TG$
C. malherbi	AU-1	$GCGGTG_4(AG)_2(TG)_{13}CACG$
C. hochstetteri	AU-1	$GCGGTG_4(AG)_3(TG)_{15}CACG$
C. unicolor	AU-1	$G_4TG_4(AG)_2(TG)_{12}CACG$
C. forbesi	AU-1	$G_4TG_4(AG)_2(TG)_{20}(CA)_2TG$
C. saisetti	AU-1	$GCGGTG_4(AG)_3(TG)_{17}(CA)_2TG$
E. cornutus	AU-1	$G_4TG_4(AG)_2(TG)_{10}TA(TG)_{17}(CA)_2TG$
E. uvaeensis	AU-1	$G_4TG_4(AG)_2(TG)_5TA(TG)_4TA(TG)_{15}(CA)_2TG$
P. varius	AU-1	$G_3TG_4AG(TG)_{12}$
B. barnardi	AU-1	$G_3TG_4(AG)_2TGAG(TG)_{10}$
P. alexandri	AU-1	$G_4TG_4(AG)_2TGAG(TG)_2AG(TG)_5$
M. pusio	AU-1	$GGAATG_4(AG)_2TGAG(TG)_2(AG)_2G_4(TG)_4AG(TG)_3$
M. undulatus	AU-1	$G_4TG_4ACAGTGAG(TG)_2AG(TG)_2$
G. pusilla	AU-L	$G_4TG_4ACAGTGAG(TG)_2AG(TG)_3$
T. haematodus	AU-L	$G_4TG_4ACAGTGAG(TG)_2AG(TG)_3$
N. hollandicus	AU-C	$G_4TAG_3(AG)_2TGAGTGTATGAG(TG)_5$
E. roseicapillus	AU-C	$G_4TG_4(AG)_2(TGAGTGTA)_2TGAG(TG)_4$
C. haematuropygia	AU-C	$G_4TG_4(AG)_2(TGAGTGTA)_2(TG)_4$

Cfor3031

C. nigra barkyli	AF	$GAGTG_7CT(GT)_3ATGT$
P. senegalus	AF	$GAGTGGAG_4(GT)_6ATGT$
P. erithacus	AF	$GAGTGGAGGCG_4(GT)_6ATGT$
C. n. chathamensis	AU-1	$G_4TGG(GT)_{10}ATGT$
C. n. novaezelandiae	AU-1	$G(GT)_{12}ATGT$
C. auriceps	AU-1	$G_4TGG(GT)_8ATGT$
C. malherbi	AU-1	$G_4(GT)_{10}ATGT$
C. e. hochstetteri	AU-1	$GG(GT)_9ATGT$
C. unicolor	AU-1	$GG(GT)_9ATGT$
C. forbesi	AU-1	$G_4TGG(GT)_{10}ATGT$
C. saisetti	AU-1	$GG(GT)_8ATGT$
E. cornutus	AU-1	$G_5(GT)_{10}ATGT$
E. uvaeensis	AU-1	$G_6(GT)_9ATGT$
P. varius	AU-1	$GAGGTG_8AGCT(GT)_3ATGT$
P. eximius	AU-1	$G_9CGGTCT(GT)_3ATGT$
P. alexandri	AU-1	$GAGTG_7CT(GT)_3ATGT$
M. pusio	AU-1	$GAGCG_7CT(GT)_3ATGT$
G. pusilla	AU-L	$GAGTCTG_5CT(GT)_3ATGT$
T. haematodus	AU-L	$GAGTCTG_5CT(GT)_3ATGT$

4.4 Discussion

4.4.1 Markers and Methods in Phylogenetic Constructions

Since the first use of microsatellite flanking region sequences to construct phylogenies by Zardoya et al. (1996), an increasing number of studies

have been published utilising this technique (for example, Ortí et al. 1997; Streelman et al. 1998; Makova et al. 2000; Zhu et al. 2000a).

Although the flanking region sequences of microsatellite loci isolated in this study generally show low resolving power (Figure 4.1), the microsatellite flanking region sequences do add to the number of loci available for construction of more accurate parrot phylogenies.

Compared with nuclear genes, mitochondrial markers have a higher rate of evolution, and evolve independently of chromosomal markers, making them popular tools for phylogenetic construction (Brown et al. 1979; Barton & Jones 1983; Moore 1995). However, there has been concern that mitochondrial DNA represents only single-gene genealogy and may not always accurately represent the evolution of the organisms examined (Pamilo & Nei 1988; Ball et al. 1990). To increase the probability of obtaining an accurate species phylogenetic tree, the use of DNA sequences from many independently evolving loci is preferred (Tajima 1983; Takahata & Nei 1985; Pamilo & Nei 1988).

The choice of algorithms for constructing phylogeny (see Felsenstein 1978; Kuhner & Felsenstein 1994; Kolaczkowski & Thornton 2004) and understanding of the most appropriate mutation model of the marker sequence (see Yang 1997; Takezaki & Gojobori 1999) are important considerations in taxonomy and evolution. Maximum parsimony (MP; Camin & Sokal 1965), maximum likelihood (ML; Felsenstein 1981), and neighbour-joining (NJ; Saitou & Nei 1987) algorithms were chosen for phylogenetic constructions in this study because they are widely accepted (Saitou & Imanishi 1989; Huelsenbeck & Hillis 1993; Tateno et al. 1994; Russo et al. 1996; Huelsenbeck 1995; Yang 1996). Differences between ML and MP trees in the microsatellite phylogenies of *Cyanoramphus*

parakeets are most likely to be caused by the differences in performance of the algorithms (Yang 1996) and lack of signal in the flanking sequences.

4.4.2 Cross-species Microsatellite Amplifications in Parrots

It has been suggested that the likelihood of successful cross-species microsatellite amplification correlates positively with phylogenetic distance in avian species (Primmer et al. 1996). The majority of the *Cfor* loci show no amplification in the AU-NZ lineage, which is believed to be the ancestral lineage of parrots, with greatest distance away from *Cyanoramphus* (Boon 2000). The amplification of these microsatellite loci generally conforms to the pattern of higher likelihood of amplification in more closely species, as predicted by Primmer et al. (1996) and Chambers & MacAvoy (2000).

4.4.3 Origin and Evolution of Microsatellites

Few studies have addressed the origin of microsatellite repeats. Messier et al. (1996) proposed a substitution event in an existing dinucleotide repeat unit can give rise to a new tetranucleotide motif. However, an alternative mechanism was also proposed (Gordon 1997) which showed slippage replication alone (without substitution) could produce the results reported by Messier et al. (1996). Phylogenetic studies have reported that microsatellites with fewer than 5 repeat units are rarely polymorphic (Strassmann et al. 1997; Zhu et al. 2000a), supporting the view that there is a threshold for microsatellite expansion (Messier et al. 1996; Rose & Falush 1998). Using data in the Human Gene Mutation Database (Krawczak & Cooper 1997), Zhu et al. (2000b) found that insertions and substitutions are common in short repeat units, and may play a role in the origin of microsatellite repeats. However, it is still unclear whether slippage replication acts on small repeat arrays. Another mechanism in

which A-rich microsatellites were generated by 3' extension of retrotranscripts was suggested as the origin of a microsatellite based on an observed association between microsatellites with retrotransposable elements (Nadir et al. 1996). The data obtained in the present study support a role for both substitutions and replication slippage in the genesis of microsatellite repeat arrays (see below).

