New Zealand Passerines: a contribution to Passerine phylogeny

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Abstract

The passerines are the largest avian order, with over 6000 species. There is strong evidence to suggest that this group arose in Australasia, with most deep lineages located in Australia, New Zealand and Papua New Guinea. The deepest lineage is the New Zealand wrens, which diverged from the main passerine lineage, possibly around 80 mya. The second split between the suboscines (mainly new world distribution) and oscines (Australasian origin) is well established. Within the oscines there are a number of small basal lineages, all located within Australasia, then around 45 mya the large division into the Passerida and the Core Corvoidea occurred. The Core Corvoidea have undergone many rapid radiations early on in their history, which has made resolving the relationships within the group complicated. The Passerida are classified into three main superfamilies, but the relationships of these three are unresolved, and the monophyly of each has been questioned.

Next generation Illumina sequencing was used to sequence the mitochondrial genomes of six native passerine species. We report a sequenced mitochondrial genome from a representative of each New Zealand passerine family, apparently a first for any country. For four new species (the fernbird, tomtit, pipit and waxeye) the mitochondrial genomes have been fully sequenced while there is partial mitochondrial genome sequence for two other species (the browncreeper and bellbird). These have been combined with the mitochondrial genome sequences of another 72 passerines including seven previously unpublished genomes. Phylogenetic trees have been produced using both maximum likelihood and Bayesian analyses, and these have been used to address a number of questions surrounding the phylogeny of the passerines.

The placements of the native New Zealand species are highlighted, and in many cases it confirms the results of earlier studies. The results suggest consideration needs to be given for formally classifying the Petroicidae, Callaeidae and Notiomystidae as basal Passerida, but whether these three families form a separate monophyletic group is still unresolved. Part of the polytomy at the base of the Core Corvoidea has been resolved. The monophyly of each of the three Passerida superfamilies have been confirmed, but it is still unclear which of the three superfamilies branched off first, and there is no support for the suggestion that the Paridae are their own fourth superfamily. A need for

the formation of an extensive collection of tissue/DNA samples from New Zealand's vertebrates has been identified, and a number of suggestions for the use of mitochondrial genome sequence when studying passerine phylogeny have been made.

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Abbreviations

BGI	-	Beijing Genome Institute
BLAST	-	basic local alignment search tool
bp	-	base pairs
BWA	-	Burrows Wheeler Alignment
CI	-	Chatham Islands of New Zealand
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
DoC	-	Department of Conservation (New Zealand)
kb	-	Kilo base (1000bp)
K/PG	-	Cretaceous Paleogene boundary
MGS	-	Massey University Genome Services
mtDNA	-	mitochondrial DNA
mya	-	million years ago
NI	-	North Island of New Zealand
NZ	-	New Zealand
PCR	-	Polymerase chain reaction
PNG	-	Papua New Guinea
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal RNA
SapExo	-	Shrimp alkaline phosphatase and exonuclease
SI	-	South Island of New Zealand
tRNA	-	Transfer RNA

1 Introduction

This introduction provides the background for this study on the phylogeny of the avian order the passerines. First, an overview is given of what is known about the phylogeny of the passerines, covering the group's origin before focusing on the different groups within the order: the New Zealand wrens, the suboscines, the deep lineages within the oscines, the Core Corvoidea and the Passerida. How this project relates to the New Zealand native passerines and their conservation is then examined, followed by a summary of the molecular techniques used to study phylogeny. Last, an outline of the project and a brief overview of the results are given.

1.1 Passerines

1.1.1 Origin

The passerines are the largest avian order; with almost 6,000 species; this order contains around sixty percent of all living bird species (1, 2). The passerines are divided into three main lineages: the New Zealand wrens, the suboscines and the oscines (see later). The passerines likely evolved in Gondwana (1, 3), and species diversity is highest in Australasia - defined as Australia, New Zealand (NZ) and Papua New Guinea (PNG). Despite northern hemisphere fossils being better known generally, the oldest passerine fossils of these birds are found in the southern hemisphere around 55 million years ago (mya) in Queensland Australia, whilst the oldest passerine fossils found in the northern hemisphere are only 34-30 million years old (1, 4). There are very few avian fossils that have been dated to before the Cretaceous Paleogene boundary (K/Pg), 66 mya (5). Some authors suggest this shows that there were few bird lineages during the Cretaceous and that the majority of the avian lineages did not evolve until the Paleogene (5). Overall, avian fossils have not preserved very well, which may be due to the structure of avian bones which are pneumatised (hollow), so are relatively light. A study using 80 complete avian mitochondrial genomes gives a divergence time of the passerines from the other birds at between 78-95 mya (5). This precedes the K/PG boundary, suggesting that the majority of the avian orders had formed by this time (5-7). Passerines were certainly present in Australasia by the Eocene (56-34 mya), and by the

late Oligocene (~23 mya) many of the current passerine lineages were present (4). The ancestor of all passerines is assumed to be insectivorous (8), but the passerines now fill many different habitats, with multiple feeding types, and occupy every continent except Antarctica.

Which group of birds are the closest relative to the passerines is controversial, and there are as many as five different hypotheses answering this question (9). The passerines were first thought to have been the sister lineage to a group of avian orders containing the Falconidae (falcons), Rallidae (rails), Gruiformes (cranes) and Cariamidae (seriemas) (10). Morphological data (11) suggested the passerines were grouped with the 'woodkings' (Piciformes and Coraciiformes). Ericson et al., (12) found, using nuclear loci, that the Passeriformes grouped with 3 other orders; the Psittaciformes (parrots), Falconidae, and Cariamidae. Support for this was found using 19 nuclear loci in Hackett et al. (13), with strong support for the closest relatives to the passerines being the Psittaciformes. Thirty independent noncoding nuclear loci used by Wang et al. (9), provided further support for the phylogeny seen in Hackett et al. Suh et al. (14), also found strong support from retro-transposon insertions for placing the parrots as the passerines closest relative. Pratt et al., (7) stated that the morphological and mitochondrial data did not support this phylogeny; instead they suggested the Cuculiformes (cuckoos) were the closest relative to the passerines, and this view was supported by Gibb (15). Pacheco et al. (5), again found no support for the Hackett phylogeny; this study using 80 avian mitochondrial genomes had weak support for the grouping of the passerines with the 'woodkings'. Overall this question is unresolved with no study having undisputable support for either scenario. It appears that what the nuclear data is suggesting (passerines with parrots) differs from what the mitochondrial and morphological data is suggesting (passerines with 'woodkings' or cuckoos). But for this study the Psittaciformes will be used as the out-group for the passerines.

1.1.2 The New Zealand wrens and suboscines

Harrison et al. (16) sequenced the mitochondria of the New Zealand rifleman *Acanthisitta chloris*; when compared to the sequence of other passerines it, as expected, fitted neither into the suboscines or oscines (Figure 1), but belongs to its own third suborder, the Acanthisitti. This is supported by multiple studies using both nuclear and mitochondrial data (1, 2, 13).

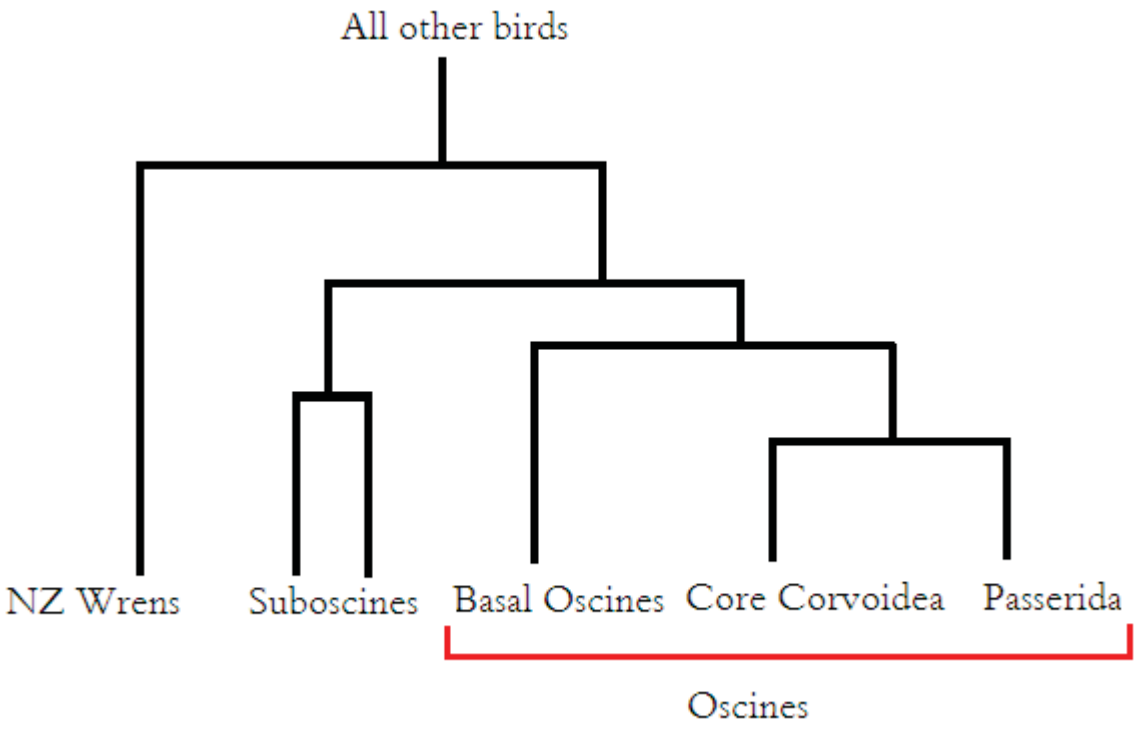

Figure 1: Simplified view of phylogeny of the passerines showing the main lineages in the order

The suborder Acanthisitti is known as the New Zealand wrens, and comprises two extant species, the rifleman (*Acanthisitta chloris*), and the rock wren (*Xenicus gilviventris*), along with four other species which have become extinct since human contact (4). Worthy et al. (4) describes a 7th species, the *Kuiornis indicator* for which fossils were found in Otago. These fossils have been dated to the early Miocene (19-16 mya), and are the oldest passerine fossils found in New Zealand. Worthy et al., (4) created a morphological tree comparing this fossil with all other Acanthisitti, based on features of the bones. The tree placed *Kuiornis indicator* as the sister taxon to all other Acanthisitti sampled by Worthy et al. (4).

The divergence of the Acanthisitti from the main passerine lineage is thought to have occurred when Zealandia split from the rest of Gondwana (1, 17), estimated to around 80 mya. The Acanthisitti is considered part of the ancient endemic component of New Zealand birds (4), lineages that have inhabited Zealandia/New Zealand since the split from the supercontinent. But recent studies (4) have suggested that Zealandia was not completely separated from the supercontinent until around 55 mya, when the Cato trough formed (18). Other groups suggest that the whole continent of Zealandia was completely submerged around 25-22 mya (19). At the very most, only ~18% of current

New Zealand was above water at that time (20), all of it being low lying. I infer from this that most, if not all, fauna and flora would have had to disperse to New Zealand since. Therefore the estimation of 80 mya being that date Zealandia spit from Gondwana may have no relevance to the divergence time of the New Zealand wrens from the rest of the passerines; and as this event has been used to calibrate more recent divergence events in the passerines (1, 21, 22) a different calibration point may need to be used for future estimations. Pratt et al., (7) used different historical points to calibrate avian divergence times and estimated the Acanthisitti split from the passerines around 61 mya. But for simplicity all the other divergence dates mentioned in this review have been estimated using the 80 mya calibration point. This is because the vast majority of passerine phylogenetic studies use the 80 mya calibration point, and I have copied these dates directly into my thesis. Therefore there is some uncertainty surrounding these dates, but they do proved a rough guide to the order of events. Ideally they would all be estimated again using more credible calibration points, but this is beyond the scope of my thesis.

Soon after the New Zealand wrens split off (between 67-81 mya) the remaining passerines diverged into two main suborders: the oscines (Passeri) and the suboscines (Tyranni) (1, 5). At this time South America was still attached to Australia, Africa and Antarctica, known as the supercontinent Gondwana, and South America did not separate until around 30-40 mya (23). The suboscines moved into what is now present day South America and around 60-65 mya they diverged into two clades (1, 5). The first, the Tyrannides, are now located within the new world, particularly South America, and is the larger of the two clades (1). The Tyrannides contain the ovenbirds, woodcreepers, antbirds, antthrushes, antpittas, tapaculos, gnateaters, gnatpittas, tyrant-flycatchers, tityras, contigas and manakins. The second, the Eurylaimides, is a smaller old world clade which originated in Africa and has expanded into Asia around the Indian Ocean (1). The Eurylaimides consists of the broadbills, asities and pittas. There are currently only two suboscines with fully sequenced mitochondrial genomes (the fuscous flycatcher and broadbill), one from each clade, and a third (golden-collared manakin) with mitochondrial transcriptome data (Table 1).

Table 1: Passerines with sequenced mitochondrial genomes. List of all passerine species (common and scientific names) with a sequenced mitochondrial genome or transcriptome, where they are classified on passerine tree, what type of data and where sourced from, including GenBank accession number.

Species	Classification	Type and source
NZ Rifleman (*Acanthisitta chloris*)	Acanthisitti, Acanthisittidae	Near complete, GenBank, AY325307
Fuscous Flycatcher (*Cnemotriccus fuscatus*)	Suboscine (Tyrani), Tyrannidae	NC_007975
Broadbill (*Smithornis sharpie*)	Suboscine (Tyrani), Eurylaimidae	NC_000879
Lyrebird (*Menura novaehollandiae*)	Oscine, Menuridae	NC_007883
Tui (*Prosthemadera novaeseelandiae*)	Oscine, Meliphagoidea, Meliphagidae	Complete, Gillian Gibb (15), KC545404
Grey Warbler (*Gerygone igata*)	Oscine, Meliphagoidea, Acanthizidae	Complete, Briar Smith, unpublished, KC545399
European Magpie (*Pica pica*)	Oscine, Core Corvoidea, Corvidae	NC_015200
Azurawinged Magpie (*Cyanopica cyanus*)	Oscine, Core Corvoidea, Corvidae	NC_015824
Rook (*Corvus frugilegus*)	Oscine, Core Corvoidea, Corvidae	NC_002069
Mongolian Ground Jay (*Podoces hendersoni*)	Oscine, Core Corvoidea, Corvidae	NC_014879
Eurasian Jay (Garrulus glandarius)	Oscine, Core Corvoidea, Corvidae	NC_015810
NZ fantail (*Rhipidura fuliginosa*)	Oscine, Core Corvoidea, Rhipiduridae	Complete, Briar Smith, unpublished, KC545405
Saddleback (*Philesturnus carunculatus*)	Oscine, Callaeidae	Complete, Gillian Gibb (15), KC545403
Hihi (*Notiomystis cincta*)	Oscine, Notiomystidae	Complete, Gillian Gibb (15), KC545400
Myna (*Sturnus tristis)*	Oscine, Passerida, Muscicapoidea, Sturnidae	NC_015195
White-cheeked Starling (*Sturnus cineraceus*)	Oscine, Passerida, Muscicapoidea, Sturnidae	NC_015237
Red-billed Starling (*Sturnus sericeus)*	Oscine, Passerida, Muscicapoidea, Sturnidae	NC_014455
Crested Myna (*Acridotheres cristatellus*)	Oscine, Passerida, Muscicapoidea, Sturnidae	NC_015613
Common hill Myna (*Gracula religiosa*)	Oscine, Passerida, Muscicapoidea, Sturnidae	NC_015898
Siberian Rubythroat (*Luscinia calliope)*	Oscine, Passerida, Muscicapoidea, Muscicapidae	NC_015074
Blue and white Flycatcher (*Cyanoptila cyanomelana*)	Oscine, Passerida, Muscicapoidea, Muscicapidae	NC_015232
Yellow-rumped Flycatcher (*Ficedula zanthopygia*)	Oscine, Passerida, Muscicapoidea, Muscicapidae	NC_015802
Grey-Capped Finch *(Carduelis sinica*)	Oscine, Passerida, Passeroidea, Fringillidae	NC_015196
Siskin (*Carduelis spinus*)	Oscine, Passerida, Passeroidea, Fringillidae	NC_015198
Indigo Bird (*Vidua chalybeata*)	Oscine, Passerida, Passeroidea, Viduidae	NC_000880
Zebrafinch (*Taeniopygia guttata*)	Oscine, Passerida, Passeroidea, Estrildidae	NC_007897
Tristram's Bunting (*Emberiza*	Oscine, Passerida, Passeroidea,	NC_015234

tristrami)	Emberizidae	
Yellow-Browed Bunting (*Emberiza chrysophrys*)	Oscine, Passerida, Passeroidea, Emberizidae	NC_015233
Jamaican Blackbird (*Nesopsar nigerrimus***)**	Oscine, Passerida, Passeroidea, Icteridae	JX516054
Chopi Blackbird *(Gnorimopsar chopi)*	Oscine, Passerida, Passeroidea, Icteridae	JX516055
Chestnut-capped Blackbird *(Chrysomus ruficapillus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516056
Yellow-hooded Blackbird *(Chrysomus icterocephalus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516060
Velvet-fronted Grackle *(Lampropsar tanagrinus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516057
Bolivian Blackbird *(Oreopsar bolivianus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516058
Pale-eyed Blackbird *(Agelaius xanthophthalmus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516059
Red-winged Blackbird *(Agelaius phoeniceus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516062
Yellow-winged Blackbird *(Agelaius thilius)*	Oscine, Passerida, Passeroidea, Icteridae	JX516069
Unicoloured Blackbird *(Agelaius cyanopus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516076
Melodious Blackbird *(Dives dives)*	Oscine, Passerida, Passeroidea, Icteridae	JX516061
Scarlet-headed Blackbird *(Amblyramphus holosericeus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516063
Common Grackle *(Quiscalus quiscula)*	Oscine, Passerida, Passeroidea, Icteridae	JX516064
Saffron-crowned Blackbird *(Xanthopsar flavus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516065
Brown-and-yellow Marshbird *(Pseudoleistes virescens)*	Oscine, Passerida, Passeroidea, Icteridae	JX516066
Bronzed Cowbird *(Molothrus aeneus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516067
Bay-winged Cowbird *(Molothrus badius)*	Oscine, Passerida, Passeroidea, Icteridae	JX516074
Yellow-tailed Oriole *(Icterus mesomelas)*	Oscine, Passerida, Passeroidea, Icteridae	JX516068
Austral Blackbird *(Curaeus curaeus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516070
Yellow-rumped Marshbird *(Pseudoleistes guirahuro)*	Oscine, Passerida, Passeroidea, Icteridae	JX516071
Brewer's Blackbird *(Euphagus cyanocephalus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516072
Golden-tufted Mountain Grackle *(Macroagelaius imthurni)*	Oscine, Passerida, Passeroidea, Icteridae	JX516073
Oriole Blackbird *(Gymnomystax mexicanus)*	Oscine, Passerida, Passeroidea, Icteridae	JX516075
Silver-eyed Mesia (*Leiothrix argentauris*)	Oscine, Passerida, Sylvioidea, Timaliidae	NC_015114
Taiwan Bulbul (*Pycnonotus taivanus*)	Oscine, Passerida, Sylvioidea, Pycnonotidae	NC_013483
Light-vented Bulbul	Oscine, Passerida, Sylvioidea,	NC_013838

(*Pycnonotus sinensis*)	Pycnonotidae	
Orphean Warbler (*Sylvia crassirostris*)	Oscine, Passerida, Sylvioidea, Sylviidae	NC_010229
Blackcap (*Sylvia atricapilla*)	Oscine, Passerida, Sylvioidea, Sylviidae	NC_010228
Reed Warbler (*Acrocephalus scirpaceus*)	Oscine, Passerida, Sylvioidea, Acrocephalidae	NC_010227
Tree Swallow (*Tachycineta bicolor***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071614
Violet-green Swallow *(Tachycineta thalassina***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071615
Golden Swallow *(Tachycineta euchrysea***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071616
Bahama Swallow *(Tachycineta cyaneoviridis***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071617
Tumbes Swallow *(Tachycineta stolzmanni***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071618
Mangrove Swallow *(Tachycineta albilinea***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071619
White-winged Swallow *(Tachycineta albiventer***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071620
White-rumped Swallow *(Tachycineta leucorrhoa***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071621
Chilean Swallow *(Tachycineta meyeni***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071622
Gray-breasted Martin *(Progne chalybea***)**	Oscine, Passerida, Sylvioidea, Hirundinidae	JQ071623
Hume's Ground Tit (*Pseudopodoces humilis*)	Oscine, Passerida, Paridae	NC_014341
Species I have worked on:		
Song Thrush (*Turdus philomelos*)	Oscine, Passerida, Muscicapoidea, Turdidae	Complete, KC545406, (24)
Fernbird (*Bowdleria punctata*)	Oscine, Passerida, Sylvioidea, Locustellidae	Complete, KC545398
Tomtit (*Petroica macrocephala*)	Oscine, Petroicidae	Complete, KC545402
Pipit (*Anthus novaeseelandiae*)	Oscine, Passerida, Passeroidea, Motacillidae	Complete, KC545397
Waxeye *(Zosterops lateralis)*	Oscine, Passerida, Sylvioidea, Zosteropidae	Complete, KC545407
Browncreeper (*Mohoua novaeseelandiae*)	Oscine, Core Corvoidea, Pachycephalidae	Incomplete (10555 bp), KC545409
Bellbird *(Anthornis melanura)*	Oscine, Meliphagoidea, Meliphagidae	Incomplete (9453 bp), KC545408
New Zealand robin (*Petroica australis)*	Oscine, Petroicidae	Complete, Gerrit Hartig, unpublished, KC545401
Golden-Collared Manakin (*Manacus vitellinus*)	Suboscine (Tyrani), Pipridae	Transcriptome (2), SRR029477–78
American Crow (*Corvus brachyrhynchos*)	Oscine, Core Corvoidea, Corvidae	Transcriptome (2), SRR029463–64
Pied Flycatcher (*Ficedula hypoleuca)*	Oscine, Passerida, Muscicapoidea, Muscicapidae	Transcriptome (2), SRR029159–61
Blue Tit (*Parus caeruleus*)	Oscine, Passerida, Paridae	Transcriptome (2), SRR029162

1.1.3 The basal oscines

The oscines remained in Australasia radiating into many different lineages before some, at least on four separate occasions, dispersed out of Australasia into the rest of the world (1). There are a number of small lineages known as the basal oscines, which radiated off the oscine tree before the large split between the Core Corvoidea and Passerida (Figure 1). The oldest of these are the family the Menuridae (13, 15), which consists of only two species, both known as lyrebirds and both are endemic to Australia. The mitochondrial genome of one of these species (*Menura novaehollandiae*) was sequenced by Slack et al. (25). These are estimated to have branched off after the K/PG boundary between 48-67 mya (5). The second division formed the lineage containing both the tree creepers (Climacteridae) and the bowerbirds (Ptilonorhynchidae), seen in Figure 2. These lineages diverged from the oscines again after the K/PG boundary (1, 13, 15), supported by multiple studies using different molecular data sets; both groups are endemic to Australia and PNG.