The majority of lineages examined at locus *Cfor1819* contained no microsatellite repeats, but have the simple sequence CAGA followed by a row of mononucleotide C repeats (Figure 4.2a; Table 4.2). However in the AU-1 lineage associated with *Cyanoramphus* species and in *Nymphicus hollandicus*, the locus contains a $(CA)_n$ microsatellite. If CAGA represents the ancestral state, then the simplest route by which $(CA)_n$ may arise is the substitution of a G for C in CAGA, forming $(CA)_2$. The subsequent expansion of $(CA)_2$ into larger numbers of repeats could be via slippage replication, perhaps facilitated by the presence of the row of flanking C mononucleotides on the 3' side. The pattern of $(C_6CA)_2$ interruptions in *B. barnardi* may be explained by an independent, additional substitution or insertion event that introduced an A within the flanking mononucleotide C repeat tracts. Alternatively, a C substitution in $(CA)_4$ can turn the motif into $(CA)_2CCCA$, and subsequent C expansion to C_6CA and duplication of the motif will result in the $(CA)_2(C_6CA)_2$ motif observed. The $(CA)_2(C_6CA)_2$ pattern also suggested that slippage may act on larger templates like C_6CA. The $CA(CCA)_n$ pattern in *Eunymphicus* species could be explained by a C insertion that follows a G to C substitution in CAGA.

The proposed model of *Cfor1819* evolution, where slippage replication expands a repeat unit after its generation through substitution or insertion, could be used to explain the origin of the repeat motifs in *Cfor2021*, *Cfor2627*, and *Cfor3031*, where substitutions and insertions can occur within the bordering strings of mononucleotides, generating new repeat

motifs. The observations from these loci support the view of Zhu et al. (2000b) that both insertions and substitutions play important roles in the origin and development of a microsatellite locus. The mechanism which assists the elongation of repeats less than the proposed 5 units "threshold" is unclear, but in the case of the 4 loci studied here, the immediately adjacent mononucleotide repeats are highly likely to act as a template for slippage replication causing the growth of microsatellite repeats.

If slippage involving bordering mononucleotides as templates causes the elongation of di- or tri- nucleotide repeats, then one might also expect the mononucleotides to grow along with the microsatellites. Thus, longer microsatellites would be expected to be associated with longer mononucleotide repeats. This is however not what is observed. It also seems that long mononucleotide repeats may accumulate nucleotide substitutions or insertions readily (see *Cfor2627* and *Cfor3031*; Table 4.2).

Two approaches are generally used to study genesis of microsatellites (reviewed by Bachtrog et al. 1999). The approach taken here follows the evolution of one or a few loci through a phylogeny (for example, Messier et al. 1996; Zhu et al. 2000a). This method relies heavily on how accurately the adopted phylogeny describes true phylogenetic relationships. It is also worth noting that shorter microsatellites may not necessarily represent young microsatellites (Streelman et al. 1998). The alternative approach, surveys the distribution of microsatellites in genomes to search for factors that influence microsatellite density. This requires knowledge from large genome projects, and assumes frequency of microsatellite genesis is directly reflected by observed microsatellite density (for example, Bachtrog et al. 1999). For parrots, neither a 100% reliable phylogeny is available, nor whole genomic information is available in parakeets. These factors remain as the present obstacles to further assess evolution models built

on parrot microsatellites. Nonetheless, the findings from *Cfor* loci provide valuable empirical data on the origin and evolution of microsatellites.

4.4.4 Future Research

Subsequent to the submission of this thesis, de Kloet & de Kloet (2005) published a global phylogeny of parrots based on spindlin gene sequences. Their study did not include many of the AU-1 lineage parrots studied in this chapter, but nevertheless provides an excellent opportunity to further investigate the evolution of *Cfor* loci when spindlin sequence data from more AU-1 parrots are obtained and incorporated into the global parrot phylogeny.

4.5   References

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Chapter 5: Does Crown Plumage Directly Reflect the Hybridisation Status of Parakeets? A Preliminary Study in Red-crowned Parakeets (*Cyanoramphus novaezelandiae novaezelandiae*) and Yellow-crowned Parakeets (*C. auriceps*) on New Zealand Mainland

5.1 Introduction

A situation that parallels the hybridisation of Forbes' parakeet with Chatham Island Red-crowned parakeet exists on the New Zealand mainland, where Red-crowned parakeets (*Cyanoramphus novaezelandiae novaezelandiae*) have been observed to pair with Yellow-crowned parakeets (*C. auriceps*; Veitch 1979; Butler 1986).

A parakeet was captured on Kapiti Island in early 2004, which had crown plumage patterns intermediate between a Red-crowned and a Yellow-crowned parakeet (Figure 5.1). The bird, which died in captivity, weighed 68 g and was banded with a metal band numbered D181961. Today, the Kapiti Island parakeet population consists solely of Red-crowned parakeets, however, Yellow-crowned parakeets are believed to have existed on Kapiti Island since the 1930s but have not been seen there since the 1960s (Colin Miskelly, NZ Department of Conservation, pers. comm.). The captured bird, D181961, could be either a hybrid between Red-crowned and Yellow-crowned parakeets, or have a mutation in the crown colour genes, representing natural variation in Red- or Yellow-crowned parakeets.

Figure 5.1   Parakeet D181961 captured on Kapiti Island displaying crown plumage characteristics intermediate between a Red-crowned and a Yellow-crowned parakeet.   Photograph courtesy of New Zealand Department of Conservation.

At the time of this study, samples from Kapiti Island were unavailable, but blood samples from a small number of Red-crowned parakeets from Poor Knights Islands, and of Yellow-crowned parakeets from Eglinton Valley were available (Boon 2000). These enabled a preliminary comparative study of the microsatellite genotypes to further test for correlation between morphology and genetics in mainland parakeets. The Eglinton population is believed to consist mainly of pure Yellow-crowned parakeets. The Poor Knights Islands population is made up of Red-crowned parakeets, but a small number of Yellow-crowned parakeets and possible hybrids between them had been reported there in the past (Chambers 1956; Sagar 1988). Therefore, the Poor Knights samples may not represent "pure" mainland Red-crowned parakeet genotypes.

The objective of this chapter is to further explore the relationship between crown morphology and genetics in *Cyanoramphus* parakeets in species other than Forbes' parakeet.

5.2 Methods

5.2.1 Extraction of DNA from Samples

Total DNA was extracted from breast muscle dissected from the corpse of bird D181961 (courtesy of Kaye Ballantyne, Victoria University of Wellington), or 5 μl of blood from 13 Poor Knights Islands Red-crowned parakeets and 16 Yellow-crowned parakeets from Eglinton Valley using High Pure DNA Template Preparation Kit (Roche), following the manufacturer's protocol.

5.2.2 Amplification and Screening of Cfor Microlatellite Loci

The target *Cfor* loci were amplified from the extracted DNA. PCRs were performed on an Eppendorf Mastercycler ep thermocycler using conditions described in Chapter 2, section 2.2.16. PCR products were genotyped and sequenced on an ABI Prism 377 sequencer (Applied Biosystems) as described in Chapter 3, section 3.2.1, and results were analysed with GeneScan Analysis and Sequencing Analysis software (Applied Biosystems) respectively.

5.2.3 Analysis of Basic Population Characteristics

Allele frequencies, expected and observed heterozygosities were calculated using the Microsatellite Analyser software (version M3.15; Dieringer & Schlötterer 2002). Because sample size bias could affect the differences in heterozygosity, a resampling of 1000 random draws was performed to allow a more balanced comparison. Allele frequency

distributions were compiled using AppleWorks (version 6; Apple Computer).

Tests for deviations from Hardy-Weinberg equilibrium (HWE) was by the Markov chain method (Guo & Thompson 1992) as implemented in GENEPOP (version 3.4; Raymond & Rousset 1995). The programme was run for 600 batches with 1000 iterations per batch, and dememorization was set at 1000. The same software was used to test for linkage disequilibrium between loci.

5.2.4 Calculation of Genetic Differentiation

Data from six loci, *Cfor0809*, *Cfor1415*, *Cfor1617*, *Cfor2021*, *Cfor2829*, and *Cfor3031* were selected for further analysis (see Chapter 3, section 3.3.3). The estimator θ_{ST} (Weir & Cockerham 1984) of F_{ST} (Wright 1951) and the estimator ρ_{ST} (Rousset 1996) of R_{ST} (Slatkin 1995) were calculated using the GENEPOP software (version 3.4; Raymond & Rousset 1995). Differentiation between Poor Knights Red-crowned parakeets and Eglinton Yellow-crowned parakeets was assessed by Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) as implemented in the GeneticStudio software (version 2.01; Dyer & Sork 2001).

5.2.5 Assignment of Individuals Using Microsatellite Allele Frequencies

Assignment of individuals to populations was made using the test implemented in the Doh software (Paetkau et al. 1995). The programme was run for 10^6 runs to assign individual D181961 into the population with the closest genotypes. Poor Knights Islands Red-crowned parakeet and Eglinton Valley Yellow-crowned parakeet samples screened in this chapter were included in the analysis together with 30 "pure" Forbes' parakeet and

13 "pure" Chatham Island Red-crowned parakeet samples (see Chapter 3, Appendix 3.2).

5.2.6 Genetic Distances Calculations

A genetic distance matrix of chord distance (D_c; Cavalli-Sforza & Edwards 1967), a statistic which makes no assumption about models of microsatellite evolution, was calculated by the Microsatellite Analyser software (version M3.15; Dieringer & Schlötterer 2002). The genetic distance matrix was then ordinated in multidimensional space by Principal Coordinate Analysis (Gower 1966) using the software PCO (Anderson 2003).

5.2.7 Mitochondrial DNA Data

The mitochondrial DNA data is kindly provided by Kaye Ballantyne (Victoria University of Wellington). The 2.5 kb mitochondrial control region was amplified using primers L16518 and H1800 (Boon et al. 2001) in 1x Expand High Fidelity PCR buffer (Roche), 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.5 μM of each primer, and 0.05 U Expand High Fidelity DNA polymerase (Roche). The cycling profile, consisting of 95°C – 3 min, 35 cycles at (95°C – 15 sec, 55°C – 30 sec, 68°C – 2 min with a cumulative increase of extension time at 20 sec per cycle), 68°C – 7 min, was run on a Perkin-Elmer model 480 thermocycler. The products were sequenced on an ABI Prism 377 sequencer (Applied Biosystems).

5.3   Results

5.3.1 HWE, Linkage Disequilibrium, and Genetic Differentiation

Most loci conformed to HWE ($P \geq 0.05$; Table 5.1), except *Cfor1415* and *Cfor3031* in the Poor Knights population, and *Cfor0809* in the Eglinton

population ($P < 0.05$). Among the loci which deviated from HWE, *Cfor1415* showed heterozygote excess, while *Cfor0809* and *Cfor3031* showed heterozygote deficiencies. Calculated expected heterozygosity values were similar with or without resampling, indicating that bias due to small population size was minimal (Table 5.1). Allele frequencies were charted in Figure 5.2. Linkage disequilibrium between loci was not observed after sequential Bonferroni correction (Rice 1989). The estimators of F_{ST} and R_{ST} were calculated to be 0.0878 and 0.1190 respectively, indicating about 9% genetic differentiation between Poor Knights Red-crowned parakeets and Eglinton Yellow-crowned parakeets. AMOVA also showed 10.2% of the genetic differentiation could be attributed to the differences between the two populations examined ($P = 0.001$).

5.3.2 Assignment of Individuals

Parakeet D181961 was assigned as a Red-crowned parakeet, similar to other members in the Poor Knights Islands Red-crowned parakeet population (Table 5.2). The probability of assigning D181961 as a Yellow-crowned parakeet was 7.15×10^{-10}, magnitudes lower than that of Red-crowned parakeet. Analysis of mitochondrial DNA control region sequences also showed that parakeet D181961 has Red-crowned parakeet maternal ancestors. Therefore, it is rather unlikely that D181961 has a recent interspecific hybridisation history.

Table 5.1   Population genetic characteristics at variable *Cfor* loci in Poor Knights and Eglinton parakeet populations. The number of alleles observed is represented by *n*; H_O and H_E represent observed and expected heterozygosities respectively. HWE (*P*) represents the *P*-value in HWE test, HWE (S.E.) represents the standard error, and H_E (R.S.) shows the mean expected heterozygosity after 1000 resamplings.