The third division within the oscines leads to the largest Australasian passerine radiation, the superfamily Meliphagoidea, broke away from the remaining oscines (26). The majority of the Meliphagoidea species are endemic to either Australia or PNG, but some have dispersed into the Pacific Islands, including New Zealand, with one species spreading up into Southeast Asia. The monophyly of this superfamily has been confirmed by multiple researchers (26), but where exactly the lineage branches off the main oscine tree has in the past been controversial. Some initially placed the group within the Core Corvoidea (27), but they are now considered to be basal oscines (15, 28), as seen in Figure 2. The superfamily is split into four families: The Maluridae, Pardalotidae, Acanthizidae, and Meliphagidae.

The Maluridae is the basal family of the four, and includes the fairy emu and grass wrens. The next two families are closely related, the Pardalotidae (pardalotes) and the Acanthizidae (Australasian warblers). The Acanthizidae are monophyletic (26, 29); they originated within Australia and split from the other Meliphagoidea during the late Oligocene (~27 mya). Within this family is the genus *Gerygone*, which contains the New Zealand species *Gerygone igata* (grey warbler), the mitochondrial genome of this species has previously been sequenced but has not been published (Table 1). The genus has undergone a major radiation within the last 5 mya (29). The closest relative of the grey warbler is the Norfolk Island species, *Gerygone modesta,* perhaps indicating a single dispersal event from Australia within the last few million years (29).

Sister to the Pardalotidae and Acanthizidae is the Meliphagidae, or the honey eaters. Gardner et al. (26) suggested that this family has had a large effect on the evolution of many flowering plants throughout Australasia. The New Zealand endemics, the tui and bellbird, form one of four clades within the Meliphagidae. The tui mitochondrial genome has previously been sequenced but has not been published (Table 1). This New Zealand clade diverged from the other clades around 19-31 mya (30). Whether this occurred in New Zealand or Australia is unresolved, but by 2.9 mya they had reached New Zealand and had split into the two species (22). No detailed study of the relationship between the four clades of the honey eaters has been carried out (26).

Two other small Australasian basal oscine lineages are the logrunners (Orthonychidae) and the Australian babblers (Pomatostomidae); these were initially placed within the Core Corvoidea by Irestedt & Ohlson (31). But Norman et al. (28) found they diverged before the Core Corvoidea and Passerida split, with the logrunners perhaps joining at the base of the Meliphagoidea (Figure 2).

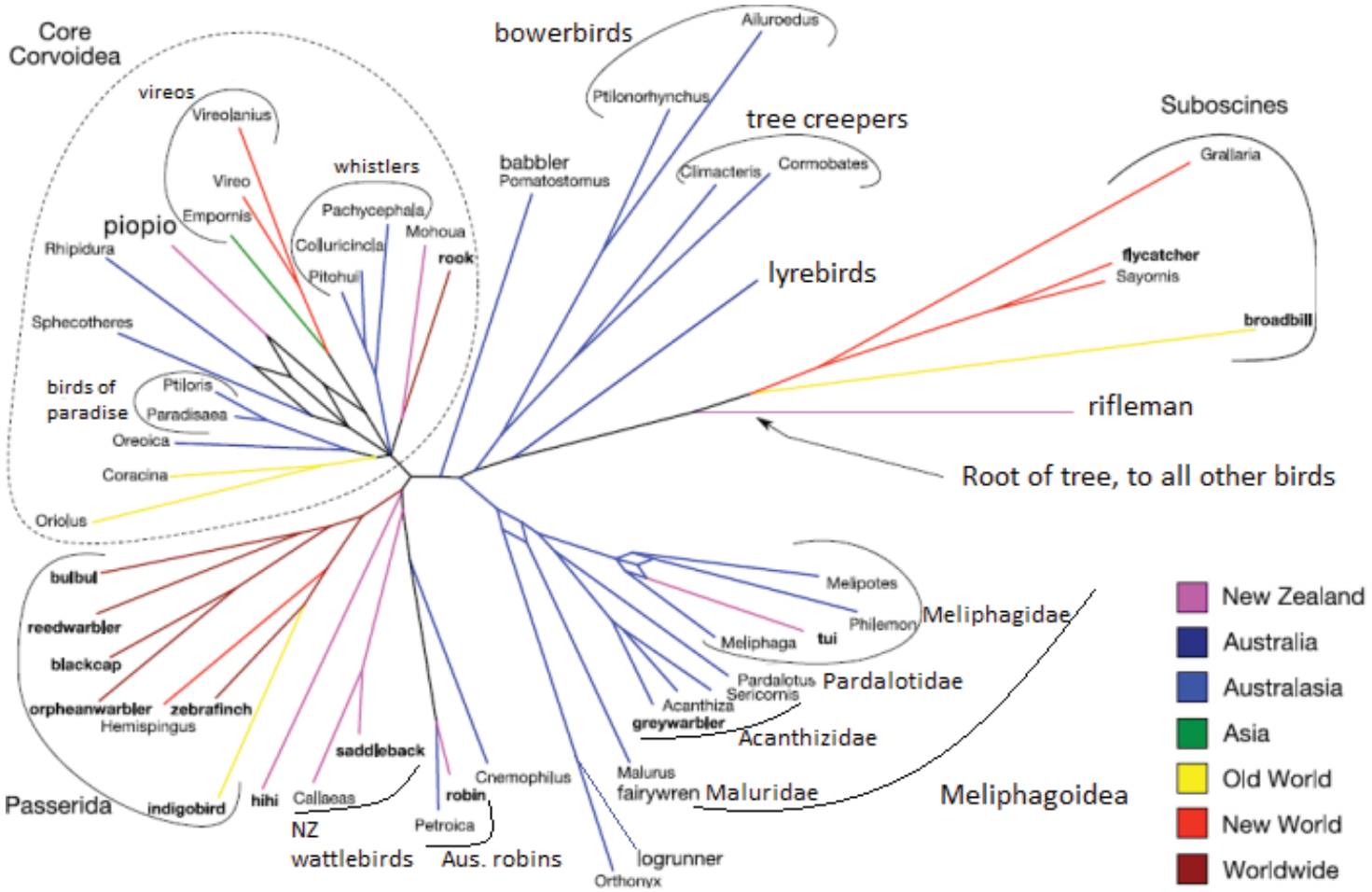

Figure 2: Network of Bayesian analysis of the passerines, based on mitochondrial data. Figure modified from Gibb (15), produced from Mr Bayes results in Splits tree. The root of the tree is on the rifleman branch. There is a large unresolved polytomy at base of Core Corvoidea. Mitochondrial data is in the form of either full mitochondrial genomes or 2-5 mitochondrial genes (15).

1.1.4 The Core Corvoidea

The oscines then split into two large lineages the Core Corvoidea and Passerida, during the mid-Paleogene, around 45 mya (13, 32). The Core Corvoidea are the most diverse lineage in Australasia (28); and Jonsson et al. (32) suggests that they radiated from the islands of Papua New Guinea. The Papuan islands first formed around the late Eocene (~34 mya), due to tectonic activity from the Australian plate moving northward. At this time we see the first radiation of the Core Corvoidea (32). Supporting this is the fact that Core Corvoidea diversity is highest in PNG, and every lineage that is outside of the Austral-Papuan region has a closely related sister lineage in PNG (32). The Core Corvoidea has dispersed out of Australasia radiating to fill many different niches, firstly reaching Asia around 40-37 mya, then Africa (29-26 mya) and the Americas (28-25 mya) (1). Jonsson et al., (32) suggests that Core Corvoidea evolution on the Papuan islands selected for species that were good at dispersing over water, unlike in Australia, which may explain why they as a lineage were so successful at dispersing throughout the world. In addition because of the mountains, there may have been more diverse habitats in PNG than in Australia. This also goes against the long held model of island biogeography where species flow (disperse) from continents to islands (sinks), but not the other way around as niche availability on continents is much smaller, so therefore it is harder for island species to establish themselves back onto the adjacent continents (32). In this case though, the islands were a conduit to the rest of the world.

A number of large radiations have occurred within the Core Corvoidea in a relatively short time frame (28). This has made the resolution of relationships within the Core Corvoidea very difficult and created what is described as a polytomy, where more than two (in this case five) lineages split apart within a short space of time (33), as seen in Figure 2 at the base of the Core Corvoidea. The basal lineage of the group are the sitella (Neosittidae) which are located within Australasia, providing further support for the Core Corvoidea originating in the region, whilst the majority of the other lineages in the group have representatives that are located elsewhere in the world (33). The New Zealand fantail (*Rhipidura fuliginosa*) is part of the large fantail family Rhipiduridae; genetic diversity in this family is relatively low (34). The family dispersed relatively recently throughout Australasia with some species reaching into Asia (30, 34). Where they are placed within the Core Corvoidea is unresolved. Some studies place them as basal to the crows, monarch flycatchers, and drongos (31, 33), whilst Gibb (15) placed

the fantails with the piopios, an extinct NZ species, and the figbirds either as a sister group to the Vireos or branching off at the polytomy site (Figure 2). More data from a range of molecular sources is needed to fully resolve this polytomy. The centre of diversity and likely origin for the Rhipiduridae family is PNG (34), and the family can be divided into six main clades. The NZ fantail is part of a clade containing species found in Australia, PNG, NZ and the Pacific Islands (34). The other clades are located in PNG or have moved north into Asia (34).

Three New Zealand species, the browncreeper, whitehead and yellowhead, make up the genus *Mohoua*; this is classified within the family Pachycephalidae, which also includes the whistlers and *Pitohui* (33, 35). The Pachycephalidae has now been found to be paraphyletic with genera spread throughout different clades within the Core Corvoidea (28). The placement of the genus *Mohoua* is unresolved; some studies place it with the whistlers (28), whilst Gibb (15) found it to branch off the crows, and Jonsson et al., (32) found the *Mohoua* diverged from the site of the large polytomy, estimating it to have occurred around the late Eocene (34 mya). A pattern that has been found in the Core Corvoidea is for the species of the mountains of Papuan islands to be related to species found in the dry areas (west of the Great Dividing Range) in Australia, with the species found in the eastern rainforests of Australia basal to these (28).

There are two types of polytomy; hard and soft. A hard polytomy is seen when there are many diversification events within a short time; and very little change in the molecular sequence occurs between each event (36). This type of polytomy may never be resolved no matter how much molecular data is used. A soft polytomy occurs when insufficient molecular data has been compared between the different lineages to indicate the order of the diversification events (36). To distinguish between the two the use of a range of molecular loci, with different evolutionary properties, is needed (36). Fuchs et al. (36) used ten loci, a mix of mitochondrial, autosomal, and sex linked, to resolve the phylogeny of the Malaconotidea, an old world clade within the Core Corvoidea. There are now six sequenced mitochondrial genomes, one transcriptome and one partial mitochondrial genome from within the Core Corvoidea (Table 1). One mechanism that does increase the chance of getting a polytomy are introgression events between the diverging lineages (36)

1.1.5 Basal Passerida?

There are a number of lineages for which it is unresolved whether they are basal groups of the Core Corvoidea or the Passerida (1, 28). One of these groups is the Australasian robins (Petroicidae). The centre of diversity of the Petroicidae is Australia and PNG with 40 of the 46 species found there (37). This group likely originated in Australia but has dispersed out to PNG, the Pacific Islands and New Zealand (30). These were previously assigned to either the Core Corvoidea (10) or the Meliphagoidea (38), both hypotheses were consistent with the centre of diversity being in Australasia. But recent work (15, 28, 31) instead found strong support for their placement at the base of the Passerida, a largely northern hemisphere centred lineage. The placement of the Petroicidae at the base of the Passerida suggests the family's evolutionary history is relatively long.

Miller and Lambert (39) investigated the relatedness of the three species native to New Zealand; The New Zealand robin (*Petroica australis*), the Chatham Island black robin (*Petroica traverseri*), and the New Zealand tomtit (*Petroica macrocephala*), along with two species found on Norfolk Island and Fiji. They analysed two regions of the mitochondrial genome, the Cytochrome-b gene and the control region, and found the black robin was sister to the tomtit and the NZ robin joined with the Pacific species and an Australian species was the out-group to all. This was unexpected as the morphology and behaviour of the black and NZ robins are very similar, compared to the tomtit, and they have always been classed as sister taxa (39). This lead Miller and Lambert (39) to suggest that the black robin is a result of an ancient hybridization event between the NZ robin and the tomtit, but nuclear data will be needed to clarify this. There is also some evidence (39) to suggest the genus *Petroica* evolved in Australia and had two invasions into New Zealand. First, a longer history for the NZ robin than the tomtit is indicated by the NZ robins being more derived from the Australian *Petroica* species than the tomtits. Also the North and South Island subspecies of the NZ robin are more genetically distinct than the two tomtit subspecies indicating the robin subspecies have been separated for a longer time. This is mostly speculation and cannot be proved without testing all Australian species in the genus.

The satin birds of the genus *Cnemophilus*, are native to the PNG mountains, and were originally placed within the Core Corvoidea with the birds of paradise when Barker et al. (1) looked at RAG-1 data. But more recent studies (15, 21) with

mitochondrial data found that they formed into a clade with the Petroicidae, at the base of the Passerida (Figure 2).

The New Zealand wattlebirds (Callaeidae), are a family made up of the three New Zealand species, the kokako and the saddleback (which are both endangered), and the huia (which became extinct during the 20th century) (21). These were originally classified as basal Core Corvoidea (1, 33) based largely on data from the nuclear gene RAG-1. But other studies (15, 21, 31) using different nuclear and mitochondrial data show very strong support for their placement as basal Passerida, either sharing a common ancestor with the Petroicidae and *Cnemophilus* around 29-41 mya (21) as seen in Figure 2, or branching off the Passerida more recently than these two (15). The New Zealand wattlebirds were thought of as being part of the ancient lineages that have a vicariant origin in New Zealand (30), primarily because the taxon is only found in NZ. However Shepherd et al. (21) showed that this taxon split from the Passerida more recently than Zealandia split from Gondwana, indicating they must have dispersed here.

The New Zealand species *Notiomystis cincta* (hihi or stichbird) was originally placed with the honeyeaters in the Meliphagoidea (27, 30). However now there is strong support for this species comprising its own family, the Notiomystidae, and diverging from the NZ wattlebird lineage around 33 mya (22). The placement of the Notiomystidae here was supported by Gibb (15) using complete mitochondrial genome data. These four lineages; the Australasian robins, the New Zealand wattlebirds, the hihi and the *Cnemophilus*, will be referred to in this study as basal Passerida. As reported here, there are now four mitochondrial genomes available from species in these groups. By analysing these along with genomes from representatives of the crown Passerida and Core Corvoidea, confirmation of their placement at the base of the Passerida branch should be found.

1.1.6 The Passerida

The Passerida is traditionally made up of three main superfamilies: the Sylvioidea, Passeroidea, and Muscicapoidea (2). The family Paridae is sometimes also classified as a fourth superfamily. The Passerida originated in Australasia around 45 mya, which involved firstly a large radiation in Australasia, followed by dispersal out into the world, followed by other large radiations resulting in the formation of many of the lineages that represent the Passerida today (1, 32, 40).

All species in the crown Passerida contain an 18bp deletion in the GADPH gene and their monophyly is undisputed (1). As mentioned above this group is split into three main super families: the Sylvioidea, Muscicapoidea, and Passeroidea. There is uncertainty about how these three superfamilies have diverged. Some studies (1, 41) state that the Sylvioidea is sister to the other two super families, with the Muscicapoidea and Passeroidea diverging later. Other studies using nuclear data (13, 40) group the Sylvioidea with the Muscicapoidea. Lastly mitochondrial transcriptome sequencing by Nabholz et al., (2) has shown the Muscicapoidea is basal to the two other superfamilies, summarised in Figure 3 from Nabholz et al. (2). There is also strong support for the split of some lineages originally placed within the Sylvioidea to form its own superfamily the Paridae (2, 40, 41). Such uncertainty leads to the question: are these true monophyletic groups? Gibb (15) used full mitochondrial genomes but only had three representatives from the Sylvioidea and two from the Passeroidea. Nabholz et al. (2) continued this by adding mitochondrial transcript data from an extra Sylvioidea, a representative from both the Muscicapoidea and the Paridae but did not receive strong support for the branching of the superfamilies as seen in Figure 3.

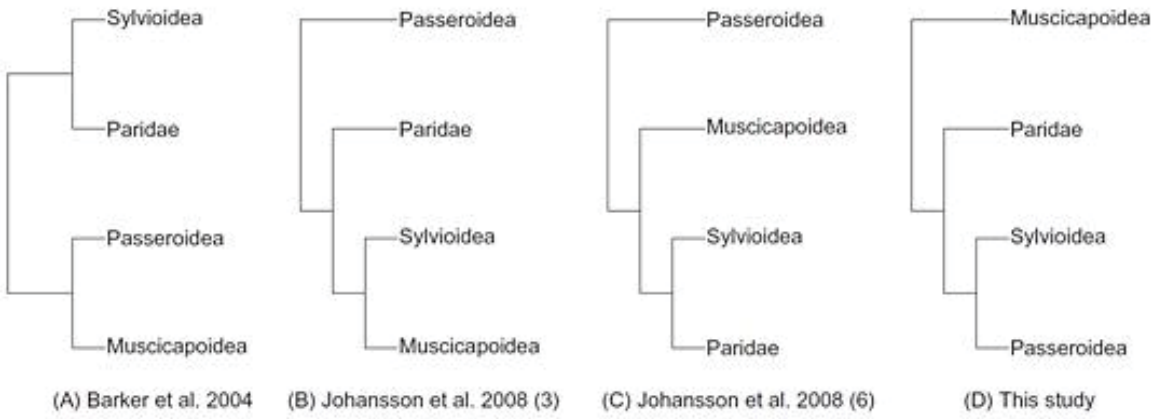

Figure 3: The differing opinions on the phylogeny of the superfamilies of the Passerida. Figure from Nabholz et al. (2). A) From Barker et al. (1) based on RAG1 and 2 genes (4165 bp) from 144 species. B) and C) are both from the Johansson et al. study (40) but differ in the number of genes used to create the tree B) with 3 genes (2315 bp) and C) with 6 genes (7288 bp). D) Is from the Nabholz et al. (2) study based on mitochondrial genomes and transcriptomes from 15 passerine species.

There are now 62 complete and one near-complete (Rifleman) mitochondrial genomes of passerines on GenBank (as of the 20/11/2012), as well as the four transcriptomes from Nabholz et al. (2). Five genomes used by Gibb (15), and the NZ fantail (unpublished) will be added to the five mitochondrial genomes I have completed:

Bowdleria punctata (fernbird), *Anthus novaeseelandiae* (pipit), *Zosterops lateralis* (waxeye), *Petroica macrocephala* (tomtit) and *Turdus philomelos* (song thrush), see Table 1. This means there are now a total of 78 passerine mitochondrial genomes to compare, including multiple representatives of each of the three superfamilies of the Passerida along with two from the Paridae. These genomes could be analysed to resolve the phylogeny of the Passerida, allowing for a greater understanding of the relationships between the superfamilies, as well as the divisions within these. All the available passerine sequences could also be analysed to resolve other questions surrounding certain parts of passerine phylogeny mentioned above.

The Sylvioidea are found throughout the world, but their diversity is centred in the Indo-Pacific area. The superfamilies phylogeny is relatively unresolved. At the base of the group a lineage made up of the tits and chickadees branches off; these are considered by some to be their own superfamily the Paridae (2), see Figure 3. This is then followed by a polytomy of four lineages (33). The three smallest include the larks and other minor groups, while the largest of the four contains the warblers, bulbuls, old world babblers and swallows. The relationships within this large group are also unresolved due to the formation of another large polytomy, indicating that the Sylvioidea radiated very rapidly and resolution will be very difficult (8, 33). The four Sylvioidea species analysed in Nabholz et al. (2) are part of the largest Sylvioidea lineage mentioned above, so do not help resolve this phylogeny. Sequenced mitochondrial genomes from representatives of the other three groups should aid in the resolution of this superfamily.

There are three native New Zealand species within the Sylvioidea superfamily. The fernbird (*Bowdleria punctata*) has been classified in its own genus (10), but other studies have suggested that it should become part of a closely related Australian warbler genus *Megalurus* (30). These have been grouped in a clade with *Acrocephalus scirpaceus* (33). The monophyly of this clade will be tested using the phylogenetic trees created in this thesis.

The second native Sylvioidea is *Zosterops lateralis*, known as the waxeye or silvereye; it is native to NZ but not endemic, with the same species found in Australia and some Pacific Islands. The waxeye first arrived in NZ in 1856, but this is thought to have been a self-introduction, so the species is classified as a native (42). Moyle et al. (17) used data from three mitochondrial and three nuclear genes from 300 individuals of

the families Timaliidae, the babblers, and the Zosteropidae to resolve the evolutionary history of these families and uncover their origins (17). The Moyle et al. (17) data shows that the two families group within the Sylvioidea, with strong support for the placement of the Zosteropidae as a sister group to the Timaliidae. The Timaliidae has one representative with a fully sequenced mitochondrial genome (the silver-eyed mesia *Leiothrix argentauris*). These two species (*Zosterops lateralis* and *Leiothrix argentauris*) should group together in the phylogenetic tree, supporting the findings of Moyle et al. (17). One calibration point for passerine phylogeny uses the age of the Solomon Islands, for the diversification of the Zosteropidae from the crown Yuhina, and is thought to have occurred a maximum of 8.8 mya (17). The Zosteropidae are thought to originate in mainland Asia, along with two of the babbler clades (17); this would be an example of passerines re-invading Australasia. The Zosteropidae are estimated to have split from the Timaliidae between 16-21 mya (17). The Zosteropidae family has been described as a great speciator (43), containing around 100 species which have all evolved within the last 10 million years.