Locus	Population	*n*	HWE (*P*)	HWE (S.E.)	H_O	H_E	H_E (R.S.)
Cfor0809	Poor Knights Is.	3	0.8208	0.0011	0.6923	0.5938	0.6086
	Eglinton Valley	4	0.0250	0.0011	0.3750	0.5907	0.5470
Cfor1415	Poor Knights Is.	9	0.0427	0.0036	1.0000	0.8554	0.8449
	Eglinton Valley	6	0.1041	0.0027	1.0000	0.8226	0.8219
Cfor1617	Poor Knights Is.	3	0.3743	0.0025	0.2308	0.3354	0.3918
	Eglinton Valley	2	-	-	0.0625	0.0625	0.1350
Cfor1819	Poor Knights Is.	5	0.1914	0.0028	0.7692	0.7908	0.7678
	Eglinton Valley	7	0.5798	0.0058	0.8125	0.8206	0.7633
Cfor2021	Poor Knights Is.	3	0.2848	0.0020	0.3077	0.3938	0.4984
	Eglinton Valley	3	0.2846	0.0019	0.5625	0.5262	0.5351
Cfor2627	Poor Knights Is.	12	0.1465	0.0090	0.7692	0.9046	0.8683
	Eglinton Valley	9	0.4787	0.0093	0.7500	0.8266	0.7532
Cfor2829	Poor Knights Is.	3	0.4955	0.0019	0.6154	0.5569	0.5461
	Eglinton Valley	5	0.2765	0.0039	0.6875	0.7117	0.7174
Cfor3031	Poor Knights Is.	7	0.0176	0.0016	0.6923	0.8369	0.8135
	Eglinton Valley	6	0.2826	0.0068	0.5000	0.5907	0.6826

Out of all 72 assignment tests (excluding D181961), three cases of misassignments were observed in the microsatellite assignment: 1) Poor Knights Islands individual CD2070 as a Yellow-crowned parakeet, 2) South-East Island individual D171531 as a Yellow-crowned parakeet, and 3) Mangere Island individual D172022 as a Red-crowned parakeet. The error rate of the microsatellite assignment is about 4.2%. The major cause of these misassignments is likely to be the birds' individual genotypes being more similar to that found in the populations they are misassigned to. No Eglinton Valley Yellow-crowned parakeet was misassigned.

Figure 5.2   Allele frequency distributions of *Cfor* loci in two populations of parakeets. Solid bars represent Poor Knights Islands population (13 individuals), shaded bars represent Eglinton population (16 individuals), and the numbers above bars represent the number of times an allele is observed.
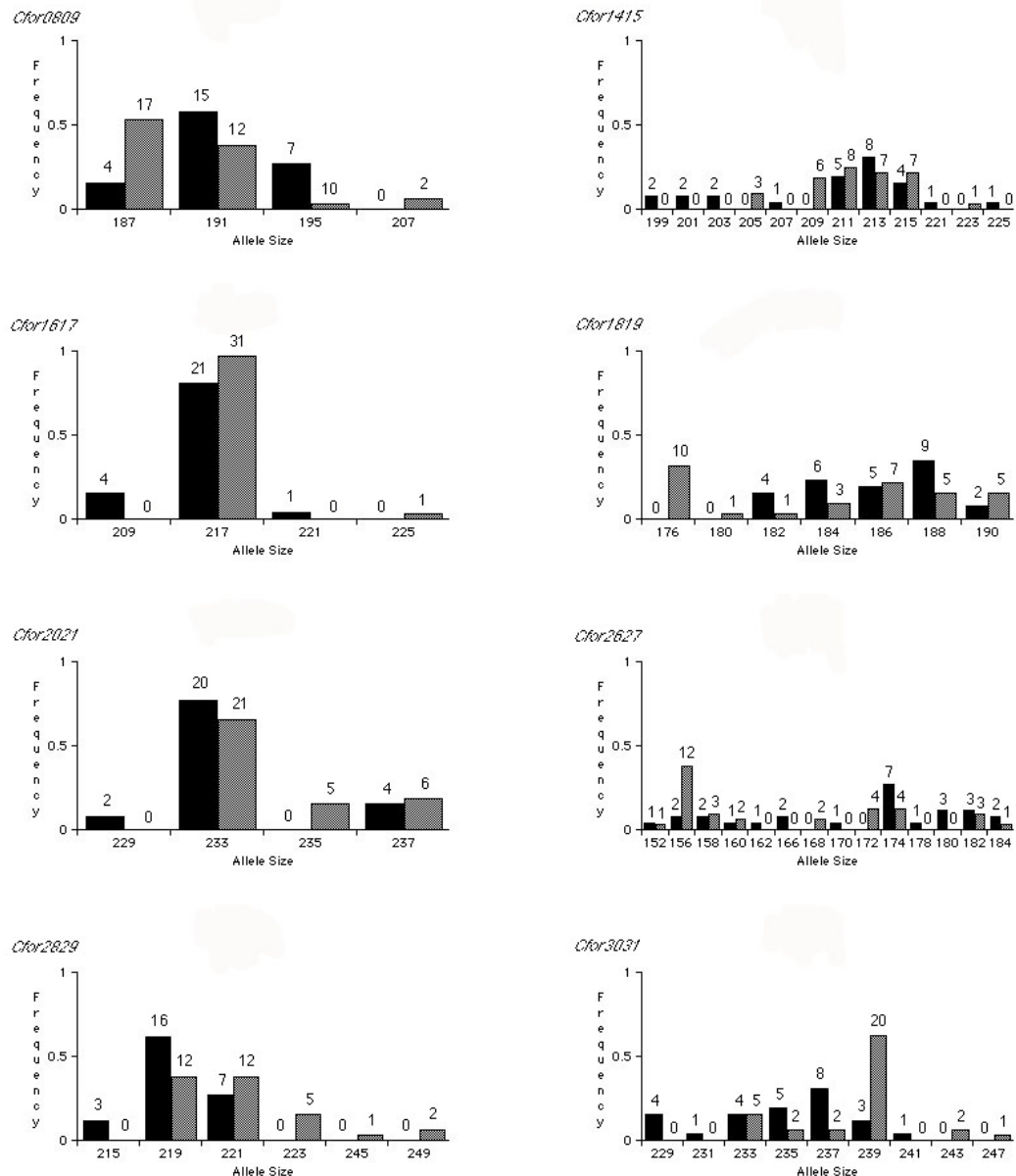
### 5.3.3 Genetic Distances Between Individuals

A principal coordinates plot of D_c distance values (Figure 5.3) shows that the Kapiti Island bird D181961 was more closely grouped to Poor Knights Islands Red-crowned parakeets than Eglinton Yellow-crowned parakeets. The first axis, PCO 1, represents 23.4% of the total variation, while the second axis, PCO 2, represents 16.8% of the total variation. Cumulatively, 40.2% of the total genetic variation was represented in the first two axes. These results supported the outcome of the assignment test.

Table 5.2 Assignment of individuals using microsatellite allele frequency and mitochondrial control region data. RCP = Red-crowned parakeet, YCP = Yellow-crowned parakeet, CIRCP = Chatham Island Red-crowned parakeet, and FBP = Forbes' parakeet. Mitochondrial DNA data was provided courtesy of Kaye Ballantyne (Victoria University of Wellington).