The last Sylvioidea native is the welcome swallow (*Hirundo neoxena*). This species is also thought to have self-introduced to New Zealand in the 1950s (42). It is therefore a native, but is not endemic, with the species distributed across Australia and other Pacific Islands. The welcome swallow is part of the family Hirundinidae, for which 10 mitochondrial genomes from different species of this family have become available (44). Therefore, the welcome swallow mitochondrial genome sequence was not considered for sequencing in this study.

The Passeroidea are found mainly in the Europe, Asia and the Americas; the majority are herbivores and include the sparrows, wagtails, finches (41). The division of the Passeroidea is also unresolved with large polytomies (33), and some traditional lineages have been found to be paraphyletic (41). But there are now 29 sequenced mitochondrial genomes, from many different lineages within the Passeroidea superfamily (Table 1). This large amount of molecular data should help resolve some of polytomies in the Passeroidea. One NZ native belongs to this superfamily; the pipit (*Anthus novaeseelandiae*). The pipit is native but not endemic to New Zealand with the species distributed across the whole of Australasia (30). The pipit is part of the worldwide distributed family Motacillidae, and the NZ pipit may represent a re-invasion back into Australasia from the outside.

The Muscicapoidea on the other hand is a relatively well resolved superfamily, with strong support for the monophyly of groups within (41). They are the smallest of the three superfamilies and are found mostly in Europe, Asia, and Africa, with one family in the Americas. They include the thrushes, starlings, old world flycatchers, and chats (41). There are no New Zealand natives in this lineage but some have been introduced by humans (30), including the song thrush (*Turdus philomelos*), for which a mitochondrial genome is now avaliable (24). In total there are 10 species with a sequenced mitochondrial genome or transcriptome from the Muscicapoidea (Table 1). The Bombycillidae (waxwings) are a family that was originally placed within the Muscicapoidea but are now suggested to be a basal group to the three superfamilies (41).

1.2 New Zealand's passerines

In New Zealand there are around 30 passerine species (45), 20 of these are native of which 17 are endemic. Some belong to an endemic family/genera (Acanthisittidae, Callaeidae, Notiomystidae, *Mohoua*, *Turnagra*), but many of the other native species have closely related species located elsewhere in the world (45), indicating that many are probably relatively recent arrivals. Studies of passerine molecular data indicate there have been 14 events of dispersal or vicariance of passerines into New Zealand (30).

A list of the conservation status of all organisms that have been recorded in New Zealand since 1800 is maintained by the Department of Conservation (42). The conservation classification status is determined for each taxon by taking into account the population size, the population trend (increasing, decreasing or stable), the breeding range, and whether the taxa has been affected by humans; a full description of how taxa are classified into these categories is detailed in Miskelly et al. (42). As of 2008 there are 428 avian taxa on the list, to subspecies/population level (42). Of these, 20 are extinct, 77 are threatened (split into 24 nationally critical, 15 nationally endangered, and 38 nationally vulnerable), 93 taxa are 'at risk' (18 declining, 10 recovering, 17 relict, and 48 naturally uncommon), 36 are native residents that are not threatened, 165 are coloniser, vagrant, or migrant, and 36 are introduced and naturalised (42). There are 12 passerine families with species native to New Zealand (42), and 51 passerine taxa; 11 taxa are extinct, 1 is nationally critical, 3 nationally endangered, 6 nationally vulnerable, 5 are declining, 3 recovering, 9 naturally uncommon, and 13 are not threatened (Table 2).

Table 2: All native New Zealand Passerine populations and their conservation status: Sorted by the family; lists scientific name, common name, each subspecies/population and their conservation status. Classification of lineages and the conservation status is defined by Miskelly et al. (42), in 2008, using the New Zealand Threat Classification System.

Family	Scientific name	Common name	Subspecies/ population	Conservation status
Acanthisittidae	*Acanthisitta chloris*	NZ rifleman	NI rifleman (granti)	At Risk – Declining
			SI rifleman (chloris)	At Risk - Declining
	Xenicus gilviventris	Rock wren		Threatened – Nationally Vulnerable
	Xenicus longipes	Bush wren	SI bush wren (longipes)	Extinct
			NI bush wren (stokesi)	Extinct
			Stead's bush wren (variabilis)	Extinct
	Traversia lyalli	Lyall's wren		Extinct
Acanthizidae	*Gerygone albofrontata*	CI warbler		Threatened – Nationally Vulnerable
	Gerygone igata	Grey warbler		Not Threatened
Callaeidae	*Callaeas cinerea*	SI kokako		Extinct
	Callaeas wilsoni	NI kokako		Threatened – Nationally Vulnerable
	Heteralocha acutirostris	Huia		Extinct
	Philesturnus carunculatus	SI saddleback		At Risk - Recovering
	Philesturnus rufusater	NI saddleback		At Risk - Recovering
Hirundinidae	*Hirundo tahitica*	Pacific swallow	Welcome swallow (neoxena)	Not Threatened
Meliphagidae	*Anthornis melanura*	Bellbird	NZ bellbird (melanura)	Not Threatened
			Three Kings bellbird (obscura)	At Risk – Naturally Uncommon
			Poor Knights bellbird (oneho)	At Risk – Naturally Uncommon
	Anthornis melanocephala	CI bellbird		Extinct
	Prosthemadera novaeseelandiae	Tui	NZ Tui (novaeseelandiae)	Not Threatened
			CI tui	Threatened – Nationally Endangered
Motacillidae	*Anthus novaeseelandiae*	NZ pipit	NZ pipit (novaeseelandiae)	At Risk - Declining

			Auckland Island pipit (aucklandicus)	At Risk - Recovering
			CI pipit (chathamensis)	At Risk – Naturally Uncommon
			Antipodes Island pipit (steindachneri)	At Risk – Naturally Uncommon
Notiomystidae	*Notiomystis cincta*	Hihi/stitchbird		Threatened – Nationally Endangered
Pachycephalidae	*Mohoua albicilla*	Whitehead		Not Threatened
	Mohoua novaeseelandiae	Browncreeper		Not Threatened
	Mohoua ochrocephala	Yellowhead		Threatened – Nationally Vulnerable
Petroicidae	*Petroica australis*	NZ robin	SI robin (australis)	Not Threatened
			Stewart Island robin (rakiura)	Threatened – Nationally Vulnerable
	Petroica longipes	NI robin		Not Threatened
	Petroica macrocephala	Tomtit	Yellow-breasted tomtit (macrocephala)	Not Threatened
			Pied tomtit (toitoi)	Not Threatened
			CI tomtit (Chatamensis)	Threatened – Nationally Endangered
			Black tomtit (dannefaerdi)	At Risk – Naturally Uncommon
			Auckland Island tomtit (marrineri)	At Risk – Naturally Uncommon
	Petroica traversi	Black robin		Threatened – Nationally Critical
Rhipiduridae	*Rhipidura fuliginosa*	Fantail	SI fantail (fuliginosa)	Not Threatened
			NI fantail (placabilis)	Not Threatened
			CI fantail (penita)	At Risk – Naturally Uncommon
Sylviidae	*Bowdleria punctata*	Fernbird	SI fernbird (punctata)	At Risk - Declining
			NI fernbird (vealeae)	At Risk - Declining
			Stewart Island fernbird (stewartiana)	Threatened – Nationally Vulnerable

			Snares fernbird (caudata)	At Risk – Naturally Uncommon
			Codfish Island fernbird (wilsoni)	At Risk – Naturally Uncommon
	Bowdleria rufescens	CI fernbird		Extinct
Turnagridae	*Turnagra capensis*	Piopio	SI piopio (capensis)	Extinct
			Stephens Island piopio (minor)	Extinct
	Turnagra tanagra	NI piopio		Extinct
Zosteropidae	*Zosterops lateralis*	Silvereye	(lateralis)	Not Threatened

The NZ pipit subspecies are all at risk, with the mainland subspecies declining despite the offshore island subspecies either recovering or being stable (Table 2), while there are different subspecies found in Australia and PNG. Fernbird populations in both the North Island (NI) and South Island (SI) are declining; the Stewart Island population is threatened and nationally vulnerable, and the two offshore populations are at risk and classified as naturally uncommon (42). This is a relatively grim situation for the species as all of the populations are at risk of extinction. The bellbird mainland subspecies is not threatened, whilst its two offshore island populations are naturally uncommon; as things stand it is very unlikely this species will become extinct (42). Both the browncreeper and waxeye are not threatened, and are relatively common (42). Within the tomtit species there are five subspecies. The NI and SI subspecies are not threatened but two of the offshore island subspecies are naturally uncommon, and the Chatham Island (CI) subspecies is nationally endangered (42). While this means the tomtit species is not likely to become extinct there is a very real chance that some of the natural diversity of the species will be lost unless measures are taken to conserve it. In general the New Zealand bird species/subspecies that are recovering are taxa that are being actively managed on predator-free offshore islands (42). An example is the saddleback which has been relocated to nine predator free islands, resulting in a population increase from 500 to around 5000 (46). Whilst no new native land species have become extinct since the 1960s, it is probable that some will go extinct in the near future (47). Many of mainland populations are threatened with extinction, their numbers are continuing to decline and their distributions are contracting (42). This would result in a loss of genetic

variation for the species which could have a large influence on how the species is able to adapt to future threats, such as disease (47).

The Department of Conservation (DoC) only have limited resources to manage the conservation of New Zealand's native birds. For the five years up to 2005, DoC received around $118.3 million in funding for conserving biodiversity on land (47). From this funding pest management, habitat restoration and species recovery programmes were performed (47), almost $10 million was spent on kiwi sanctuaries and $17 million on recovery programmes for other species. A variety of methods are used including transferring bird populations to predator-free off-shore islands, onsite breeding programmes, and rearing in captivity and releasing into managed environments (46). One question is: how do the government and DoC decide which species to conserve? The focus has been on conserving species level diversity, in particular recovery programmes for species that are facing extinction (47). Understanding the phylogeny of the Passerines is important for making informed decisions around funding the conservation of each species. To insure the greatest diversity both above and below species level is conserved, DoC first needs to have a clear understanding of the diversity of New Zealand's species. Phylogenetic trees can be used as a tool to assess how unique each species is, and how to allocate conservation resources.

1.3 Use of molecular data in phylogeny

What are the best molecular markers to use in these phylogenetic studies? Earlier studies used single genes such as the nuclear genes RAG-1, RAG-2, myoglobin (1), the mitochondrial genes Cytochrome *b* (CytB), or areas in the control region (39), or a combination of these markers (31, 41, 48). Each marker is not ideal for resolving all levels of phylogeny. Treplin et al. (41) states that much of passerine classifications has been based around RAG-1 when in fact this gene has low resolving power when there have been rapid radiations, which is what is seen in the oscines. Barker et al. (1) used RAG-1, and studies since have disagreed with their placement of many of the basal Passerida and Core Corvoidea (15, 31, 41), and other genes work better. Treplin et al. (41) found that the nuclear ZENK gene had good resolving power for divergences between 10-60 mya.

As the technology we use to sequence DNA and analyse molecular data improves we have seen the use of more molecular data per species. Next generation sequencing

allows large amounts of molecular data to be sequenced from a single sample, and is cheaper than the conventional capillary sequencing. Which makes resolving complex radiations (e.g. polytomies) achievable and cost effective (49). Hackett et al. (13) used 19 nuclear nuclear loci from 169 bird species to look at the deeper relationships between birds, but even with this amount of data Pratt et al. (7) found that the evidence from both morphological data and full mitochondrial genome sequence did not agree with all of Hackett's placements, e.g. the placement of the parrots as closest relative to the passerines. Pratt et al. (7) states using smaller sequences such as single genes or intron sequences do not result in good estimates for times of divergence. Nabholz et al. (2) observes that phylogenies produced from combinations of nuclear markers are weakly supported for passerine relationships.

Mitochondrial genomes are becoming a popular molecular marker for resolving phylogeny. Data from complete mitochondrial genomes (5, 7, 15), or the mitochondrial transcriptome (2) have been used by many of the more recent studies to achieve good resolution of passerine phylogeny. The circular mitochondrial genome is fast evolving, doesn't undergo recombination, and it is relatively easy to amplify its DNA (50, 51), making it a useful marker for phylogenetic studies. The mitochondrial genome is expected to have a substitution rate which can be used as a molecular clock (51, 52). As the mitochondrial genes are critical to the functioning of the entire cell there is a constraint that prevents them from evolving too rapidly and changing their function (51). This constraint means that non-synonymous substitutions should rarely happen, while the synonymous substitution rate is expected to be relatively high due to the relatively inefficient mitochondrial DNA repair system (51). This leads to the assumption that the vast majority of mutations seen in the mitochondrial genome are neutral (51, 53). Also as the genome is asexual it is assumed there will be an irreversible accumulation of slightly deleterious mutations (54), the process known as Muller's ratchet.

Many phylogenetic studies using the mitochondrial genome do not take into account the different evolutionary rates of different regions of the genome can have. The different rates can cause a bias in the phylogenetic analysis, to avoid this bias some studies don't analyse the full genome (55). Partitioning the molecular data allows the use of all the heterogeneity by applying separate evolutionary models to the different parts of the mitochondrial genome (55). The partitioning that worked best in the study by Powell et al. (55) was based on the codon position in protein coding genes, RNA

secondary structure, and whether the region is coding or non-coding. The partitioning used by Pratt et al. (7) will be applied to the molecular data in this study, and is the same as is recommended by Powell et al. (56).

There are a number of potential sources of error which can have an effect on resolving phylogeny, including: introgression, incomplete lineage sorting, base composition, gene duplication/orthologs, long branch attraction and sampling error. Individual gene trees often look different to the true species tree. This can be caused by gene flow after the initial lineage splitting event (29, 36, 51), known as introgression. Introgression is normal and has been shown to occur naturally in many different animals including birds (57, 58), beetles (59), mice (60), and even humans (61). A study on the passerine family the Parulidae found that the phylogenetic tree produced using mitochondrial DNA did not match with the plumage colour characteristics (58). Lovette (58) found that the colourful yellow plumage trait was found in distantly related species within the family. Lovette (58) suggests there possibly was gene flow after the lineages had initially diverged and the yellow plumage trait was inherited from the distant relative. Incomplete lineage sorting can also cause problems for resolving phylogeny (62); this is where the phylogeny produced from a single gene does not match the overall or true phylogeny, it is caused by genetic drift.

The use of multiple loci, under different evolutionary constraints (such as a combination of mitochondrial, autosomal and sex-linked genes) may help resolve the problems caused by introgression and incomplete lineage sorting (29, 36). Overall, mitochondrial data from protein, rRNA, and tRNA coding genes has been found to be very informative in resolving phylogeny (29). A study using mitochondrial genomes from ten species from a swallow genus *Tachycineta* found that analyses of data from complete protein-coding sequence was not enough to fully resolve the phylogeny of the lineage (44), complete resolution could only be achieved by including data from non-coding regions in the analyses. The group also found no evidence for positive selection occurring on the mitochondrial genome (44), indicating there was selection for preserving the function of the mitochondrial genes.

Base composition of the molecular data can also have a large effect on phylogenetic trees. Nabholz et al. (63) found that GC content variation had a large effect on the placement of taxa in phylogenetic trees. They found that a higher recombination rate was associated with a higher GC content. When studying the relationship between

different avian orders it was found that when GC variation was not accounted for, the parrots did not group as the sister lineage to the passerines (63). This was due to the relatively low GC content of the parrots and ratites. But when the GC variation was accounted for, by using RY coding for the third codon (replacing all A and G with R and all T and C with Y), there was strong support for the parrots as the sister group to the passerines (63).

Some studies have used the presence of a transposon, found in the control region of many birds, or the duplication of the control region to determine phylogeny (5, 64, 65). Often the non-coding control region is duplicated in avian species (5), a duplication appears to have occurred on multiple occasions within the passerines. Within the 78 species with sequenced mitochondrial genomes, 26 species, belonging to five distinct lineages, have a duplicated control region. The duplication is likely caused by slipped strand mis-pairing, often due to the presence of repeats at either of ends of the junctions of the control regions (66). Mitochondria are thought to be under pressure to be compact (66), so the elimination of these extra copies of genes/regions is assumed to be selected for, with mutations making extra copies non-functional followed by the elimination of the pseudogene. This process is known as the tandem duplication/random loss model of mitochondrial genome rearrangement (66). The study by Schirtzinger et al. (66) demonstrated that control region duplication has occurred on at least six occasions in the parrot lineage. There has been no evidence for any loss of a duplicated control region, giving no support for the hypothesis that there is selection for eliminating the duplicate (66). It would be interesting to see if there is any evidence of duplication elimination occurring in the passerines? Or if each distinct lineage with a duplicated control region is due to a separate duplication event?

When studying phylogeny there are restrictions on how much molecular data one can use, because it is both expensive to collect the data (nucleotide sequence), and the larger the data set the more computer hours the phylogenetic software takes to run the analysis. Therefore there is a need to strike a balance between two possible strategies on how much molecular data is used from each the species. The first is to have more data from a few taxa, and the second is to have more taxa but a smaller amount of data from each. There are advantages and disadvantages to both. With fewer taxa a problem is long branch attraction; this is where two sequences that are highly dissimilar to the other sequences in the analysis cluster together even though this may not represent the true phylogeny. One way to stop this is to “break up” these long branches by including

sequence from relatively close relatives of the two sequences. With more taxa with smaller amounts of data from each another problem arises that is sampling error. If an error is made in the sequencing of the DNA and a base is changed this can affect the resolution of the phylogeny by creating an artificial signal. The effect the signal has is increased when only small amounts of sequence from each species are compared as there are fewer nucleotides to compare. Overall increased taxon sampling in the form of more species with more molecular data from each, such as multiple species with sequenced mitochondrial genomes, will increase the accuracy of the phylogenetic analysis.

1.4 Project outline

The passerines are diverse and numerous and understanding their history is far from complete. Many studies have gone some way to explaining the phylogeny of the order as a whole, whilst others have focused on specific taxa. The fact that passerines have undergone such large and rapid radiations have made resolving their history very complicated, and we may never fully understand it. But with every extra study the phylogenetic tree is taking form, providing a clearer understanding of the evolution of the group. A number of deep areas are now well established such as the Gondwanian origin of the passerines and the Australasian origin of the oscines, the division into the three suborders, and the placement of a number of the basal oscines. But many of the more recent divergences require more research to resolve conflicting results between different studies. These conflicts include the placement of many of the basal Passerida, the polytomy seen at the base of the Core Corvoidea, and the relationships between the three superfamilies of the Passerida.

New Zealand has 20 native passerine species, 17 of which are endemic, these belong to 12 families (three endemic). A number of these species are threatened or at risk of extinction, and fully understanding the diversity of the native passerines is important for the conservation of the group.

The aim of this project is to have a fully sequenced mitochondrial genome from each of New Zealand's native passerine families. These will be used as a molecular catalogue for all of New Zealand's passerine families, which should provide an insight into the diversity of the native passerines, and will provide more information for conservation. Phylogenetic trees using the genomes of New Zealand species along with all other available passerine genomes will be constructed. These trees will be used to

address a number of questions surrounding passerine phylogeny (discussed above), and also the placement of a number of New Zealand taxa within the order.

Objective 1: Construct a sequenced mitochondrial genome from each of New Zealand's passerine families.

Objective 2: Use these genomes along with all other available genomes to construct a robust phylogeny for the passerines, which will then be used to address a number of questions surrounding the avian order.

1.5 Overview of results

The mitochondrial genomes of the fernbird, pipit, tomtit, and waxeye have successfully been sequenced. The mitochondrial genome of both the browncreeper and bellbird has been partially sequenced. All have been annotated and are submitted to GenBank. They have been aligned with 66 available passerine mitochondrial genomes from GenBank and seven other unpublished passerine genomes sequenced by others of my research group. Phylogenetic trees using both maximum likelihood and Bayesian analyses have been constructed. These trees have been used to address a number of the questions surrounding the phylogeny of the passerines such as the placement of the Australasian robins, NZ wattlebirds and the hihi as Basal Passerida.

2 Materials and Methods

2.1 DNA extraction

The fernbird, pipit, tomtit, and browncreeper were provided by the Museum of New Zealand Te Papa Tongarewa in Wellington as tissue preserved in alcohol. The sample for the waxeye was provided by the Wairarapa DoC centre in Masterton, and was from frozen tissue (Table 3), it was collected in 2002. The bellbird was provided by Phil Cassey and John Ewen from Tiritiri Matangi Island in the form of a blood sample, collected in 2006. Total DNA was extracted from tissue (25-50 mg) or blood of each bird using the High Pure™ PCR Template Kit (Roche Applied Science, Mannheim, Germany), following the manufacturers' protocol. An ethanol precipitation reaction was used in some of the samples to remove any ethanol carryover. DNA concentrations were measured using the Nanodrop (Thermo Scientific), following the manufacturers' instructions. DNA was also extracted from the tui, fantail and harrier hawk, but was not used for next generation sequencing.

2.2 DNA amplification

For each species where genomic DNA was extracted in sufficient quantity, long range PCRs were performed to amplify the mitochondrial DNA. A region of ~1kb, from 12S-16S was then amplified from one of the long range products to confirm the identity of the sample.

For long range PCRs the Roche Expand Long Template PCR system was used with the following reaction mixture: 35.75µL sterile Milli-Q H_2O, 5µL PCR Buffer 3 (supplied with polymerase), 1µL forward primer (10µM), 1µL reverse primer (10µM), 2.5µL dNTP mix (10µM), 0.75µL Expand Long Template Enzyme mix (3.75U), and 5µL of template DNA. The reaction was mixed and run on a PCR machine using the following thermal cycle programme: Lid temperature 93°C; 1. 93°C 02:00; 2. 93°C 00:30; 3. 52°C 00:30; 4. 68°C 10:00; repeat steps 2-4 x9; 5. 93°C 00:30; 6. 52°C 00:30; 7. 68°C 10:00; repeat steps 5-7 with 00:20 added to step 7 each cycle; 8. 68°C 20:00°C; 9. 10°C hold.