Population	Sample	Microsatellite Assignment		mtDNA Assignment
		Assignment	Assignment Probability	
Poor Knights	CD2063	RCP	2.56×10^{-6}	RCP
	CD2067	RCP	1.18×10^{-7}	RCP
	CD2068	RCP	7.34×10^{-5}	RCP
	CD2070	YCP	4.07×10^{-5}	RCP
	CD2071	RCP	3.59×10^{-6}	RCP
	CD2073	RCP	8.51×10^{-11}	RCP
	CD2075	RCP	4.14×10^{-6}	RCP
	CD2078	RCP	4.95×10^{-10}	RCP
	CD2080	RCP	5.22×10^{-6}	RCP
	CD2087	RCP	1.63×10^{-6}	RCP
	CD2091	RCP	1.33×10^{-6}	RCP
	CD2093	RCP	9.37×10^{-5}	RCP
	CD2113	RCP	2.79×10^{-7}	-
Eglinton	FT3323	YCP	8.53×10^{-10}	YCP
	FT3325	YCP	7.17×10^{-6}	YCP
	FT3326	YCP	1.08×10^{-6}	YCP
	FT3327	YCP	1.99×10^{-4}	YCP
	FT3328	YCP	6.42×10^{-6}	YCP
	FT3329	YCP	6.07×10^{-5}	YCP
	FT3330	YCP	1.12×10^{-9}	YCP
	FT3331	YCP	6.51×10^{-5}	YCP
	FT3334	YCP	5.24×10^{-6}	YCP
	FT3336	YCP	2.55×10^{-7}	-
	FT3337	YCP	2.67×10^{-4}	YCP

	FT3339	YCP	3.50×10^{-4}	YCP
	FT3346	YCP	5.78×10^{-6}	YCP
	FT3347	YCP	1.45×10^{-5}	YCP
	FT3348	YCP	3.22×10^{-4}	YCP
	FT3349	YCP	5.17×10^{-7}	YCP
South-East Is.	D171526	CIRCP	2.22×10^{-4}	CIRCP
	D171527	CIRCP	6.53×10^{-3}	CIRCP
	D171531	YCP	1.50×10^{-3}	CIRCP
	D171533	CIRCP	1.24×10^{-2}	CIRCP
	D171535	CIRCP	6.56×10^{-4}	CIRCP
	D171536	CIRCP	4.69×10^{-3}	CIRCP
	D171586	CIRCP	1.79×10^{-3}	CIRCP
	D171587	CIRCP	7.93×10^{-6}	CIRCP
	D171588	CIRCP	1.84×10^{-3}	CIRCP
	D171589	CIRCP	7.07×10^{-5}	CIRCP
	D171593	CIRCP	4.99×10^{-4}	CIRCP
	D171596	CIRCP	1.01×10^{-4}	CIRCP
	D171597	CIRCP	5.13×10^{-6}	CIRCP
Mangere Is.	BW-G	FBP	1.56×10^{-4}	FBP
	G-BO	FBP	5.87×10^{-3}	FBP
	G-BR	FBP	6.03×10^{-2}	FBP
	G-RY	FBP	1.25×10^{-2}	FBP
	GW-W	FBP	6.03×10^{-2}	FBP
	OY-G	FBP	3.09×10^{-3}	FBP
	RG-G	FBP	5.05×10^{-4}	FBP
	WO-G	FBP	1.56×10^{-4}	FBP
	WR-G	FBP	6.02×10^{-2}	FBP
	W-WB	FBP	1.21×10^{-4}	FBP
	D171514	FBP	3.01×10^{-3}	FBP
	D172011	FBP	1.25×10^{-2}	FBP
	D172022	RCP	3.77×10^{-6}	FBP
	D172042	FBP	6.03×10^{-2}	FBP
	D174714	FBP	7.00×10^{-3}	FBP
	D174717	FBP	7.01×10^{-3}	FBP
	D174718	FBP	2.66×10^{-2}	FBP
	D175003	FBP	4.37×10^{-2}	FBP
	D175013	FBP	5.32×10^{-4}	FBP
	D175103	FBP	4.14×10^{-4}	FBP
	D175104	FBP	6.42×10^{-3}	FBP
	D175111	FBP	6.02×10^{-2}	FBP
	D175114	FBP	2.83×10^{-3}	FBP
	D175128	FBP	2.66×10^{-2}	FBP
	D175135	FBP	1.25×10^{-2}	FBP
	D175181	FBP	1.31×10^{-3}	FBP
	D175187	FBP	6.02×10^{-2}	FBP
	D175190	FBP	6.03×10^{-2}	FBP
	D175207	FBP	6.02×10^{-2}	FBP
	D175214	FBP	6.02×10^{-2}	FBP
Kapiti	D181961	RCP	2.89×10^{-7}	RCP

Figure 5.3   Principal coordinate analysis of Cavalli-Sforza & Edwards (1967) chord distances between individual parakeets.

△ Poor Knights ● Eglinton ◇ Kapiti Island
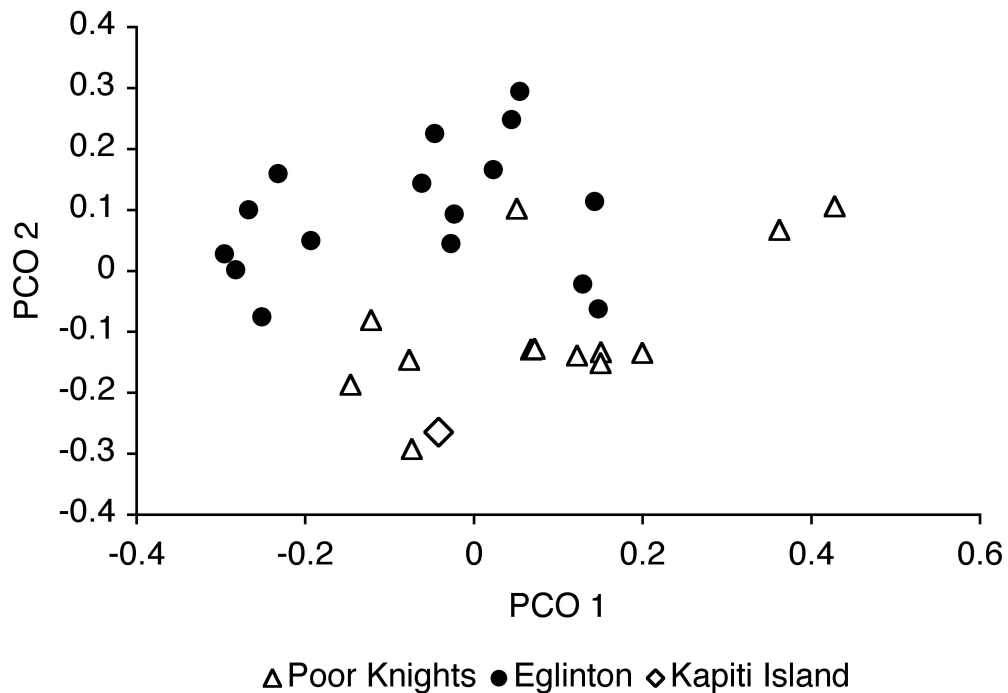
5.4   Discussion

5.4.1   Relationship Between Morphological Characteristics and Genetics

There is no empirical genetic evidence that the crown plumage pattern observed in parakeet D181961 is the result of hybridisation. The bird has typical Red-crowned parakeet mitochondrial control region sequence (Kaye Ballantyne, Victoria University of Wellington, pers. comm.; Table 5.2), and the mitochondrial DNA data rules out the further possibility that this bird represents a new species. If hybridisation had occurred in recent generations, then one would expect microsatellite allele frequencies of sample D181961 to be shifted towards the Yellow-crowned parakeet end of the genotype spectrum and to a degree directly related to how recently the hybridisation took place. However, neither microsatellite assignment test nor the genetic distance data for microsatellites suggest that this is the case. Although rather unlikely, it cannot be ruled out that D181961 may

have retained Yellow-crowned parakeet type crown morphology alleles inherited from a very ancient hybridisation event. A more detailed study of the situation would require samples from the Kapiti Island population, which are currently unavailable.