For short range PCRs, Roche Taq DNA polymerase was used with the following reaction mixture: 36.25µL sterile Milli-Q H_2O, 5µL 10x PCR buffer (supplied with

polymerase), 2µL forward primer (20µM), 2µL reverse primer (20µM), 1µL dNTP mix (10µM), 0.25µL Taq polymerase (1.25U), and 3µL of template DNA. The reaction mixture was mixed and run on a PCR machine using the following thermal cycle programme: Lid temperature 94°C; 1. 94°C 03:00; 2. 93°C 00:30; 3. 52°C 00:30; 4. 72°C 02:00; repeat steps 2-4 x34; 5. 72°C 05:00°C; 9. 10°C hold.

Primer combinations were found by examining previous avian mitochondria studies and using the laboratory primer database, (full list of primers used is in Supplementary Table 1). During the extractions and PCRs, 1% (w/v) agarose gels in 1x TAE (Tris Acetate EDTA) were run and visualised on a Gel Doc™ (Bio-Rad®) using SYBR®-Safe (Invitrogen). A negative control was always included to check for contaminants. A 1kb+ ladder (Invitrogen) was included to estimate size and concentration of PCR products. 5µL of the PCR products were purified enzymatically for sequencing using 2µL shrimp alkaline phosphatase and 1µL exonuclease (USB®); this digested excess dNTPs and single stranded primers left in the reaction.

2.3 Capillary sequencing and confirmation of species

The short range PCR products were used as templates for the v3.1 BigDye® Terminator Cycle Sequencing reactions (Applied Biosystems). Products were sequenced in the forward and reverse direction using primers that generated the short range product. The sequencing reactions and clean up were performed by the Massey University Genome Service centre (MGS). Products were then sequenced on the ABI 3730 automated sequencers (Applied Biosystems), at MGS. Sequences were viewed and aligned in Geneious, versions 5 and 6 (Biomatters). By running the sequence on the programme nucleotide BLAST (Basic Local Alignment Search Tool) (67), the sequence was used to confirm that the DNA belonged to the correct species. In some cases there were no sequences available from the New Zealand species so if the DNA sequence matched a close relative of the expected species it was assumed the DNA was from the correct species.

2.4 Illumina Sequencing

A Qubit® Flurometer (Life Technologies™) was used to measure the quality and quantity of the DNA samples, and whether there was any protein or RNA contamination, before being sent for sequencing. Three DNA samples were then sent to the Beijing Genome Institute (BGI). The pipit and tomtit DNA were very degraded and

were run as single samples on their own lane. The fernbird's DNA was good quality and quantity and was mixed in with other DNA (from a frog, mollusc and fish). These four species DNA were not indexed because it was assumed there was sufficient divergence in their mitochondrial DNA sequence that later separation would be possible. The samples were sequenced using the Illumina® Solexa HiSeq® 2000 sequencing pipeline. A hard drive containing the paired end short 100bp reads was received back from BGI; these reads were in fastq format. After base calling, the low quality and ambiguous base calls were filtered out using the Chastity filter. The read data was then available to us for genome assembly. The quality of the reads were assessed using the software Fast QC, which gives an overview of any problems with the reads such as read quality, content and identifies if there were any over represented sequences indicating contamination.

The waxeye was prepared as five long range PCR products spanning the entire mitochondrial genome, using the following primer combinations: Av1753F12S-Av5201tmetR, Av4165nd1F-Av7662co1R, Av7318co1F-Av10116co3R, Av9942co3F-Av16137tproR, and Av15671cytbF-Av2150R12S. These waxeye products were run on a 1% w/v in 1x TAE agarose gel and the Zymoclean™ Gel DNA Recovery Kit was used to extract the PCR products. The DNA concentrations of these clean PCR products were measured using Nanodrop (Supplementary Table 2). Before being sent for Illumina sequencing at New Zealand Genomics Limited (NZGL) the products were combined together so that there was an equal concentration of each in the mixed sample.

REPLI-g® (Qiagen®) was used on the browncreeper and bellbird complete genomic DNA to amplify the total genome, because the DNA was degraded and low in concentration. The waxeye, browncreeper and bellbird were then sent to NZGL for Illumina sequencing, each was given a unique index and the samples were run together in the same lane. The Qubit® Flurometer was used again to measure the DNA concentrations and contamination levels of these samples. They were sequenced using the Illumina MiSeq®, the bellbird and browncreeper were prepared as an Illumina TruSeq® DNA libraries, whilst the waxeye was an Illumina Nextera® library. TruSeq® DNA library preparation involves the use of magnetic beads for the shearing of DNA into fragments. It has higher accuracy but takes significantly longer and requires 50ng of total DNA. Nextera® library preparation involves enzymatic fragmentation which is quicker but less accurate, though it requires only 1ng of total DNA.

The read data from NZGL was returned as 150bp paired-end reads, in fastq format. The quality of the reads were assessed using SolexaQA (68), Fast QC (69), and FastQ Screen (70). The raw reads were then processed by using the software BWA (Burrows-Wheeler Aligner) (71), and reads that map to the PhiX genome were removed. The PhiX genome is a small virus genome which is added to the DNA before sequencing at a very low level. It is used after sequencing to quickly estimate error rates.

2.5 Genome assembly

2.5.1 Pipit and tomtit

The mitochondrial genomes of the pipit and the tomtit were assembled as follows. First, the FastX toolkit was used to remove the adapters from the short reads. The software BWA (Burrows-Wheeler Aligner) (71) was used to map the short reads to a reference genome. The zebrafinch (*Taeniopygia guttata*) and Tristram's bunting (*Emberiza tristrami*) were chosen as the reference genomes for the pipit because all three belong to the superfamily Passeroidea, and they have been separated for less than 30 million years (1). The rook (*Corvus frugilegus*) was chosen as the reference genome for the tomtit because the Australasian robins were thought to be part of the Core Corvoidea (1), and when the 1kb region sequenced from the tomtit sample was run through the program BLAST the closest match was the rook. Very few reads mapped to both the reference genomes (see results), indicating there were too many sequence differences between the species.

Velvet (72) was then used for de novo assembly of the genome: first, the paired reads were joined together using the shuffling function. Velvet is a de novo sequence assembler that uses a De Bruijn based assembly method. A K-mer sweep was performed which identified a K-mer length that produced the highest N50 for the set of reads. A K-mer sweep is where the 100bp reads are broken into smaller fragments (K-mers) of a certain length, to find the optimal K-mer length to assemble the set of reads (73). The N50 is a statistic which estimates the mean contig size for the assembly (73). K-mers of 67 were best for the pipit, and 71 for the tomtit (Table 8 and Table 11), using an expected coverage of auto (which cuts off contigs with coverage less than half the median coverage). The contigs produced by Velvet at this K-mer length, were run through BLAST against GenBank identifying any that matched with other passerines on GenBank (see Table 9 and Table 12 for contigs found).

BWA was then used to map the short 100bp paired reads to these contigs. These assemblies were exported and viewed in the mapping viewer program Tablet (74), to see the coverage of the contigs. Supercontigs were then made in Geneious. The supercontigs were used for mapping in BWA, and the process was repeated until the entire mitochondrial genome was assembled. The entire tomtit genome was assembled using this process, but the pipit still contained 2 large gaps. The pipit reads were reassembled using Velvet with a K-mer size of 41 and an expected coverage of 5; because the DNA was degraded, the use of smaller K-mers and lower coverage was expected to improve the number of contigs formed. All the contigs were run through BLAST as before, and new contigs were found (Table 10). These were then added to the supercontigs produced for the pipit above, and was used as a reference genome for mapping more reads in BWA. Annotation of the protein coding genes and rRNAs were done by comparison to a reference genome; *Emberiza tristrami* was used for the pipit, and the rook (*Corvus frugilegus*) was used for the tomtit. The annotation of the tRNAs was done using the online software DOGMA (75).

2.5.2 Fernbird

The fernbird mitochondrial genome was extracted from the mixture of the four species DNA reads by Bennet McComish. He firstly trimmed the adapters from the ends of the reads, removed any short reads by using the software cutadapt (76), and checked the quality of the reads with Solexa QA (68). A K-mer sweep of the data using the software Velvet was then completed. It was found that K-mers of 37 and a minimum coverage of 18 produced the best contigs and had the highest N50 value. These contigs were then run through the program BLAST to find contigs that closely matched bird sequences on GenBank. These selected contigs were put into Geneious to view and to make supercontigs.

The software BWA was then used to map the short pair-end reads to the supercontigs. This data was then exported into the mapping viewer Tablet and the supercontigs were extended in Geneious, which were then used again to map more of the reads in BWA. This method was not able to extract the entire mitochondrial genome out of the mixed reads so Velvet was re-run with a higher K-mer value of 63 and a lower minimum coverage of 5. These contigs were run through BLAST as before, and some of the missing regions of the genome were found in the contigs. These contigs were then imported into the Geneious file and added to the supercontig to produce a

sequence spanning the entire mitochondrial genome, except for one small gap in the first control region. The fernbird's genome was then annotated using Geneious, with the reed warbler's (*Acrocephalus scirpaceus*) genome used as reference for the protein and rRNA coding genes and the online software DOGMA as above to find the tRNAs. The reed warbler was used as the reference genome for the assembly and annotation of the fernbird mitochondrial genome because the two genera, *Bowdleria* and *Acrocephalus*, have been grouped together in a clade within the Sylvioidea (33). The fernbird data from the earlier capillary sequencing also matched closest to the reed warbler in a BLAST comparison.

2.5.3 Waxeye, browncreeper and bellbird

The reads from the waxeye, browncreeper and bellbird were 150bp paired-end reads in fastq format. The following species were used as reference genomes for the assembly: for the waxeye – the silver-eyed mesia (*Leiothrix argentauris*), for the browncreeper - the rook (*Corvus frugilegus*), and for the bellbird - the tui (*Prosthemadera novaeseelandiae*). The silver-eyed mesia was used for the waxeye because their families (the Timaliidae and the Zosteropidae, respectively) are considered sister families (17). The rook was used for the browncreeper as both belong to the Core Corvoidea. Finally, the tui was used for the bellbird as both belong to the Meliphagidae (22).

The reads were mapped to the reference genome using the software BWA and Bowtie 2 (77). With Bowtie 2 the reads were mapped using local alignment which maps differently than the default end to end alignment. Local alignment allows reads to map if part of the read matches the reference genome, even if the ends of the read are different. End to end alignment aligns the total read to the reference genome, so if part of the read differs significantly to the reference genome the whole read will not align. Local alignment works well when the reads are long (such as 150 bp) and when the template DNA was degraded, as was the case with the browncreeper. A K-mer sweep for each species was then run on Velvet and Velvet was used for de novo assembly of the reads at the optimal K-mer length. All contigs produced were run on BLAST against GenBank, any that matched passerines were added to the supercontigs made from BWA and Bowtie 2. BLAST2go (78) was used to run the contigs produced in Velvet for the bellbird reads at K-mer length of 97bp and the browncreeper reads at a K-mer length of 35bp.

2.5.4 Completing the mitochondrial genomes

The mitochondrial genome of the tomtit was complete after assembling the Illumina reads. The pipit mitochondrial genome had two gaps after assembly, one in the Cox3 gene and the other in the 5' region of the control region. The Cox3 gap was closed by PCR using total pipit genomic DNA as the template and the primers Av10307Cox3F, Av10647Cox3F, and Av10884nd3R. Sequencing was done as above using these primers. New primers were designed in Geneious: PipitnD6F and PipitcontrolR, and the control region gap was covered as above. Five regions within the genome had low coverage (reads are from only one DNA strand), two were located adjacent to the gaps mentioned above. The other three existed within the genes Cox1, CytB and ND6. Capillary sequencing was to confirm these sequences, using the primers Av7318Cox1F, Av9025tlysR, Av15671cytbF, Av16137tproR, PipitnD6F, and PipitcontrolR.

The fernbird had a small gap in the 5' region of the first control region; this gap was closed by first using PCR with the following primers, Av15671cytbF-Av16137tproR, on total genomic DNA. This PCR product was then sequenced in the forward and reverse directions by the MGS on the ABI3730 sequencer. The sequence was edited in Geneious and the control region gap closed. Similarly capillary sequencing was also used to confirm two regions in the 12S gene where coverage was very low, using the primers Av510dloopF, Av1249tpheF, Av1272tpheR, Av2150R12S.

The waxeye had two large contigs spanning a large region of the mitochondrial genome; one from the second control region to Cox3 and the other from CytB into the first control region. Two gaps remained, the first around 4000bp in length from Cox3 to CytB and the other around 1500bp from the first control region to the second control region. Both total genomic DNA and long range PCR products were used as templates for short range PCRs. These products were then sequenced using the same methods discussed above by the MGS on the ABI3730 sequencer. Six new primers were designed in Geneious for the covering of the gaps in the waxeye mitochondrial genome; WeND5F, WeCytBF, WeCytbR, WeCR1F, WeCR1R and WeND6R. A full list of the primers used is found in Supplementary Table 1.

For the bellbird the Illumina sequencing produced only two reads that mapped to the tui, and no contigs were made by Velvet that matched passerines, it was therefore assumed the Illumina sequencing of the Bellbird DNA sample did not work. Therefore the bellbird genome was sequenced using capillary sequencing, using the same DNA

extraction as before as the template for short range PCR. The full list of primers used is found in Supplementary Table 1.

The browncreeper genome was in multiple contigs with many gaps. The DNA extracted from the tissue samples was very degraded and was low in concentration, all was used when preparing the Illumina Nextera sequencing library so none remained for PCR and further capillary sequencing. To complete this genome new tissue will need to be obtained.

Gerrit Hartig sequenced the majority of the New Zealand robin (*Petroica australis*) using capillary sequencing. Two gaps remained one in the ND4 gene and the other from within the first control region to tPhe; these two gaps were covered by myself using long and short range PCR and capillary sequencing. Two new primers were designed using Geneious: AvtGluRobR and AvND6RobF to partially cover the second gap.

2.5.5 Transcriptome data

Nabholz et al. (2) used brain transcriptome sequenceing reads data from the study by Kunstner et al. (79) to assemble the sequence of the mitochondrial protein coding genes of four passerines. These four were the American crow - *Corvus brachyrhynchos* (SRR029463–64), pied flycatcher - *Ficedula hypoleuca* (SRR029159–61), golden-collared manakin - *Manacus vitellinus* (SRR029477–78), and blue tit - *Parus caeruleus* (SRR029162). The birds' brain transcriptomes was sequenced using the 454 transcriptome sequencing technologies (Roche). I used these transcriptome reads to assemble the sequence of the rRNA and some of the tRNA genes from these four species. The reads were downloaded from the GenBank read database as .sra files. The sra toolkit was used to extract the read files using the software fastq.pileup. The reads were imported into Geneious, and the genes were assembled using the Geneious functions multiple align, map to reference, and de novo assembly. The following close relatives were used as reference genomes: the rook for the American crow, the yellow-rumped flycatcher for the pied flycatcher, the ground tit for the blue tit, and the fuscous flycatcher for the golden-collared manakin. These rRNA and tRNA gene sequences were added to the protein coding genes to form nearly complete mitochondrial transcriptome sequences of the four species for use in the phylogenetic analysis.

2.6 Phylogenetic analysis

All 79 passerine genomes (Table 1) and four parrot genomes; kakapo (*Strigops habroptilus*), lovebird (*Agapornis roseicollis*), cobalt-winged parakeet (*Brotogeris cyanoptera*), and cockatiel (*Nymphicus hollandicus*) were aligned using Geneious. The four parrots used were selected from the phylogeny of Pacheco et al. (5), covering the full diversity of parrots with mitochondrial genomes available. The sequences were aligned at the amino acid level for protein coding genes and based on the stem and loop secondary structure for the RNA genes. The data set contains 12 protein coding genes, 2 rRNAs and 21 tRNAs. Stop codons (often incomplete), ND6 (light-stranded encoded), gaps and ambiguous sites adjacent to the gaps were not included in the alignment. These sites have been shown to be the most heterogeneous and, therefore, typically do not perform well in phylogenetic studies when the species are not closely related (5, 52). Where species genomes are incomplete the missing nucleotides were filled using question marks (?). The full data had 13588bp from each species. The data set had five partitions: 1) first codon of all 12 protein coding genes, 2) second codon, 3) third codon, 4) stems of rRNA and tRNA coding genes, 5) loops of rRNA and tRNA coding genes, this is the same as is used by Pratt et al. (7). RY coding was used for the third codon of protein coding genes, these are the most variable of the five partitions (65, 80), and has been shown to decrease the differences in nucleotide composition and increase the proportion of observable changes in along each branch of the phylogenetic tree (5).

Phylogenetic analysis was done using the CIPRES Science Gateway (81). RAxML-HPC2 (82) was used for maximum likelihood (ML) analysis with bootstrapping, with default parameters set. The GTR-CAT model was used for the bootstrapping phase and GTR-GAMMA for the final tree inference. MrBayes (83) was used for Bayesian analysis. 10 million generations were run, sampling every 2500th generation, with a burn in phase of the first 10% of trees. Sampling of the Monte Carlo Markov Chain from MrBayes were assessed using Tracer v1.5 (84) to check for convergence, and the trees were summarized using the software TreeAnotator (85). The trees were viewed, and edited using Fig Tree v1.40, with Splits Tree version 4 (86) used for constructing consensus networks. Subsets of the taxa set were run using both RaxML and MrBayes using the above methods.

Initially both maximum likelihood and Bayesian phylogenetic analyses were run using all 83 species (Table 1). Various subsets of these species were used for additional

ML analyses using RaxML to confirm results seen from the full data set trees. All used 13588bp from each species. The following subsets were used: The full set removing any species that have uncalled bases (76 species remain - Supplementary Figure 4). All Passerida including the four New Zealand basal Passerida (63 species - Supplementary Figure 6). Then all Passerida removing *Ficedula hypoleuca* and *Lampropsar tanagrinus*, as these had large amounts of uncalled bases and had close relatives already in the set (61 species - Supplementary Figure 7). All Sylvioidea and including Parus caerulus (19 species - Supplementary Figure 10).

3 Results

3.1 DNA extraction and next generation sequencing sample preparation.

DNA was extracted from 9 birds (Table 3). DNA extractions from the kokako, shining cuckoo and sooty shearwater were not successful. The extractions from the fernbird, waxeye, tui, and fantail had very high DNA concentrations (over 100ng/µl), whilst the pipit and browncreeper DNA concentration was very low (around 10ng/µl) (Table 3). Confirmation of extraction by gel electrophoresis (Figure 4) shows that DNA can be seen from each bird species. The fernbird, tomtit, pipit, harrier hawk, tui, fantail and waxeye are all bright, high molecular weight DNA bands, indicating high DNA concentration and most of the DNA is in long fragments (Figure 4A). The browncreeper DNA has low molecular weight and is a smear, indicating the DNA is degraded into fragments 1kb or smaller (Figure 4B). The bellbird DNA is a high molecular weight but is a smear, indicating the DNA is degraded but in 2kb+ fragments (Figure 4C). Both the browncreeper and bellbird smears were dull which meant the DNA concentrations were low.

The BLAST (67) results show that the nucleotide sequence from the fernbird extraction matched closest to the reed warbler, which is a passerine from the Sylvioidea (Table 3), the superfamily in which the fernbird is classified. The tomtit matched the tomtit 12S gene sequence on GenBank. The pipit matched with Tristram's bunting, and both are classified in the Passeroidea superfamily. The browncreeper matched with the rook, both in the Core Corvoidea. The waxeye matched the silver-eyed mesia, with both being classified in the Sylvioidea superfamily. The tui matched the tui sequence on GenBank, while the fantail matches the fantail and the bellbird the bellbird. The harrier hawk matched another member of the order Falconidae - the western marsh harrier (Table 3). These results confirm that the DNA extracted was from the correct species, and could then be used for Illumina Solexa sequencing. The tui, harrier hawk and fantail DNA samples were not used any further in this study and are stored for future use.

Table 3: The species extracted for this study. Samples of muscle tissue collected from three sources. Extracted DNA concentration of sample measured on the Nanodrop in ng/µl. DNA was used to make 1kb product from part of the mitochondrial genome (12S-16S regions); this region was sequenced using capillary sequencing on an ABI3730 sequencer (MGS). The sequence was run on BLAST against GenBank. Results give species with closest match to extracted sequence.

Species	Provided by, source and year collected	Extracted DNA concentration (ng/µl)	1kb sequence closest match (Blast)
Fernbird (*Bowdleria punctata*)	Te Papa, Wellington Codfish Island, 1997	189	Reed warbler (*Arcocephalus scirpaceus*)
Tomtit (*Petroica macrocephala*)	Te Papa, Wellington, DM88862, no collection data	46.2	Tomtit (*Petroica macrocephala*)
Pipit (*Anthus novaeseelandiae*)	Te Papa, Wellington, OR.011943, Auckland Islands, 1966	10.7	Tristram's bunting (*Emberiza tristrami*)
Browncreeper (*Mohoua novaeseelandiae*)	Te Papa, Wellington, OR.022865, Southland, 1983	11.8	Browncreeper (*Mohoua novaeseelandiae*)
Waxeye (*Zosterops lateralis*)	Wairarapa DoC, 2002	114	Silver-eyed mesia (*Leiothrix argentauris*)
Tui (*Prosthemadera novaeseelandiae*)	Wairarapa DoC, 2008	165	Tui (*Prosthemadera novaeseelandiae*)
Fantail (*Rhipidura fuliginosa*)	Wairarapa DoC, Masterton, 2004	107.7	Fantail (*Rhipidura fuliginosa*)
Harrier Hawk (*Circus approximans*)	Wairarapa DoC, L-38051, 2008	39	Western marsh-harrier (*Circus aeruginosus*)
Bellbird (*Anthornis melanura*)	P Cassey & J Ewen, C77620, Tiritiri Matangi Island, 2006	20.2	Bellbird (*Anthornis melanura*)

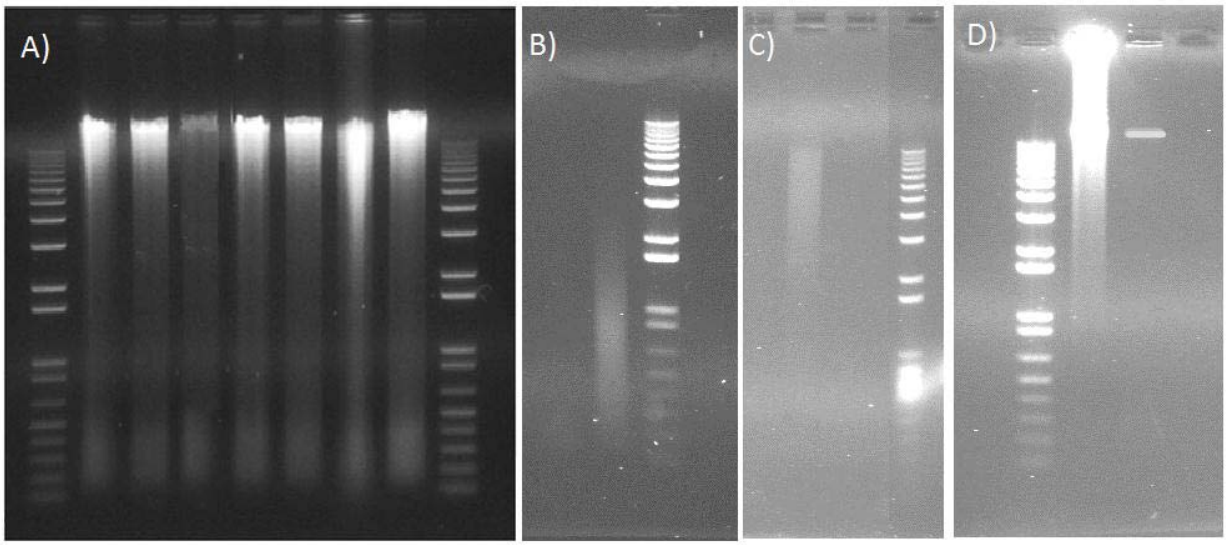

Figure 4: Electrophoresis of DNA extractions. A) Lanes 1 and 9: 1kb+ ladder, lanes 2-8: Total DNA of the fernbird, tomtit, pipit, harrier hawk, tui, fantail, and waxeye. B) Lane 1: Total DNA of browncreeper, lane 2: 1kb+ ladder. C) Lane 1: Total DNA of bellbird, lane 2: blank, lane 3: 1kb+ ladder. D) DNA after REPLIg®: Lane 1: 1kb+ladder, lane 2: Bellbird, lane 3: Browncreeper.

The quality and concentration of the DNA samples were re-checked using a Qubit® Fluorometer by the MGS, prior to the library construction. The Qubit® results (Table 4) differ significantly from the Nanodrop results (seen in Table 3). The reason for this is unresolved. Firstly the Qubit results were measured two months after the Nanodrop results, so the fernbird and tomtit samples may have degraded in this time, but why the pipit concentration has increased is unknown. REPLI-g® was used on the bellbird and the browncreeper to amplify the genomic DNA; this caused an increase in DNA concentration from 20.2 to 54.5 ng/µl for the bellbird, and an increase from 11.8 to 20 ng/µl for the browncreeper (Table 3 and Table 5). The bellbird sample contained significant levels of RNA (Table 5), which may have been sequenced with the bellbirds DNA during Illumina sequencing. The TruSeq® library prep requires 50 ng of total DNA, while Nextera® requires only 1 ng, so all three samples had sufficient DNA for Illumina sequencing (Table 5).

Table 4: Samples used for Illumina HiSeq sequencing at BGI. The pipit and tomtit DNA were sent as single samples, whilst the fernbird DNA was mixed with frog, mollusc and fish DNA. For the three bird species a full genomic DNA sample was used. The concentration of DNA in the sample was determined by a Qubit® Fluorometer. The volume is the amount of each sample sent, and total DNA is calculated from concentration and volume. The total of the mix in bottom row is the concentration after the samples are combined together.

Species	Template	Qubit (ng/µl)	Volume (µl)	Total DNA (ng)
Pipit	Genome	30.0	50	1500
Tomtit	Genome	36.8	50	1840
MIX:				
Frog	Genome	8.37	50	418.5
Fernbird	Genome	69.9	20	1398
Mollusc 1	Genome	33.4	20	668
Fish	Genome	55	30	1650
Total		37.2	120	4464

Table 5: Samples used for Illumina MiSeq sequencing at NZGL. The five waxeye long range PCR products were combined together. DNA and RNA concentrations were measured, using Qubit, before and after the library sample preparation step. The Volume used and total DNA is the amount used for the library prep. The DNA concentrations before library prep for the bell bird and browncreeper are after REPLI-g®. RNA concentration is a measure of contamination.

Sample	DNA Conc. pre library prep (ng/µl)	RNA Conc. pre library prep (ng/µl)	Library sample prep method	Volume used (µl)	Total DNA (ng)	DNA Conc. post library prep (ng/µl)	RNA Conc. post library prep (ng/µl)
Bellbird	54.5	4.2	Illumina TruSeq®	40	2180	7.35	<0.02
Browncreeper	20	<0.02	Illumina TruSeq®	25	500	0.701	<0.02
Waxeye (combined LRs)	1.08	<0.02	Illumina Nextera®	15	16.2	1.42	<0.02

3.2 Illumina sequencing results

The sequencing data was received back in fastq format. For both the pipit and tomtit there were over 1 billion bases after cleaning (removing of uncalled base reads), whilst the fernbird mix had over 3 billion bases of clean reads (Table 6). There were 195 million bases of read data for the waxeye, 285 million for the browncreeper, and 375 million for the bellbird (Table 6). Around 1 million bases of data is required to provide sufficient reads to recover a mitochondrial genome, indicating there should be enough read data here for each of the genomes. One problem is that the majority of the reads sequenced here will be from sources of DNA other than the target mitochondrial genome, such as the nuclear genome and because of the age of some of the samples we also expect some microbial contaminants.

Table 6: Amount of data received back from BGI and NZGL after Illumina sequencing. Fernbird mix is the mix mentioned in Table 4. For the BGI reads: Raw data is read data after base calling. Clean data is the read data after the use of a Chastity filter to remove ambiguous base calls and low quality reads. For the NZGL reads: Clean data is after the reads have been processed by removing any reads mapping to the PhiX genome The reads from BGI are short paired 100bp reads, the NZGL reads are short paired 150bp reads.

Sample	Raw data (million bases)	Clean data (million bases)
BGI reads (Illumina HiSeq)		
Pipit	2877	1857
Tomtit	1660	1147
Fernbird mix	4553	3366
NZGL reads (Illumina MiSeq)		
Waxeye		195
Browncreeper		285
Bellbird		375

The quality of the pipit, tomtit, waxeye, browncreeper and bellbird reads were assessed using the software Fast QC, with results summarized in Table 7. The basic statistics look at the number of reads, size of reads and GC content overall, the sets of reads from the five species were all found to be good quality. The per base sequence quality scores indicate the quality of each base over the length of the 100-150 base reads. For all sets of reads the quality is lower at the start and the end of each read, which indicates trimming may need to be done. But for four of the species the quality score still remains good for the majority of the reads, and have passed this quality assessment, only the bellbird has a warning, with the quality particularly poor after the 100th base mark. All the species reads passed the per sequence quality score, indicating the vast majority of the reads are good quality. Per base sequence content indicates if the position along the read influences the base call. The pipit had no position base call bias, but the other four all had a warning or failed, with the 10 bases at the beginning of the reads having a slight base bias, which may indicate contamination. The per base GC content is the proportion of G/C at each position on the read, the browncreeper and bellbird had a warning while the waxeye failed indicating there were positions along the reads where there was an unusual G/C content. For the per sequence GC content there was a warning for the tomtit and browncreeper, with the waxeye and bellbird failing, this was because on average the reads had a lower or higher GC content than would have been expected. This usually indicates there is a specific contaminant in the sample (a sequence that appears multiple times in the library).

Table 7: Fast QC quality scores of pipit, tomtit, waxeye, browncreeper and bellbird reads. The Fast QC results for the Illumina sequencing reads from BGI (pipit and tomtit) and NZGL (waxeye, browncreeper and bellbird). Results are summarised from both the paired first and second reads. The software measures a number of characteristics of the reads, and gives it a quality score based on the average of all reads in the set. Good quality (Pass) means the average of all the reads is above a certain level, with bad quality (Fail) being below a certain level. There is also a middle region where the quality is at a level where it may or may not cause problems when assembling (Warning). The explanation of each measure of quality is described above.

	Pipit	Tomtit	Waxeye	Browncreeper	Bellbird
Basic Statistics	Pass	Pass	Pass	Pass	Pass
Per base sequence quality	Pass	Pass	Pass	Pass	Warning
Per sequence quality scores	Pass	Pass	Pass	Pass	Pass
Per base sequence content	Pass	Warning	Fail	Warning	Warning
Per base GC content	Pass	Pass	Fail	Warning	Warning
Per sequence GC content	Pass	Warning	Fail	Warning	Fail
Per base N content	Pass	Pass	Pass	Pass	Pass
Sequence length distribution	Pass	Pass	Pass	Pass	Pass
Sequence duplication levels	Fail	Warning	Fail	Warning	Fail
Over represented sequences	Pass	Pass	Warning	Warning	Warning
K-mer content	Pass	Warning	Fail	Fail	Fail

All five species passed the per base N content indicating none of the reads contained uncalled bases in the form of 'N's. Sequence length distribution measures the length of the reads, and all reads from the five species were the correct length (100 or 150 bases). Sequence duplication levels indicate if there are any 100bp reads that occur more times than would be expected, it is a measure of how unique the reads are. If many duplicated reads occur it is an indication of possible contamination. The pipit, waxeye, and bellbird failed this test with a unique to duplicate read ratio of around 10 unique reads for every 7 duplicated reads, whilst the tomtit and browncreeper had a warning with a ratio of 10:3. This means that when assembling the mitochondrial genome contaminant reads will need to be filtered out, but it should not cause too many problems. For the waxeye, browncreeper, and bellbird there was a warning for over-represented sequences (an individual sequence making up over 0.1% of all reads in the library), these are likely to be adapter sequences added in the library prep and may need to be trimmed before genome assembly. K-mer content identifies any 5 base K-mers that are over-represented; the tomtit, waxeye, browncreeper and bellbird all had some which may interfere with the genome assembly.

The waxeye, browncreeper, and bellbird also had Fast Q screen and Solexa QA (68) quality assessment. A Fast Q screen checks for contamination. It was set to screen all the reads to see if any matched *E. coli*, yeast, PhiX, Illumina adapters, and cloning vector sequences. The results show that none of the reads match these five sources of sequence contamination (Figure 5). The Solexa QA programme gives a number of outputs, which are comparable to the results from Fast QC, but are measured in different ways. First, there is a measure of quality over the length of the reads; the bellbird had a decrease in the quality towards the ends of the reads. This decrease in quality confirms the warning given for per base sequence quality from Fast QC for the bellbird (Table 7). Second, the next measure is the error rate over the read length, for all three species there was a very low error rate with only a very slight increase in error rate at the last base of the length. And last, a measure of the length of the reads with all being 150 bases long confirming sequence length distribution result (Table 7).

The fernbird was assembled by Bennet McComish. Adapters were removed from all the reads from the mix using the software Cutadapt (76). The reads were then run on Solexa QA, software that measures the quality of the reads. It estimated the probability that an error in base calling occurred along the read, and graphs the average for all reads in the set. The probability of a base calling error occurring was 0 until around the 90^{th} base in the read where it began to increase to under 0.05 by the 100^{th} base (Figure 6). This means that for the first 90 bases of the read we can be very confident the base call was correct, but after the 90^{th} base some bases may be called incorrectly. To remove these errors the software Quality trimmer (87) was used which removed any bases in a read where the confidence in the base calling was below the threshold ($p=0.005$). This trimmed all the bases after the low confidence base call. After this the majority of reads were still 100 bases long (Figure 7).

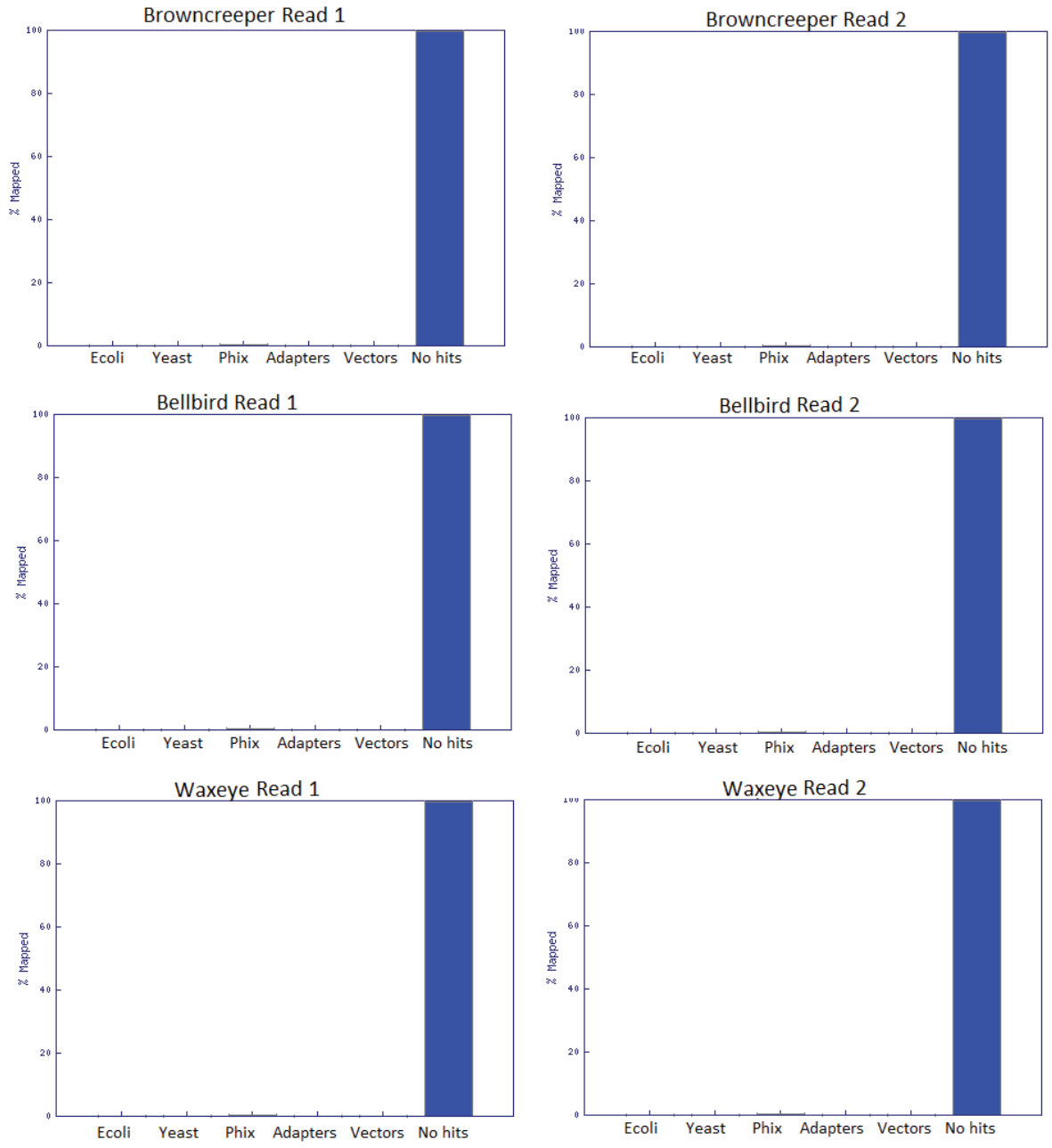

Figure 5: Fast Q screen results for the browncreeper, bellbird, and waxeye reads. The program Fast Q screen compares all reads to sequence from *E coli*, yeast, Phix, Illumina adapters, and cloning vectors, checking for sources of contamination in the reads. The paired data is shown by two sets for each species (read 1 and read 2). None of the reads in any read set have matched the potential sources of contamination shown here by all reads being in the no hits column.

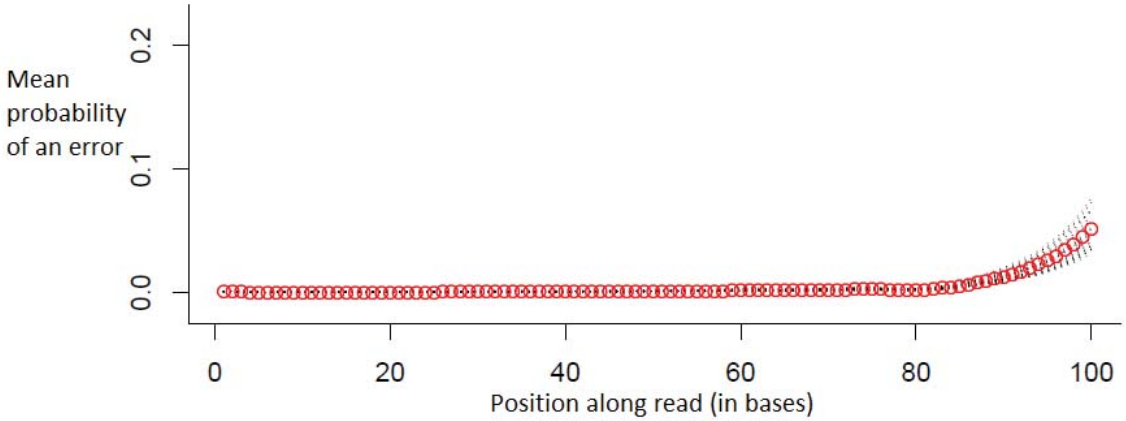

Figure 6: Probability of base calling error along reads in fernbird mix. Error probability at around 0 until around the 90th base when it increases to around 0.05 by the 100th base.

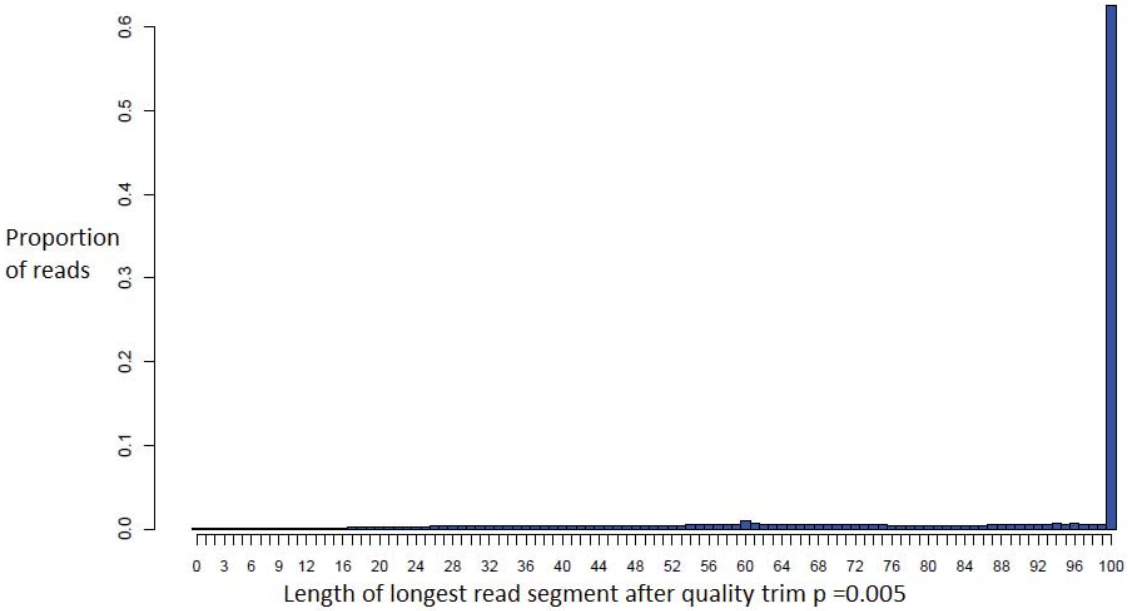

Figure 7: Length of fernbird mix reads after quality trim. Reads trimmed when the confidence of the base call is below threshold p=0.005. All remaining bases after low quality bases were removed by the software Quality trimmer.

3.3 Genome assembly

3.3.1 Pipit

The processed pipit reads were run on the software BWA (71) which maps the reads to the reference genome, the zebrafinch (*Taeniopygia guttata*). Only 344 reads mapped to the zebrafinch genome, covering around 12% of the mitochondrial genome. The mapping results were viewed in Tablet and a consensus sequence was extracted, that when run on BLAST matched to Passerines. The fact that only a small proportion of the genome was extracted using mapping is probably an indication that the pipit genome is too divergent from the reference genome used.

The de novo assembly software Velvet (72) was then used. For the pipit a K-mer sweep was performed using an expected coverage set at auto. The highest N50 value was seen at the K-mer length of 67 (Table 8), so the resulting contigs were looked at further. The contigs were all run on BLAST against GenBank; the majority were contaminants matching bacterial and fungal sequences. Eight contigs were found that matched Passerines (Table 9), covering 15435 bases of the genome. All genes except ND6, tRNAs Pro and Glu and the control region were recovered and there were also a number of gaps within the genes ND1, Cox1, Cox3, and CytB. Contigs made using a K-mer length of 41 were then run on BLAST to see if any additional regions of the genome could be found. 5 Contigs matched passerines spanning a total of 16468 bases (Table 10). These contigs extended into the control region, removed the gap in the ND6 gene and shortened the gap in the CytB gene, but still a number of gaps remained.

Table 8: K-mer sweep of the pipit reads. Performed using shuffled paired reads from the pipit data, on the software Velvet with expected coverage set to Auto. K-mer is the length in bases of the K-mer. Estimated coverage is the average coverage (in bases) of all the contigs, estimated by Velvet. N50 is a statistic describing the quality of the assembled contigs (higher numbers equal better quality). Max contig length is the length in bp of the longest contig made by Velvet.