Taylor et al. (1986) performed breeding experiments between Yellow-crowned parakeets and Orange-fronted parakeets (*C. malherbi*). They showed that hybrids can display intermediate crown plumage patterns as well as parental phenotypes. Therefore, hybrids can be morphologically identical to parental species (see also Chapter 3).

An alternative explanation of the crown morphology observed in bird D181961 is a mutation occurring in the crown colour control pathway genes on Red-crowned parakeet genotype background. It has also been suggested that production of red carotenoid pigments in birds, which give red colouration on feathers, may be related to environmental factors such as diet, and the metabolic abilities of the birds (Hill 1996). Therefore, crown colouration could be affected by both genetic and environmental factors, and may not be a very reliable indicator of species identities in parakeets.

5.4.2 *Genetic Variation in Island and Mainland Parakeet Populations*

Comparing the number of observed alleles between the Poor Knights Islands and Eglinton Valley populations with the Mangere Island and South-East Island populations studied in Chapter 3, equal or higher number of alleles are observed in Poor Knights Islands and Eglinton Valley populations at all loci (Table 5.3). These differences may be species differences, or they may represent lower diversity in isolated island populations, consistent with the literature survey by Frankham (1997) that showed most island populations have less genetic variation

than mainland populations as measured by allele diversity and heterozygosities using various nuclear genetic markers.

Although the Poor Knights Islands are about 20 km from the adjacent mainland, genetic exchange with mainland populations is possible (Sagar 1988).

Increased genetic variation may increase the adaptive potential of populations to changing environments. Ayala (1965) suggested that pre-existing genetic variation is critical for short-term evolutionary changes. Compared to Yellow-crowned and mainland Red-crowned parakeets, Forbes' and Chatham Island Red-crowned parakeets may be more vulnerable to environmental changes, and thus the Chatham Islands species may require more attention in management.

Table 5.3 Comparison of number of observed alleles between Chatham Islands, Poor Knights Islands and New Zealand mainland parakeet populations.

Locus	Mangere Is. (250 samples)	South-East Is. (35 samples)	Poor Knights Is. (13 samples)	Eglinton Valley (16 samples)
Cfor0809	4	3	3	4
Cfor1415	7	5	9	6
Cfor1617	2	2	3	2
Cfor1819	4	4	5	7
Cfor2021	2	1	3	3
Cfor2627	5	4	12	9
Cfor2829	3	2	3	5
Cfor3031	7	5	7	6

5.5   References

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Chapter 6: Conservation of Forbes' Parakeet – Present and Outlook

6.1 Summary

Results from this study clearly show that Forbes' parakeets have hybridised extensively with the Chatham Island Red-crowned parakeets (see Chapter 3). Despite the extensive hybridisation, genetic differentiation studies using microsatellite markers showed a morphological class of birds that is still genetically distinct from Chatham Islands Red-crowned parakeets. Therefore, "pure" or near-"pure" Forbes' parakeets and a lot of their original genetic variation still remain to be conserved in the Mangere Island parakeet population. Cryptic hybrids (comprising F_n hybrids and backcrosses) with Forbes' parakeet morphology exist in large proportions (77.9% of all Forbes' parakeet morphotype birds tested), and are by definition impossible to identify based solely on crown plumage. In contrast, all of the clear morphological hybrids show their genetic hybridisation history.

Using both genetic and morphological criteria, a Forbes' parakeet is defined as a bird which has morphological score MP1 (morphological true Forbes' parakeet), mitochondrial DNA haplotype 3 (MT3), and assigned as a Forbes' parakeet (MS1) in microsatellite assignment tests (see Chapter 3, section 3.3.6). Of 203 birds tested in this study, 17.8% of birds fall into this category. Being able to establish guidelines for defining a "true" Forbes' parakeet ("pure" or near-"pure") greatly facilitates identification of parakeets and associated management efforts.

Microsatellites isolated in this study are believed to evolve through either a SMM (Ohta & Kimura 1973) or a TPM (Di Rienzo et al. 1994) mode. This

indicates mutation patterns vary for different microsatellite loci, and no single universal model exists to date. The origin of four of the dinucleotide microsatellite loci isolated appears to be linked to flanking single nucleotide repeat tracts. The data support slippage-replication as the main mechanism that generates polymorphisms in microsatellites (Schlötterer & Tautz 1992), and a role played by substitutions and insertions in generation of new repeat themes (Messier et al. 1996; Zhu et al. 2000).

The tools developed in this study are also widely applicable in other parakeets and parrots. The microsatellite markers isolated here will very likely contribute to the study of various population genetics aspects in other parrots in the future.

6.2   Present Status of Forbes' Parakeet

6.2.1 Inbreeding and Genetic Bottlenecks

Many New Zealand land birds have experienced substantial decline over the last millennium, with many endemics already lost (such as the moa), or facing extinction (McDowall 1969). Inbreeding depression and genetic bottlenecks have been extensively studied by conservation geneticists because of the associated risk of extinction (for example, Frankham & Ralls, 1998; Saccheri et al. 1998). Worldwide, many small island populations are significantly inbred, and are more prone to the undesirable effects of inbreeding depression (Frankham 1998). Conversely, it has also been noted that while some abundant species have minimal variability, some endangered species have high levels of genetic variability comparable with healthy populations (Amos & Harwood 1998). Therefore, genetic variability, though important in enhancing the potential in adaptation, may not necessarily enhance the survival of a population (Lande & Shannon 1996). Inbreeding coefficients are defined in terms of

the probability of identity in state of different pairs of genes (Rousset 2002). Departures from HWE and homozygote excesses have been frequently used as a sign of possible inbreeding (Robertson & Hill 1984). However, a recent simulation study has suggested that correlation between heterozygosity and inbreeding is strong only when a large number of microsatellite loci (~200) can be used in estimating heterozygosity (Balloux et al. 2004). Inbreeding depression has been suggested as a cause of reduced fitness and juvenile mortality, for example, in the Japanese quail and ungulates (see Sittmann et al. 1966; Ralls et al. 1979; Ralls & Ballou 1982). Studies with experimental *Drosophila* populations also show that inbreeding and loss of genetic diversity are related to decrease in disease resistance (Spielman et al. 2004).