K-mer	Estimated Coverage	N50	Max contig length (bp)	Total number of contigs
21	2.8	728	15615	25128
31	3.5	877	18118	23651
41	4.1	1678	20042	10261
51	5.1	1744	19446	9565
61	6.2	2589	26814	4760
63	6.4	2587	26812	4610
65	6.5	2627	26810	4438
67	6.7	2683	26808	4180
69	6.8	2618	26806	4045
71	7.0	2616	26804	3891

Table 9: Pipit contigs using K-mers 67 bases in length. Contigs from Velvet at a K-mer length 67 (expected coverage Auto), that matched Passerines when run on BLAST against GenBank. Node is the node id number. BLAST match is the species that was the closest matched to the contig. Length is length of the contig in base pairs. Mean coverage is the mean coverage of the reads mapped to the contig when used as reference sequence in BWA. Reads are the number of reads that formed each contig. A full list of the mitochondrial genes and regions contained within each contig is provided. Genes were identified using the *Emberiza tristrami* as reference, (P) indicates only part of the gene/RNA/region were present.

Node	BLAST match	Length (bp)	Mean Coverage	Reads	Mitochondrial genes and regions within contig
879	*Carduelis sinica*	3329	27	901	Control region (P), tRNA-Phe,12S, Val, 16S, Leu, ND1 (P)
1498	*Emberiza tristrami*	3407	36	1207	ND1 (P), Ile, Gln, Met, ND2, Trp, Ala, Asn, Cys, Tyr Cox1 (P)
2288	*Carduelis spinus*	2832	31.7	901	Cox1 (P), Ser, Asp, Cox2, Lys, ATP8, ATP6, Cox3 (P)
2006	*Emberiza tristrami*	1210	17.4	212	Cox3 (P), Gly, ND3, Arg, ND4L, ND4 (P)
2691	*Carduelis sinica*	526	15.2	81	ND4 (P)
3623	*Carduelis spinus*	3505	25	931	ND4 (P), His, Ser, Leu, ND5, CytB (P)
2723	*Taeniopygia guttata*	626	12.9	82	CytB (P), Thr

Table 10: Pipit contigs using K-mers 41 bases in length. Contigs from Velvet at a K-mer length 41 (expected coverage auto), that matched Passerines when run on BLAST against GenBank. Node is the node id number. BLAST match is the species that was the closest matched to the contig. Length is length of the contig in base pairs. Mean coverage is the mean coverage of the reads mapped to the contig when used as reference sequence in BWA. Reads are the number of reads that formed each contig. A full list of the mitochondrial genes and regions contained within each contig is provided. Genes were identified using the *Emberiza tristrami* as reference, (P) indicates only part of the gene/RNA/region were present.

Node	BLAST match	Length (bp)	Mean Coverage	Reads	Mitochondrial genes and regions within contig
1654	*Vidua chalybeata*	6277	25.3	1590	Control region (P), tRNA-Phe,12S, Val, 16S, Leu, ND1, Ile, Gln, Met, ND2, Trp
3595	*Protonotaria citrea*	1306	47.0	625	Asn, Cys, Tyr, Cox1 (P)
4745	*Emberiza tristrami*	2832	31.6	898	Cox1 (P), Ser, Asp, Cox2, Lys, ATP8, ATP6, Cox3 (p)
1624	*Emberiza chrysophrys*	4802	24.6	1184	Cox3 (p), Gly, ND3, Arg, ND4L, ND4, His, Ser, Leu, ND5, CytB (p)
5011	*Emberiza chrysophrys*	1251	18.8	217	CytB (P), Thr

A supercontig of the contigs recovered was made in Geneious; a consensus sequence was extracted filling the gaps with 'N's. This consensus sequence was then used as a reference genome for mapping reads in BWA, which extended the regions covered. The process was repeated a number of times until all genes were covered and only two gaps remained one within the Cox3 gene, and one in the control region just after tRNA Glu (Figure 8). When compared to both *E. tristrami* and the zebrafinch, the gap in Cox3 appeared to be only 2bp long, but no reads were found to cover the gap. The two gaps were closed using PCR and capillary sequencing. There were five regions where coverage was low (reads were from only one DNA strand), two were located at the edge of the gaps mentioned above. The other three existed within the genes Cox1, CytB and ND6, the base calling of these sites were confirmed using capillary sequencing, this means that all bases have been sequenced in both directions. The genome length to date is 16798 bases long, and has a mean coverage depth of 24 reads (Std. dev. 17 reads), and the maximum coverage depth is 114 reads. The genes were annotated using *Emberiza tristrami* as the reference genome, and the DOGMA software was used to find the tRNAs. The total genome is 16798bp long and the gene arrangement is the same as was first reported for *Gallus gallus*.

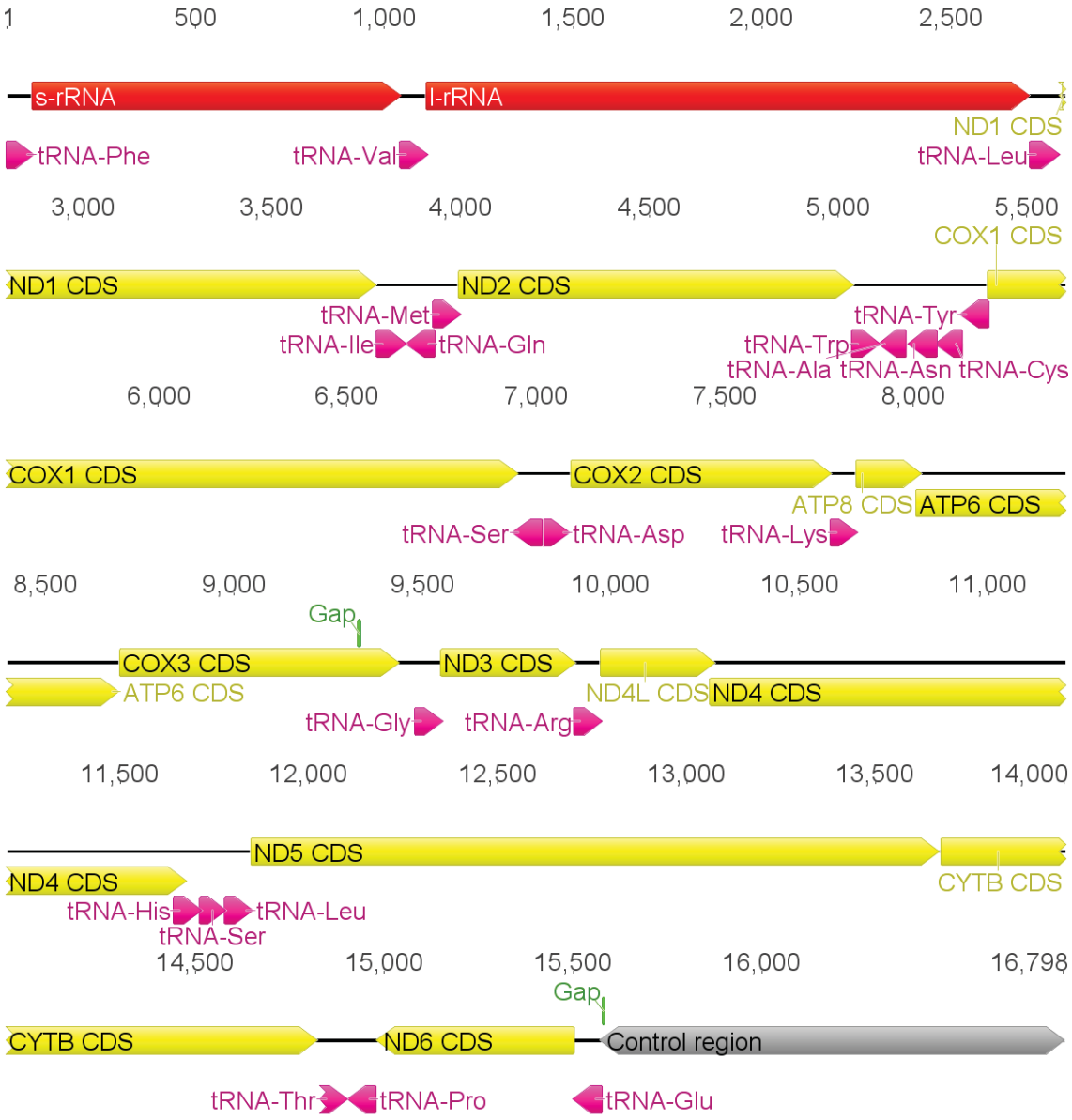

Figure 8: Pipit Mitochondrial Genome. Assembled using Velvet, BWA and Geneious. Annotated using *Emberiza tristrami* as the reference genome, and DOGMA to find the tRNAs. Two gaps remained after assembly of Illumina HiSeq reads, one in Cox3 coding region, and one in the non-coding control region. These were later covered using capillary sequencing by MGS.

3.3.2 Tomtit

The rook (*Corvus frugilegus*) was used as the reference genome for the tomtit reads. The tomtit reads were trimmed, removing the ends of the reads. They were then run on the software BWA which maps the reads to a reference genome. Only 613 reads mapped to the rook genome, covering only 16% of the genome. The mapping results were viewed in Tablet and a consensus sequence was extracted, that when run on BLAST matched to Passerines. Again this is probably an indication that the rook is too far diverged from the tomtit to be used as a reference genome.

A K-mer sweep was then performed in Velvet using an expected coverage set at auto. The highest N50 value was seen at a K-mer length of 71 (Table 11). This was the highest K-mer length available in Velvet at the time (November 2011), and as the N50 is still relatively low at 71 (244), the optimal K-mer length is most likely higher than this. The contigs made at 71 were then run through BLAST against GenBank; the majority were contaminants matching bacterial and fungal sequences. Three contigs were found that matched Passerines (Table 12), covering 16661 bases of the genome.

Table 11: K-mer sweep of the tomtit reads. Performed using shuffled paired reads from the tomtit data, on the software Velvet with expected coverage set to Auto. K-mer is the length in bases of the K-mer. Estimated coverage is the average coverage (in bases) of all the contigs, estimated by Velvet. N50 is a statistic describing the quality of the assembled contigs (higher numbers equal better quality). Max contig length is the length in bp of the longest contig made by Velvet.

K-mer	Estimated Coverage	N50	Max contig length (bp)	Total number of contigs
21-51	Doesn't work			
61	1.8	141	12891	47490
65	2.9	151	17771	12615
71	4.68	244	28131	6653

Table 12: Tomtit contigs using K-mers 71 bases in length. Contigs from Velvet at a K-mer length 71 (expected coverage Auto), that matched Passerines when run on BLAST against GenBank. Node is the node id number. BLAST match is the species that was the closest matched to the contig. Length is length of the contig in base pairs. Mean coverage is the mean coverage of the reads mapped to the contig when used as reference sequence in BWA. Reads are the number of reads that formed each contig. A full list of the mitochondrial genes and regions contained within each contig is provided. Genes were identified using the *Corvus frugilegus* as a reference genome, (P) indicates only part of the gene/RNA/region were present.

Node	BLAST match	Length (bp)	Mean Coverage	Reads	Mitochondrial genes and regions within contig
861	*Luscinia calliope*	13105	133.1	17459	ND6 (P), tGlu, non-coding region 2, tPhe,12S,t Val, 16S, tLeu, ND1, tIle, tGln, tMet, ND2, tTrp, tAla, tAsn, tCys, tTyr Cox1, tSer, tAsp, Cox2, tLys, ATP8, ATP6, Cox3, tGly, ND3, tArg, ND4L, ND4, tHis, tSer, tLeu, ND5 (P)
445	*Gracula religiosa*	2258	130	2949	ND5 (P), CytB, tThr, non-coding region 1 (P)
4609	*Petroica traversi*	1298	103.8	1348	tThr (P), non-coding region 1, tPro, ND6 (P)

All genes and a duplicated control region were recovered, but there were two gaps within the genes ND5 and ND6. A super contig of the three contigs was made in Geneious; a consensus sequence was extracted filling the gaps with Ns. This was then used as a reference genome for mapping reads in BWA, which extended the genome into the gaps. The process was repeated a number of times until the entire genome was recovered (Figure 9). The genome length is 16861 bases long, and has a mean coverage depth of 129 reads (Std. dev. 32), and the maximum coverage depth is 264 reads. The genes were annotated using the rook (*Corvus frugilegus*) as the reference genome, and DOGMA software was used to find the tRNAs. All of the tomtit genes appear to be the same length as those of the rook. The control region is located between the genes tThr and tPro. A duplicated piece of the control region, 190bp long, is found between tGlu and tPhe, this small duplication is seen in the New Zealand robin (*Petroica australis*) as well. There are two regions where the coverage was relatively low (within 12S rRNA and ND2 gene); the base calling of this region was confirmed using PCR and capillary sequencing.

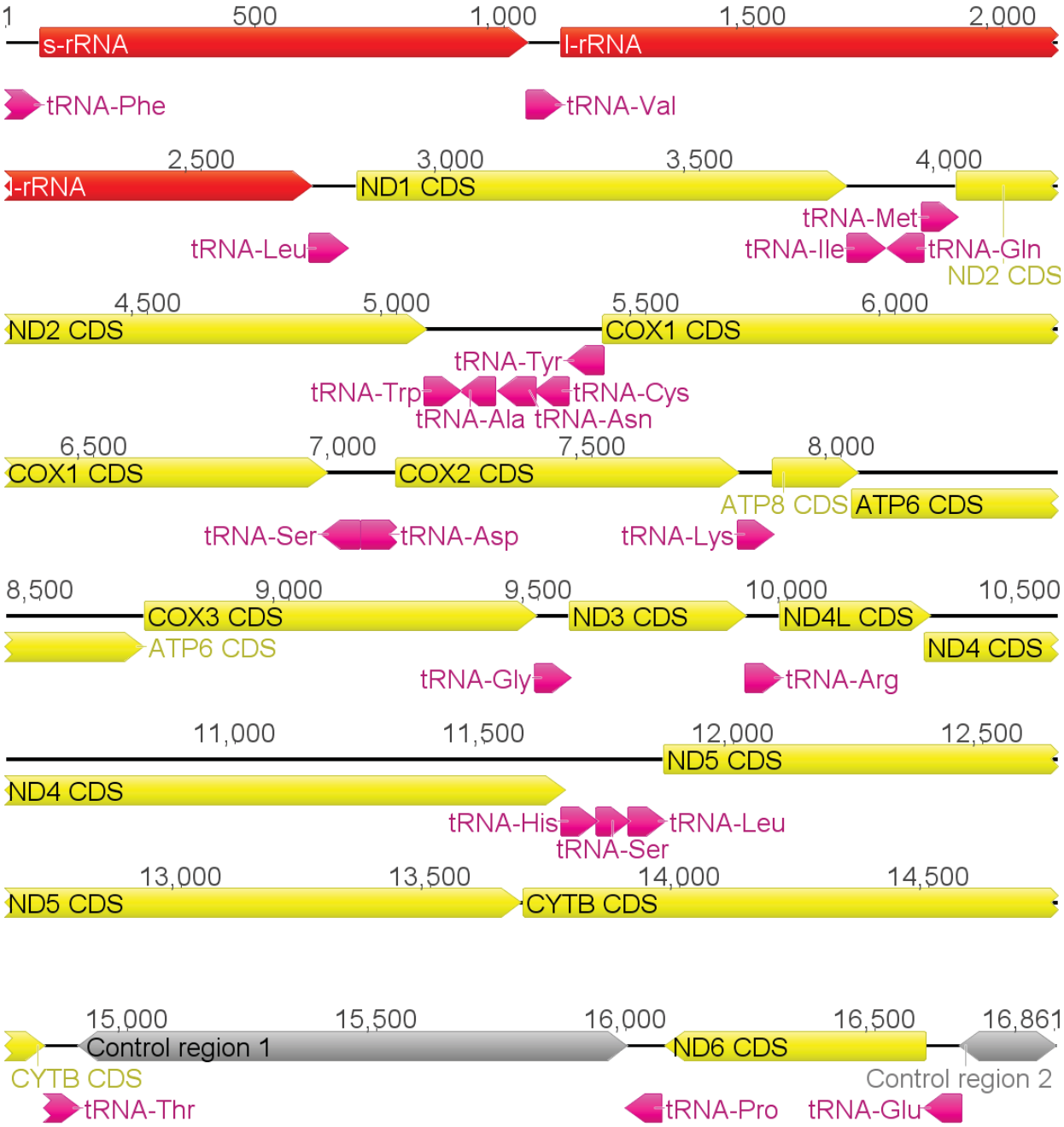

Figure 9: Tomtit mitochondrial genome. Assembled using Velvet, BWA and Geneious. Annotated using *Corvus frugilegus* as the reference genome, and DOGMA to find the tRNAs.

3.3.3 Fernbird

For the fernbird mitochondrial genome a K-mer sweep in Velvet was performed using an expected coverage set at 18. The highest N50 value was seen at the K-mer length of 37. The contigs made at 37 were then run on BLAST against GenBank. A number of contigs were found that matched Passerines, but they did not cover the entire genome. Bennet next tried the contigs generated using a K-mer length of 63 with an expected coverage of 12. When run on BLAST more contigs were found that matched Passerines. The contigs from these two K-mer lengths were combined in Geneious, and a super contig spanning 17991 bases was generated (Figure 10). A single gap remained in the first control region next to tThr (Figure 10), the length of this gap was unknown. This gap was later covered using PCR and capillary sequencing done by the MGS. This supercontig was then used as a reference genome for mapping in BWA. The genome has a mean coverage depth of 54.2 reads (standard deviation of 21), and the maximum coverage depth is 153 reads. There are two regions within the 12S rRNA where the coverage was low; the base calling of these sites were checked using PCR and capillary sequencing. The genes were annotated using the Taiwan bulbul (*Pycnonotus taivanus*) as the reference genome, and DOGMA software to find the tRNAs. There is a duplicated control region, with the first found between tThr and tPro, and the second between tGlu and tPhe, this gene arrangement is seen in many of the passerines, and was first identified in the falcons (88).

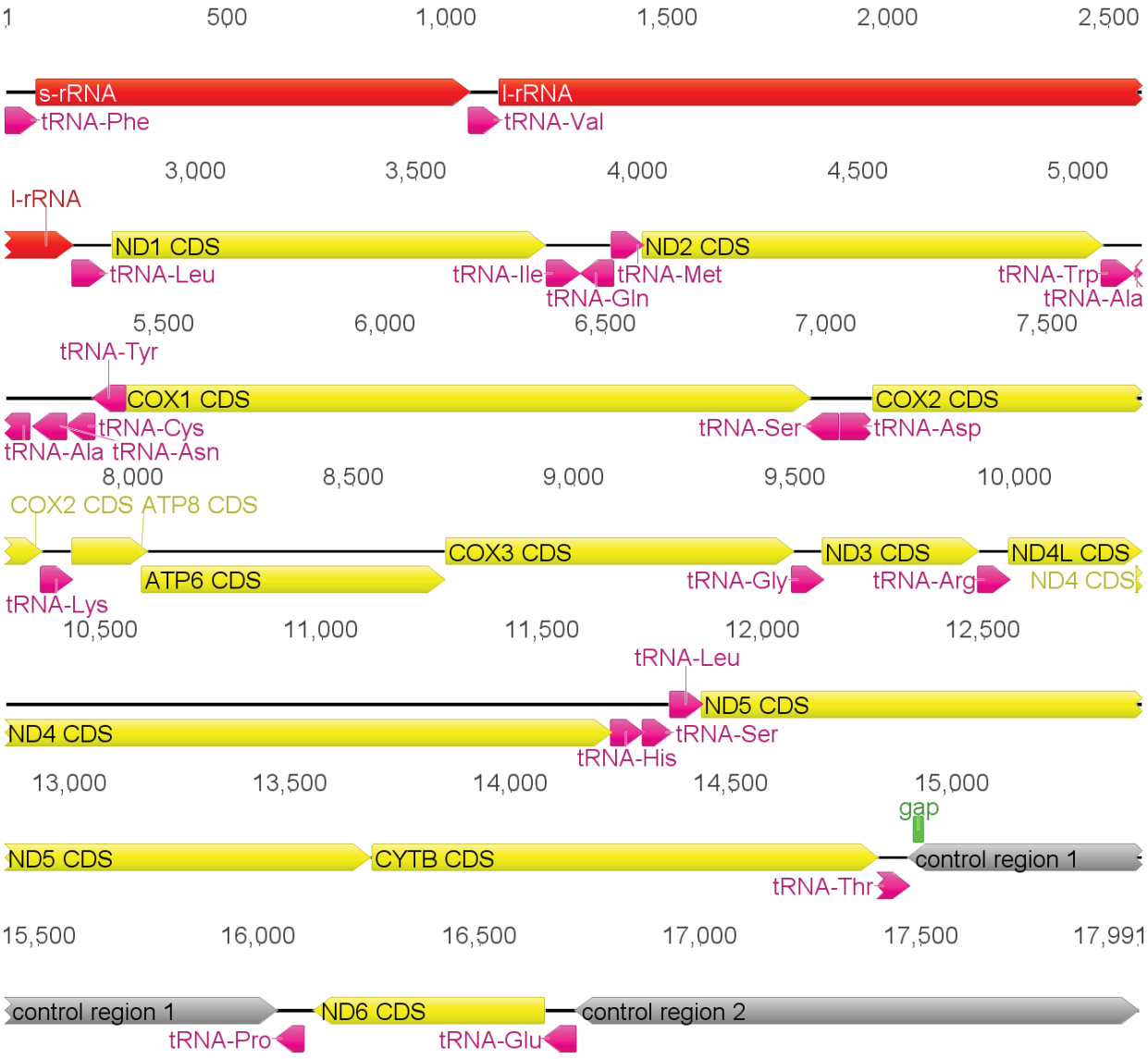

Figure 10: Fernbird mitochondrial genome. Assembled using Velvet, BWA and Geneious. Annotated using *Pycnonotus taivanus* as the reference genome, and DOGMA to find the tRNAs. One gap remained in the first control region (unknown length) after assembling the Illumina reads. This gap was covered using capillary sequencing by MGS.

3.3.4 Waxeye

The silver-eyed mesia's (*Leiothrix argentauris*) mitochondrial genome was used as reference genomes for the assembly of the waxeye gnome. The processed reads were mapped to the reference genome in BWA. Only around 3000 reads initially mapped to the reference genome. The contigs made from BWA and the reference genome continued to be used in BWA to map extra reads, extending out the contigs. This method produced two large contigs spanning from the second control region to Cox3 and from CytB into the first control region. Overall 655,771 reads in total had mapped to the two supercontigs, with an average coverage depth of 8986 reads. Two gaps

remained the first around 4000bp in length from Cox3 to CytB and the other around 1500bp from the first control region to the second control region.

A de novo assembly of the reads was then tried using the software Velvet. A K-mer sweep was performed using Velvet; this identified the K-mer length that produced the highest N50 for the set of reads. K-mers of 127 was best for the waxeye (Table 13), using an expected coverage of auto (which cuts off contigs with coverage less than half the median coverage). Other expected coverage values were tried but Auto was found to have the highest N50. The contigs produced by Velvet at this K-mer length, were run through BLAST identifying any that matched with other passerines (not shown). All matched within the supercontig made using BWA, so we were unable by this method to cover the two gaps in the mitochondrial genome. However the two gaps in the waxeye genome were covered using PCR and capillary sequencing, a full list of primers used is found in Supplementary Table 1. The completed waxeye mitochondrial genome spans 17,973bp and contains a duplicated control region (Figure 11). A duplicated control region is also found in the waxeye mitochondrial genome, and is the same gene arrangement as the fernbird.

Table 13: K-mer sweep of the waxeye reads. Performed using shuffled paired reads from the waxeye data, on the software Velvet. K-mer is the length in bases of the K-mer. Expected coverage set at Auto means contigs are cut when coverage reaches less than half the median coverage. N50 is a statistic describing the quality of the assembled contigs (higher numbers equal better quality). Max contig length is the length in bp of the longest contig made by Velvet.

K-mer	Expected coverage	N50	Max contig length (bp)	Total number of contigs
101	Auto	101	5730	5200
121	Auto	584	5553	185
127	Auto	1699	6151	43
127	4	750	6151	83
127	10	765	2481	77

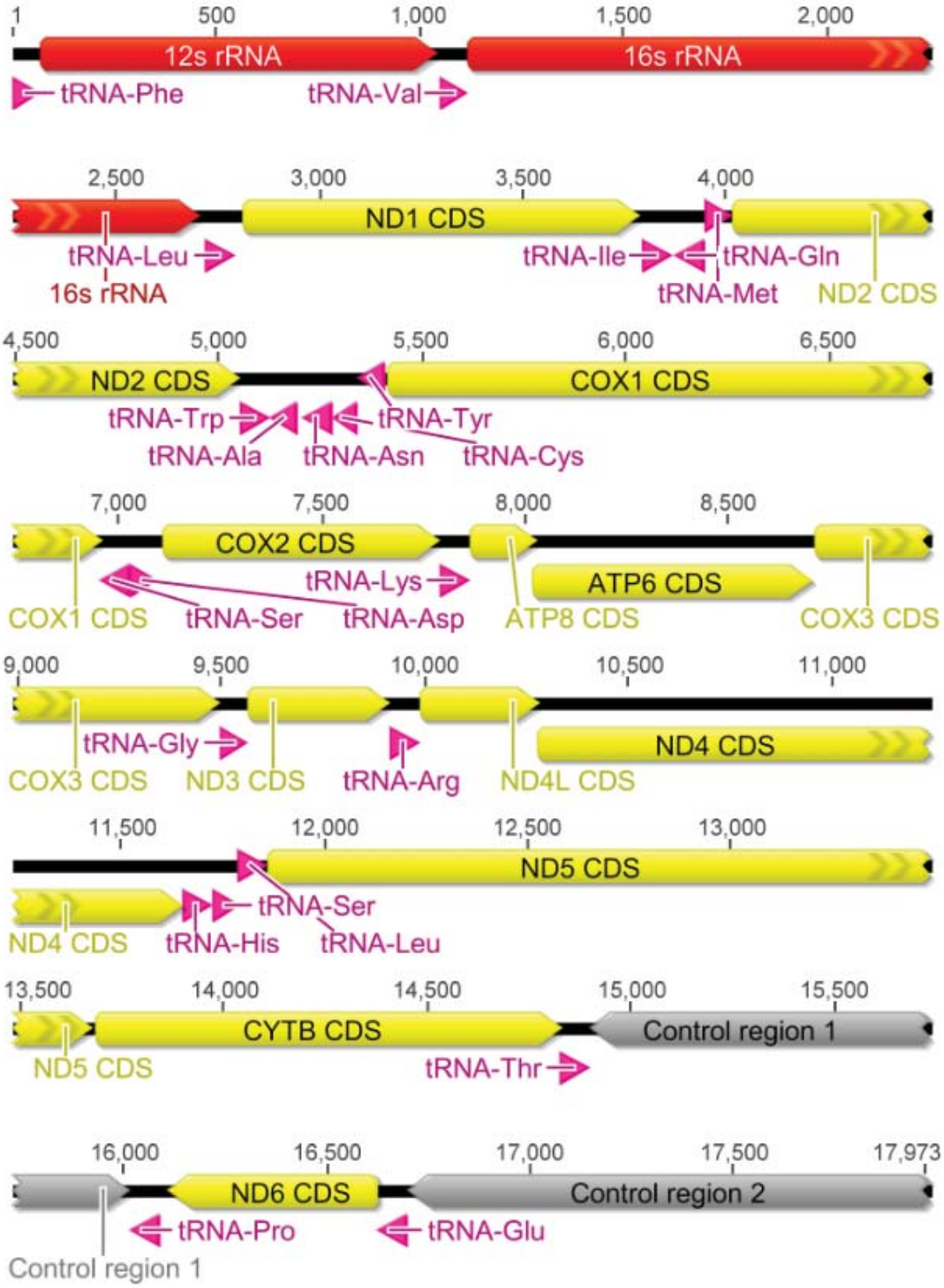

Figure 11: Waxeye mitochondrial genome. Assembled using Velvet, BWA and Geneious. Annotated using *Leiothrix argentauris* as the reference genome, and DOGMA to find the tRNAs. Two gaps remained after assembling the Illumina reads; the first from Cox3 to CytB, and the second from Control region 1 to Control region 2. These gaps was covered using capillary sequencing by MGS.

3.3.5 Browncreeper

The rook (*Corvus frugilegus*) was used as the reference genome for the browncreeper reads. BWA was used to map the processed reads to the reference genome; the contigs made from BWA were then used to map extra reads. After multiple runs in BWA 105 reads were aligned into around 20 small contigs covering many different regions of mitochondrial genome. After BWA another mapping software, Bowtie 2 (77), was used on the reads. Bowtie 2 was run using local mapping, this is where any section along the 150bp reads can be mapped to the reference genome, and if the ends are different they are trimmed. Local mapping differs from the default end to end mapping where the ends of the reads are mapped first, if these don't match the reference genome the whole read does not map. The use of local mapping extended the contigs produced from BWA for the browncreeper, with 253 reads formed into 21 contigs with a mean coverage depth of 3.5 reads.

The K-mer sweep of the browncreeper reads found the highest N50 at K-mers 127 bp in length (Table 14). When contigs were run on BLAST against GenBank, no sequences were found to match avian mitochondrial data. A low expected coverage value of 5 was tried at K-mer lengths of 127 and 97, and the contigs produced by Velvet were run on BLAST. Three from the K-mer length of 97 matched passerine mitochondrial DNA (not shown), they match what had already been produced using BWA and Bowtie 2.

Overall 10,555 bases of the browncreeper genome has been sequenced, with sequence from 2 rRNA, 17 tRNA and 11 protein coding genes, along with over 1000bp of the control region. As no DNA from the browncreeper remained the gaps were not covered using PCR and capillary sequencing.

Table 14: K-mer sweep of the browncreeper reads. Performed using shuffled paired reads from the browncreeper data, on the software Velvet. K-mer is the length in bases of the K-mer. Expected coverage set at Auto means contigs are cut when coverage reaches less than half the median coverage. N50 is a statistic describing the quality of the assembled contigs (higher numbers equal better quality). Max contig length is the length in bp of the longest contig made by Velvet.

K-mer	Expected coverage	N50	Max contig length (bp)	Total number of contigs
97	5	287	1701	4450
107	Auto	631	6876	1101
115	Auto	788	6868	713
121	Auto	2151	21874	312
127	Auto	2940	21986	183
127	5	831	6041	453

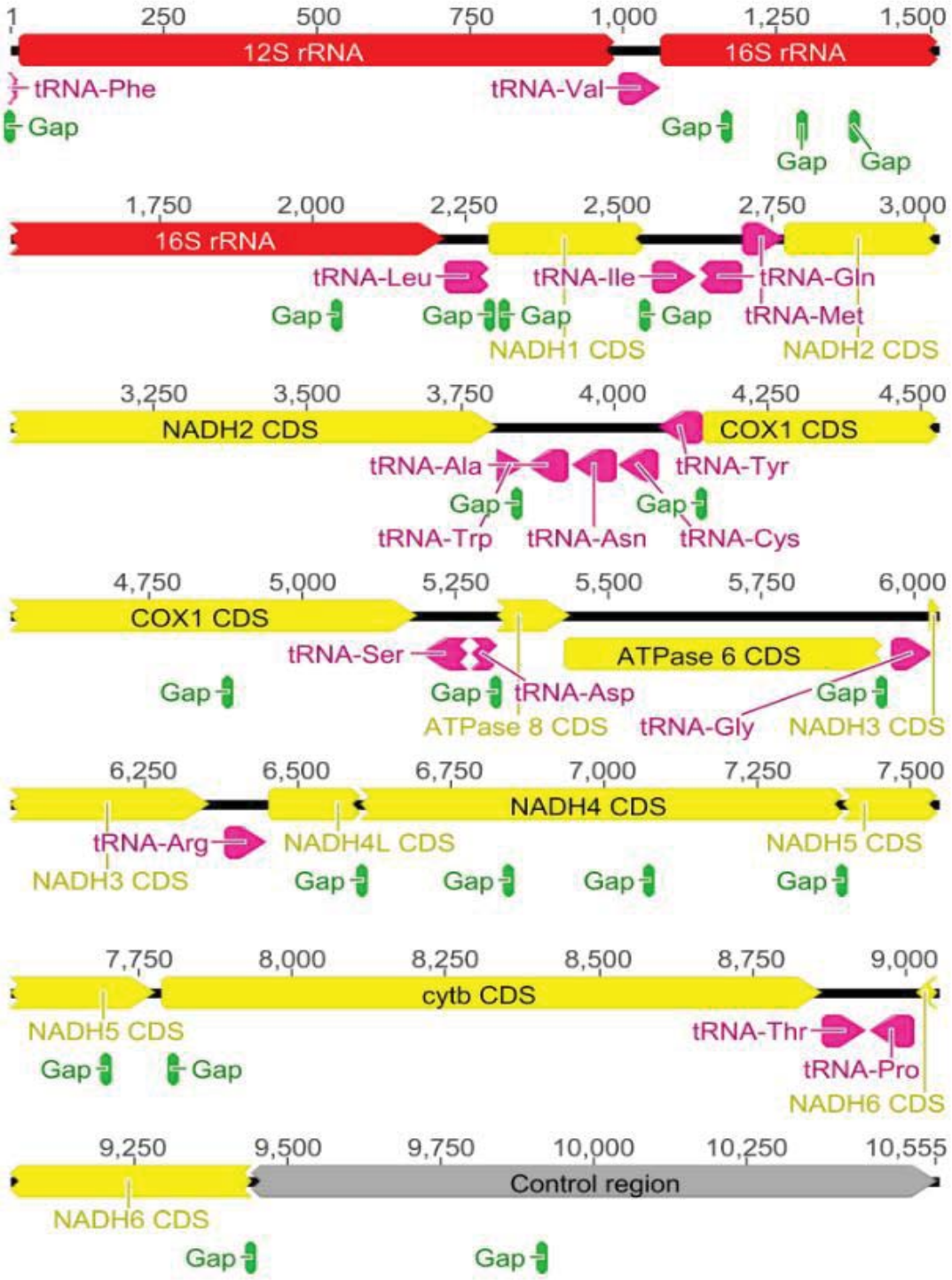

Figure 12: Browncreeper partial mitochondrial genome. Assembled using Velvet, BWA, Bowtie2 and Geneious. Annotated using *Corvus frugilegus* as the reference genome, and DOGMA to find the tRNAs. Twenty one gaps remain after assembling the Illumina reads, indicated here by the green symbol, the size of each is unknown.

3.3.6 Bellbird

For the bellbird the tui (*Prosthemadera novaeseelandiae*) was used as a reference genome. The reads were run on BWA, but only two reads mapped to the tui genome. The reads were then run through Bowtie 2, using local mapping, but only the same two reads were found. Two bellbird mitochondrial genes had been previously sequenced, the 12S rRNA and the NADH2 gene, these were tried as reference genomes in both BWA and Bowtie 2, and no reads mapped. The K-mer sweep in Velvet of the bellbird reads found the highest N50 at K-mers of 57 (Table 15). The program Blast2GO (78) was used to run the contigs produced through BLAST against GenBank. No passerine contigs were found, a summary of the species distribution of the BLAST results for K-mers of 97bp in length shows the contigs matched bacteria (Figure 13A). These results suggested that none of the "bellbird" reads were actually from bellbird DNA, but were made from contaminant bacterial DNA instead. With the browncreeper BLAST2GO was used to run all the contigs produced from the browncreeper reads as a comparison; it found the vast majority of contigs also matched bacteria, but a small proportion were passerine indicating some of the reads were from browncreeper DNA.

Table 15: K-mer sweep of the bellbird reads. Performed using shuffled paired reads from the bellbird data, on the software Velvet. K-mer is the length in bases of the K-mer. Expected coverage set at Auto means contigs are cut when coverage reaches less than half the median coverage. N50 is a statistic describing the quality of the assembled contigs (higher numbers equal better quality). Max contig length is the length in bp of the longest contig made by Velvet.

K-mer	Expected coverage	N50	Max contig length (bp)	Total number of contigs
57	Auto	359	4256	66781
97	Auto	267	1533	8077
107	Auto	241	1792	5620
127	Auto	138	2033	1466

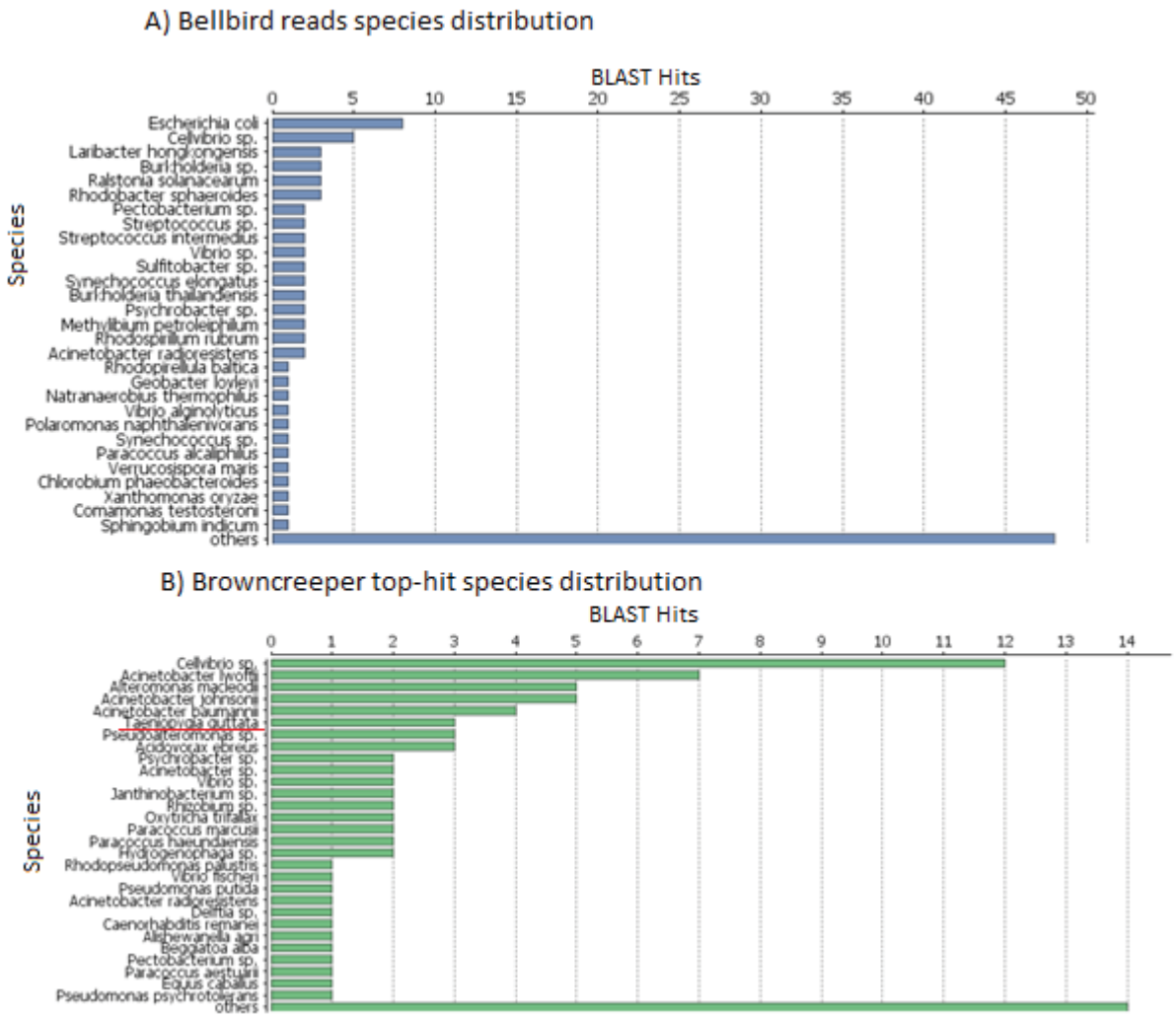

Figure 13: Bellbird and Browncreeper Blast2GO results from Velvet contigs. Contigs run through BLAST against GenBank. A) The species distribution of the "bellbird" contigs when run on BLAST. Made at K-mer length of 97bp. B) Browncreeper contigs top matching species distribution. Made at K-mer length of 35bp. The only avian DNA identified here is the zebrafinch (*Taeniopygia guttata*) highlighted in the browncreeper reads.

Short range PCR using total genomic DNA and capillary sequence was then used to sequence the bellbird genome. A partial mitochondrial genome (9453bp) of the bellbird has been sequenced (Figure 14). 2 rRNA, 14 tRNA, and 10 protein coding genes are at least partially sequenced, while nine gaps remain un-sequenced. It would have been good to have another Meliphagidae mitochondrial genome (because it is a basal oscine), but it does not affect our aim of having one mitochondrial genome from every New Zealand family of passerines. It will be a future project to complete the bellbird genome.

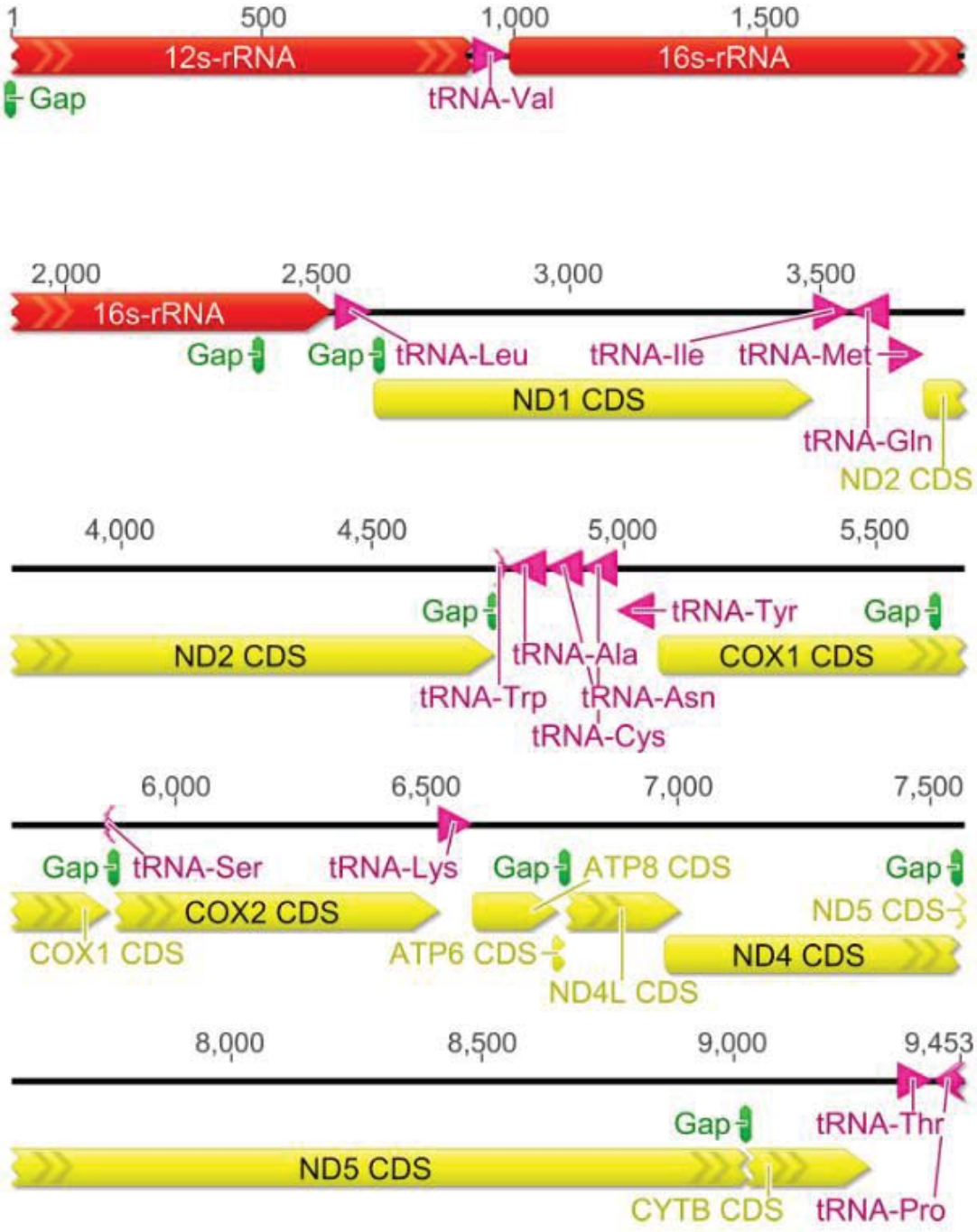

Figure 14: Bellbird partial mitochondrial genome. Assembled using Geneious. Annotated using *Prosthemadera novaeseelandiae* as the reference genome, and DOGMA to find the tRNAs. Nine gaps remain after capillary sequencing, indicated here by the green symbol, the size of each gap is unknown.