Field studies of the Mangere Island Forbes' parakeet population showed morphological Forbes' parakeet pairs have smaller clutch size and considerably lower hatching rate than hybrids and Chatham Island Red-crowned parakeet pairs (Denise Fastier, NZ Department of Conservation, pers. comm.). Inbreeding in the Forbes' parakeet population (see Chapter 3, section 3.3.5) may be a plausible explanation for the apparent reduction in fitness. In addition, the Mangere Island parakeets have a lower microsatellite allelic diversity compared to mainland New Zealand parakeets (see Chapter 5, section 5.4.2). The reduced genetic diversity could be attributed to founder effects, inbreeding, or past genetic bottlenecks. Because of the low genetic diversity in the Mangere Island population, it may be more susceptible to newly introduced diseases (Spielman et al. 2004). Therefore, increasing attention in preventing disease in this population is vital to its survival. The present situation where all of the world's remaining Forbes' parakeets exist in one single population on Mangere Island has also put this species in high risk of extinction.

A sharp loss in genetic diversity can sometimes be detected in bottlenecked populations (Nei et al. 1975), for example, in the Cheetah (O'Brien et al. 1985). The severe loss of genetic diversity may increase the vulnerability of the population to extinction (O'Brien et al. 1985). The Forbes' parakeet population is believed to have experienced a reduction in population size when Mangere Island was cleared for farming (Taylor 1975), but the exact population numbers immediately before and after clearing of Mangere Island have not been documented. The results of the microsatellite bottleneck tests do not suggest a recent genetic bottleneck in the Mangere Island parakeet population (Chapter 3, section 3.3.5). Despite the fact that there is no detectable genetic bottleneck the Forbes' parakeet population is very likely to have experienced a population bottleneck judging by the low numbers observed (Taylor 1975).

6.2.2 Hybridisation

Although hybridisation may have helped to sustain the Forbes' parakeet morphotype during times with low population numbers, the proportion of hybrids at present has certainly outnumbered Forbes' or near-Forbes' parakeet types (Chapter 3, section 3.3.6). It has been proposed that factors bringing ecologically divergent parental species together often change the environment to form habitats for hybridisation (Templeton 1981). Long term coexistence of parent species and hybrids may be explained by adaptive advantages of hybrids in temporally changing environments (Lewontin & Birch 1966; Spaak & Hoekstra 1997). The hypothesis that hybrids perform better in a modified environment (Anderson 1948) suggests the present Mangere Island habitat may not be one that confers advantages to "pure" Forbes' parakeets.

Hybridisation can be a route to introduce genetic variability for adaptive radiation (Seehausen 2004) and can make a positive contribution to

evolution (Barton 2001). However, further hybridisation, which is likely to result in more extensive mixing of the gene pool, does not appear to help the conservation of "pure" Forbes' parakeets and may eventually lead to the extinction of Forbes' parakeet genotypes. The rarity of "pure" Forbes' parakeet types also suggests hybridisation is slowly displacing the parental genotypes, and constitutes a threat to the long-term survival of the parent species as in other cases documented (see Rhymer & Simberloff 1996; Huxel 1999; Wolf et al. 2001).

Past cullings of hybrid and Chatham Island Red-crowned parakeets without genetic testing have not eliminated "pure" Forbes' parakeets, but the measure failed to stop hybridisation involving cryptic hybrids. At present, it is uncertain whether crown colouration plays a role in mating choice in parakeets. By eliminating parakeets with extra red feathers in the crown, the culling practice essentially encourages matings between any surviving Chatham Island Red-crowned parakeets or hybrid parakeets with morphological Forbes' parakeets, and consequently increasing the number of cryptic hybrids in the population. Therefore, hybrid culling may not be an optimal solution to the problem.

6.3   Conservation Considerations

6.3.1 How Many Evolutionarily Significant Units Make Up the Mangere Island Parakeet Population?

Since the recognition of Forbes' parakeet as a full species (Triggs & Daugherty 1996; Boon et al. 2000), increasing efforts have been directed towards its conservation (see Greene 2000). Whether the mix of Forbes' parakeets and its hybrids on Mangere and Little Mangere Islands represent a single unit for conservation will need to be considered.

With the increasing need of conserving natural genetic diversity and resources (Frankel 1974), the Evolutionarily Significant Unit (ESU) concept was proposed to provide a rational basis for setting priorities in conservation that reflect underlying genetic diversity (Ryder 1986). Among numerous definitions proposed for ESU (for example, Moritz 1994; Vogler & DeSalle 1994; Crandall et al. 2000), the definition of Moritz (1994) is most clearly based on the use of molecular techniques.

Boon et al. (2001) showed that the Mangere Island parakeet population is made up of birds from four different mitochondrial haplogroups: Forbes' parakeet haplogroups (MT1 – 3), and Chatham Island Red-crowned parakeet haplogroup (MT4), with Forbes' parakeet haplogroup MT3 apparently monophyletic to other parakeet species. Birds with haplogroup MT3 makes up about 55% of the Mangere Island population sampled (Chapter 3, section 3.3.8).

Significant divergence of microsatellite allele frequencies was observed between "pure" Forbes' parakeets and Chatham Island Red-crowned parakeets in this study. The combined mtDNA, microsatellite, and morphological data suggest that Forbes' parakeets are genetically distinct from Chatham Island Red-crowned parakeets, and "pure" or near-"pure" mitochondrial haplogroup MT3 Forbes' parakeets certainly form a single genetically distinct ESU. Both mitochondrial and nuclear genetic data also suggest non-hybrid birds of Chatham Island Red-crowned parakeet haplotype form another distinct ESU.

The origin of mitochondrial haplotype MT2 birds is unknown. MT2 birds on South-East Island all showed morphology of Chatham Island Red-crowned parakeets, while those on Mangere Island can have Forbes' parakeet crown morphology. The data support the view of Boon (2000) that the presence of MT2 birds with Forbes' parakeet morphotype is a

result of recent hybridisation. Haplotype MT2 birds may originally have been part of the Mangere Island population and some members have migrated to South-East Island (Ballantyne et al. 2004), or they may be originally part of Chatham Island Red-crowned parakeet population on South-East Island. Evaluation of these contrasting hypotheses will require larger scale haplotype screening of parakeets from South-East Island and the main Chatham Island.

Mitochondrial haplogroup MT1 birds have not been found on South-East Island, and all MT1 parakeets screened showed either Forbes' parakeet or hybrid crown morphologies (Chapter 3, section 3.3.8). This group of birds, found within the Red-crowned parakeet clade in mitochondrial control region phylogeny (Boon 2000), has apparently hybridised with Forbes' parakeet in the past as Boon (2000) suggested.

6.3.2 Management of Hybrids

The main aim of managing hybridisation is to minimise the harmful effects caused to the parental species (Rhymer & Simberloff 1996). However, the positive role of hybridisation in evolution should also be taken into account when making conservation management policies (Dowling & Secor 1997). Because hybridisation can be an important source of genetic variation (Lewontin & Birch 1966), different management strategies are recommended for natural and human induced hybrids, and natural hybrids may deserve some form of protection (Allendorf et al. 2001).

Consideration should also be given to the effects of the presence of a large number of hybrids on the parent species. While maintaining hybrids may preserve genetic diversity on the island, ultimately it could also lead to the total loss of the remaining Forbes' parakeet types. On the other hand, if all hybrids are removed (removal of 81% of the population), a

substantial decline in the population will occur, and there will also be a genetic diversity loss through another human induced bottleneck. Therefore, a strategy needs to be developed to control the number of hybrids in the population while maintaining a maximum amount of existing genetic variation.

Management of hybrid parakeets is also subject to practical constraints. Genetic testing of every bird on the island may not be an economically favourable prospect and would certainly require a large investment in human resources. Field studies, therefore, can best rely on morphological identification in most cases. The main problem faced here is that morphological intermediacy does not always infer hybridity. This is also a problem frequently encountered by plant taxonomists (Wilson 1992). This study has shown that parakeets with morphological types MP2 and MP3 can have Forbes' parakeet genetics, and the MP1 class consists of a proportion of cryptic hybrids (Chapter 3, section 3.3.8). Past culling of mostly MP3 – MP5 parakeets without genetic testing has not eliminated "pure" Forbes' parakeets, but has not efficiently eliminated hybrids either, especially cryptic hybrids showing Forbes' parakeet morphology MP1.

The immigration of Chatham Island Red-crowned parakeets also poses a major problem in the conservation of Forbes' parakeets. Immigration of Chatham Island Red-crowned parakeets to Mangere Island is usually from the neighbouring Pitt Island and South-East Island. Although the main Chatham Island is about 40 km from Mangere Island, possibly within the dispersal range of parakeets, no birds have been observed flying from that direction. Another consideration is whether Chatham Island Red-crowned parakeets should be removed from Mangere Island. Existing Chatham Island Red-crowned parakeets on Mangere Island could breed with Forbes' parakeet types and hybrids, but they could also mate with Chatham Island Red-crowned parakeet immigrants, lowering the chance

of formation of new Forbes' parakeet and Chatham Island Red-Crowned parakeet pairs. Eliminating existing Chatham Island Red-crowned parakeets on Mangere Island would mean all new migrants would have to breed with Forbes' parakeets or hybrids. Instead of hybrid culling, a preferred long-term solution would be reforestation of the habitat to favour Forbes' parakeets.

6.3.3 Prospect for the Longer Term Survival of Forbes' Parakeet

Considering genetic implications and practical limitations, the following strategies are suggested for future conservation of Forbes' parakeets:

✎ A proportion of hybrids, especially those in morphological groups MP1 and 2, should be maintained to preserve genetic diversity in the population and to avoid accidental removal of predominantly Forbes' parakeet genotypes. To minimise the threat of losing Forbes' parakeet genotypes through hybridisation, a threshold of hybrid proportion should be decided for restarting of hybrid removal or when hybrid numbers increase quickly. A consensus was reached in a technical meeting at the Wellington conservancy of the New Zealand Department of Conservation in November 2004 that culling of hybrids will resume if morphological hybrids (MP3 and MP4) and Chatham Island Red-crowned parakeets make up more than 25% of the Mangere Island population, or when morphological hybrids (MP3 and MP4) reach 10% of the population, or when Forbes' parakeet x Chatham Island Red-crowned parakeet pairs are observed.

✎ If culling is resumed, genetic assessment of culled birds should be carried out to assess the effectiveness of culling.

✎ Birds with unclear morphological identities should be genetically tested before they are culled.

✎ Establishing new populations of Forbes' parakeet will greatly reduce the risk of extinction of this species. Founding of new Forbes' parakeet populations elsewhere should only include parakeets which are genetically tested as Forbes' parakeets, and a sufficient number to represent reasonable amount of genetic variation.

✎ Periodical genetic testing (for example, every 5 years) should be carried out on a subsample of the Mangere Island population to monitor the changes in genetic proportions. This is particularly important if hybrids have been eliminated during the period, because the effects of hybrids removal to the remaining population could be assessed.

✎ Reforestation of the Mangere Island habitat is urgently needed to favour Forbes' parakeets.

6.4 Future Research

6.4.1 Chatham Island Parakeets

With the presently available molecular tools, various other ecological problems in the Chatham Islands parakeet populations could be studied. Knowledge of the mating system of Mangere Island parakeets would certainly help to inform management decisions in lowering hybridisation rates. Being Mendelian markers, microsatellites could be used to analyse pedigree and kinship through the study of the pattern of allelic inheritance (Blouin 2003). Also of interest is the correlation between genetic variation and offspring viability. Along with blood or feather samples from viable offspring, tissues from non-viable offspring, such as eggs that failed to hatch, can be used as a source of DNA for genetic testing to determine whether hybrids are more or less fit than their parents. Data from fitness studies can be used to assess post-mating barriers between the two species.

The origin of various mitochondrial DNA haplogroups (Boon et al. 2001) has not been determined. Of most concern is haplogroup MT2, which is found on both Mangere and South-East Islands. Haplogroup MT2 birds on South-East Island are morphologically indistinguishable from Chatham Island Red-crowned parakeets. A larger scale survey of Chatham Island Red-crowned parakeets from the main Chatham Island and South-East Island would help resolve the mystery. If MT2 birds originate from pure Chatham Island Red-crowned parakeet populations, then the suggestion by Boon (2000) that MT2 parakeets with Forbes' parakeet morphotypes are the results of recent hybridisations can be confirmed.

6.4.2 Other Parakeets and Parrots

There has been a suggestion that genetic difference between parents predicts reproductive compatibility (Edmands 2002). Since Forbes' parakeets and Chatham Island Red-crowned parakeets are the most divergent pairing in all New Zealand parakeets (Boon et al. 2001), then one would expect all pairs of New Zealand *Cyanoramphus* parakeets to have the potential to hybridise. Hybrids between other New Zealand *Cyanoramphus* parakeets have been observed, for example, hybrids between mainland Red-crowned and Yellow-crowned parakeets (Butler 1986). The markers developed in this study along with mitochondrial DNA markers from Boon et al. (2001) could be used to identify genetic hybrids, and to assess correlation between morphotypes and genotypes in other *Cyanoramphus* species.

It appears that some of the microsatellite loci isolated in this study are present in a wide range of parrot species (see Chapter 4, section 4.3.1). There are many more parrot species in the world that have not been surveyed in this study due to unavailability of samples. Adding more taxa into the existing collection would enhance the phylogenetic trees built and

offer more insights on the origin and evolution of microsatellites and parrots.

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