3.4 Transcriptome reads

Using the brain transcriptome sequenceing reads data from Kunstner et al. (79), the sequence of some of the mitochondrial genes have been assembled, with Nabholz et al. (2) assembling the protein coding genes, and myself assembling the rRNA and tRNA genes. The full list of which genes have been extracted from each species is found in Table 16. Around 14,000 bp from each of the four species has been extracted.

Table 16: Mitochondrial genome genes extracted from the brain transcriptome reads. Reads were sequenced using the 454 transcriptome sequencing by Kunstner et al. (79). The protein coding genes were assembled by Naholz et al. (2), and the tRNA and rRNA were assembled by myself. A full list of the mitochondrial genes assembled from the transcriptome reads is given for each species. The full gene sequence has been assembled unless indicated as being only partial (P).

Species	Mitochondrial genes assembled from reads	Total bases
Golden-collared manakin - *Manacus vitellinus*	tPhe (P), 12S, 16S, tLeu, ND1, tMet (P), ND2, tTyr, Cox1, tSer, tAsp, Cox2, tLys, ATP8, ATP6, Cox3, tGly, ND3, ND4L (P), ND4, tHis (P), ND5 (P), CytB	14,287
American crow - *Corvus brachyrhynchos*	12S, 16S (P), tLeu (P), ND1, tIle, tGln (P), ND2, Cox1, tSer, tAsp, Cox2, ATP8 (P), ATP6, Cox3, tGly, ND3, ND4L (P), ND4, ND5 (P), CytB, ND6, tGlu (P)	14,061
Pied flycatcher - *Ficedula hypoleuca*	12S, 16S, ND1, tIle (P), ND2, tTyr, Cox1, tSer, Cox2, ATP8 (P), ATP6, Cox3 (P), tGly, ND3, ND4L (P), ND4 (P), ND5, CytB	14,563
Blue tit - *Parus caeruleus*	tPhe, 12S, tVal (P), 16S, tLeu, ND1, tIle, tGln, tMet (P), ND2 (P), Cox1, tSer, tAsp, Cox2, tLys, ATP8, ATP6, Cox3, ND3, ND4 (P), ND5, CytB	14,314

3.5 Phylogenetic analysis

The results of both the maximum likelihood (Figure 15) and Bayesian (Figure 16) analysis phylogenetic trees are discussed, focusing on certain parts of the tree using maximum likelihood analyses consensus networks. Analyses were run using the full species data set (83 species with 13588bp of mitochondrial data from each) and smaller species sets (see section 2.6). Maximum likelihood phylogenetic analyses were conducted using RaxML (82), producing a set of possible trees and a "best tree" summarized from this set showing support for each node in the form of a posterior probability bootstrap. Initially five million generations were run on MrBayes (83), sampling every 2500th generation, but Tracer v1.5 suggested that the effective sample size (ESS) was too small. 10 million generations, still sampling every 2500th generation, was found to produce a large enough ESS so was therefore used for all MrBayes analyses. All the trees produced after a burn in of 1 million generations were then summarized into the Bayesian analysis phylogenetic tree (Figure 16).

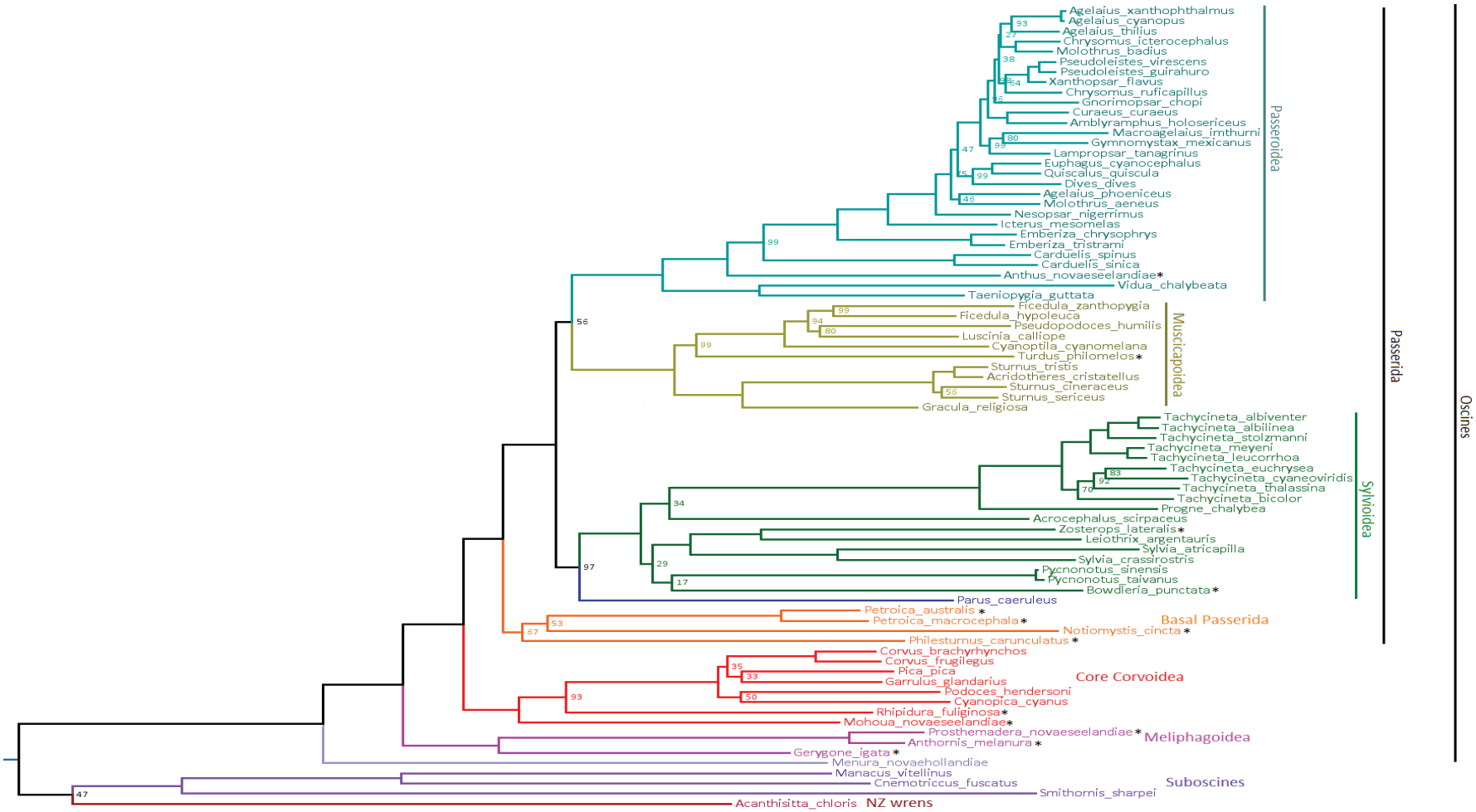

Figure 15: Maximum likelihood phylogenetic analysis tree from all 79 passerine species using mitochondrial genome data (13588bp). Four parrots were include as an out-group but have been removed to improve viewing. All nodes have full posterior probability bootstrap support (100) unless indicated. Produced from maximum likelihood analysis results from RaxML in Fig Tree v1.40. The 13 asterisks (*), signal the species mitochondrial genome has been sequenced by our group and is unpublished.

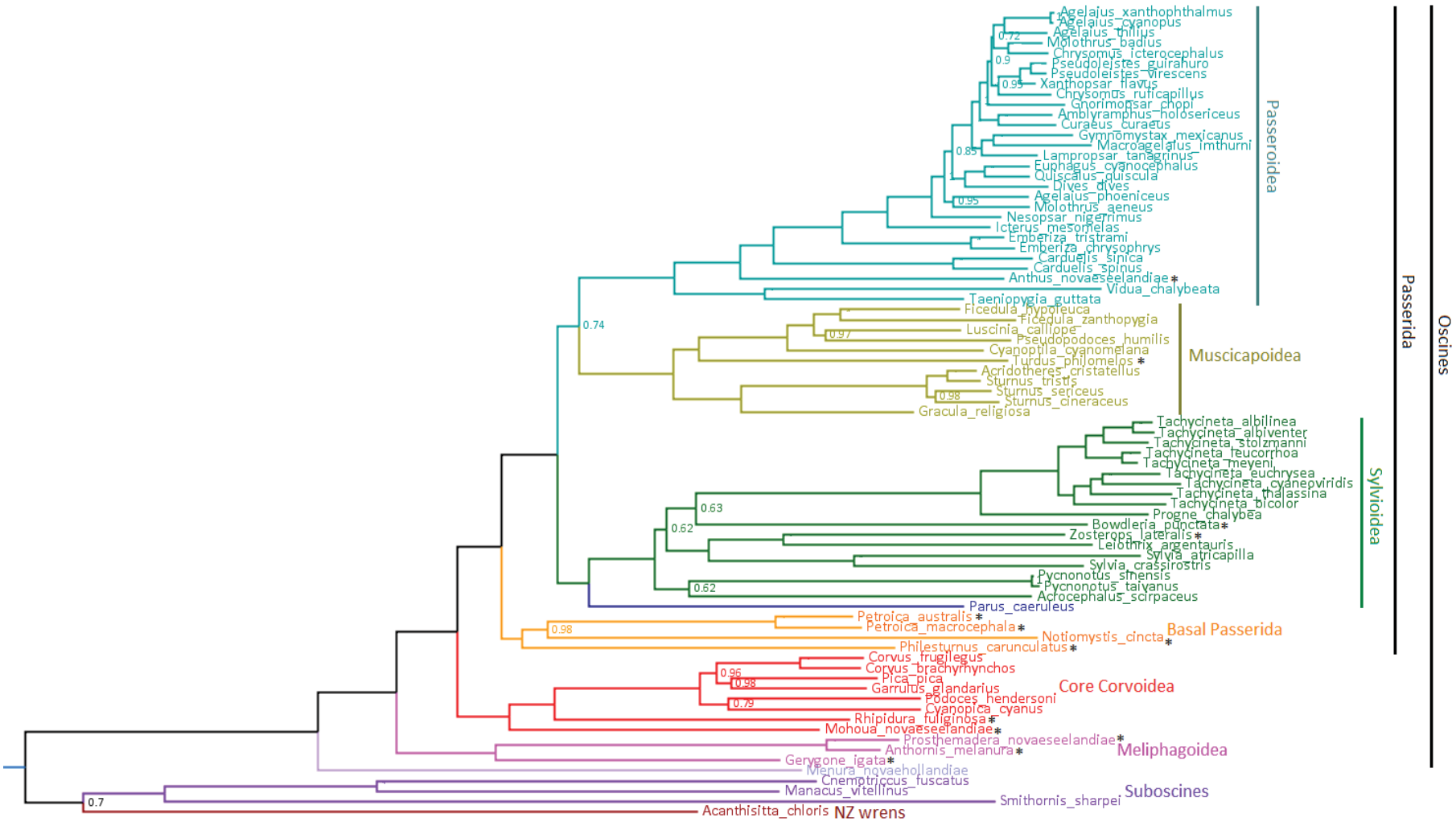

Figure 16: Bayesian analysis phylogenetic tree from all 79 passerine species using mitochondrial genome data (13588bp). Four parrots were included as an out-group but have been removed to improve viewing. All nodes have full posterior probability bootstrap support (1) unless indicated. Produced from Bayesian analysis results from MrBayes in Fig Tree v1.40. The 13 asterisks (*), signal the species mitochondrial genome has been sequenced by our group and is unpublished.

The parrots have all grouped together (Supplementary Figure 1), with full bootstrap support for the branching pattern seen in Pacheco et al. (5). There is also undisputed support for the monophyly of the passerines (Figure 15). The diverging of the three main sub-orders, the Acanthisitti, suboscines and oscines, has not been resolved in either the maximum likelihood (Figure 15) and the Bayesian (Figure 16) analyses. Both trees have weak support for the grouping of the suboscines with the rifleman (*Acanthisitta chloris*). The consensus network from the maximum likelihood analysis (Supplementary Figure 2) demonstrates this, with around a third of the trees produced grouping the rifleman with the suboscines, while almost half have the rifleman as sister to the oscines and suboscines.

There is full bootstrap support for the suboscines as a group (Figure 15 and Figure 16). The suboscines have diverged into the two expected lineages; the Tyrannides from the Americas (*Cnemotriccus fuscatus* and *Manacus vitellinus*) and the Eurylaimides from Africa and Asia (*Smithornis sharpei*). The oscines have also all grouped together as expected with complete bootstrap support (Figure 15 and Figure 16). The first to diverge from the oscine branch is the lyrebird (*Menura novahollandiae*), followed by the three Meliphagoidea. The grey warbler (*Gerygone igata*) is sister to the two closely related honey-eater species the tui (*Prosthemadera novaseelandiae*) and bellbird (*Anthornis melanura*).

The next branching event is the splitting of the two large oscine lineages: the Core Corvoidea and the Passerida. The Core Corvoidea as a whole has full support in both the maximum likelihood and Bayesian analysis phylogenetic trees (Figure 15 and Figure 16). The first to diverge from the lineage is the New Zealand native the browncreeper (*Mohoua novaeseelandiae*), followed by the other native, the fantail (*Rhipidura fuliginosa*), both of these events are fully supported. At the crown of Core Corvoidea is the crow group represented by six species. There is complete support for them as a monophyletic group, but little support for any of the divergence events within the group, apart from the pairing of the two *Corvus* species (Supplementary Figure 3). The pairing of *Cyanopica* and *Podoces* is seen in both the maximum likelihood (Figure 15) and Bayesian (Figure 16) trees, with weak support (50 and 0.79). *Pica* and *Garrulus* also are paired together (33 and 0.98), with some support for grouping this pair with the two *Corvus* (35 and 0.98). When taxa with uncalled bases were removed (including the Core Corvoidea: *Corvus brachyrhynchos* and *Mohoua novaeseelandiae*), the branching

pattern of the crows changed (Supplementary Figure 4), but again no node has strong support.

Note that the Australasian robins (*Petroica australis* and *Petroica macrocephala*), the New Zealand wattlebirds (*Philesturnus carunculatus*) and the hihi (*Notiomystis cincta*) have all branched off at the base of the Passerida. There are three questions surrounding the three families (Petroicidae, Callaeidae and Notiomystidae) that make up the "basal Passerida". First, there is full support from both the maximum likelihood and Bayesian phylogenetic trees (Figure 15 and Figure 16) for the placement of these three families at the base of the Passerida. Second are the three families monophyletic? There is only weak support (67%) from the maximum likelihood tree (Figure 15), but there is full support from the Bayesian analysis (Figure 16). Third what is the exact order of branching of the three families? There is full bootstrap support for the joining of the two *Petroica* together. The Bayesian analysis (Figure 16) has very strong support (0.98) for the Petroicidae pairing with the Notiomystidae, but the consensus network (Supplementary Figure 5) shows only 53% of the trees produced from the maximum likelihood analysis have the robins grouping with the hihi. Another 39% have the Callaeidae and Notiomystidae paired together (Supplementary Figure 5), and the Petroicidae are the sister group. When all taxa with uncalled bases are removed from the data set the bootstrap support for the monophyly of the "basal Passerida" increases slightly from 67% to 70% (Supplementary Figure 4). The support for the grouping of the Petroicidae with the Notiomystidae also increases from 53% to 60%. When running the 63 Passerida species including these four basal Passerida, the support for the four together increases to 100%, but support for Petroica with the Notiomystis decreases to 54% (Supplementary Figure 6).

The monophyly of the Passerida without the basal Passerida is fully supported from both the maximum likelihood and Bayesian analysis phylogenetic trees that the three superfamilies of the Passerida all group (Figure 15 and Figure 16). But how the three superfamilies split apart is still unresolved. There is weak support (56% and 0.74) for the Passeroidea grouping with the Muscicapoidea, with the Sylvioidea as the basal group. But as the maximum likelihood consensus network shows a significant percentage (33%) of the trees produced in RaxML also support the Passeroidea pairing with the Sylvioidea (Supplementary Figure 8). A number of different maximum likelihood trees were made, the first removed any taxa with uncalled bases (Supplementary Figure 4), which again grouped the Passeroidea with the

Muscicapoidea, but support had dropped to only 54%. When only the Passerida species were run (Supplementary Figure 6) the Passeroidea now paired with the Sylvioidea with weak support (59%), and when two species were removed (*Ficedula hypoleuca* and *Lampropsar tanagrinus*, as they had uncalled bases) the support for the Passeroidea and Sylvioidea grouping together improved to 73% (Supplementary Figure 7). What all these different trees show is that how the three superfamilies have diverged is still unresolved, and increasing the number of species used in these phylogenetic analyses has not improved the resolution. However, so far each of the three superfamilies appears to be monophyletic.

The Paridae were considered by some studies (2) as their own superfamily, there are only two species classified in this lineage used in this study; *Parus caeruleus* and *Pseudopodoces humilis*. The two species are placed in different locations on the phylogenetic trees (Figure 15 and Figure 16) suggesting the Paridae are not monophyletic. In this *Pseudopodoces humilis* is found within the Muscicapoidea with full support from both trees. This suggests *Pseudopodoces humilis* has been wrongly classified within the Paridae and should instead be classified as part of the Muscicapoidea. The only other Paridae used in this study is *Parus caeruleus* (blue tit) which has branched off at the base of the Sylvioidea. The support for this is very strong in both trees (Figure 15 and Figure 16). As this is the superfamily in which the Paridae were originally classified, I find no support for them as their own superfamily. Although based on the branch length in both analyses, they are clearly a very deep lineage within the Sylvioidea.

There is full support from both trees for the monophyly of the Sylvioidea (Figure 15 and Figure 16). At the base of the Sylvioidea is a polytomy, with the two different phylogenetic trees giving different branching patterns (Figure 15 and Figure 16). There is only weak support for the majority of the nodes in this superfamily; this is demonstrated by the consensus network of the maximum likelihood phylogenetic tree (Supplementary Figure 9). Only two groupings have strong support; the first is the large group containing the nine *Tachycineta* species and *Progne chalybea*, the other is the grouping of the New Zealand native *Zosterops lateralis* (waxeye), with *Leiothrix argentauris* and the two *Sylvia* species. Five different lineages have split apart from polytomy site (Supplementary Figure 9), one of the lineages consists of the New Zealand fernbird (*Bowdleria punctata*). There does appear to be weak support for the grouping of the four species above with the two *Pycnonotus* species (Supplementary

Figure 10). The Bayesian analysis tree has the *Tachycineta* fully resolved (Figure 16), but in the maximum likelihood tree there is an unresolved site in the *Tachycineta* (Supplementary Figure 9). The maximum likelihood trees produced by using only Sylvioidea taxa improved the resolution, with strong support for a number of divergence events (Supplementary Figure 10). There is now full bootstrap support for the divergence pattern seen in the Bayesian analysis tree (Figure 16)

The monophyly of the Muscicapoidea is fully supported, with the 11 species that were classified in the superfamily all grouping together (Figure 15 and Figure 16). The Muscicapoidea in general is well resolved with full support for the majority of branching events from both the maximum likelihood and Bayesian phylogenetic trees (Figure 15 and Figure 16). There is a division at the base into two groups. The two *Ficedula* species have paired together as expected, these group with another pair made of *Luscinia calliope* and *Pseudopodoces humilis*, which as mentioned above appears to have been wrongly classified as part of the Sylvioidea family the Paridae. *Cyanoptila cyanomelana* branches below these four followed by the song thrush (*Turdus philomelos*), which as expected is found within this superfamily. In the other half of the Muscicapoidea there is a group of four species made of the three *Sturnus* species and *Acridotheres cristatellus*. Branching off basal to these four is *Gracula religiosa*. How the *Sturnus* and *Acridotheres* species diverge is unresolved in the full maximum likelihood tree (Figure 15). The consensus network shows just over half of the trees produced in RaxML supported splitting the foursome into two pairs (Supplementary Figure 11), with the *Acridotheres* pairing with *Sturnus tristis*, but around 40% of the other trees had *Sturnus sericus* as the sister taxa of this pair. What is definite is that *Acridotheres cristatellus* lies within the genus *Sturnus*, questioning the monophyly of the *Sturnus*.

There are 29 Passeroidea species analysed here have all grouped together supporting the monophyly of the superfamily (Figure 15 and Figure 16), and most of the divergences within are fully supported by both the maximum likelihood and Bayesian analyses. At the base of the superfamily the pair *Taeniopygia guttata* and *Vidua chalybeata* diverge from the other species. The next to branch off is the New Zealand native the pipit (*Anthus novaeseelandiae*), followed by the two *Carduelis* species, then the two *Emberiza* species, leaving the large Icteridae family which consists of the remaining 22 species. The consensus network (Supplementary Figure 12) of the maximum likelihood analysis has strong support for most divergence events in this

large family, but at a number of sites there is uncertainty, this is likely due to the relatively short time between each divergence event.

4 Discussion

4.1 DNA extraction and Illumina sequencing

The Illumina sequencing worked well for the fernbird, tomtit, pipit and waxeye samples, with the majority of their mitochondrial genomes being sequenced. But the Illumina sequencing did not work very well for both the browncreeper and bellbird, and it was not possible to assemble the full mitochondrial genomes of these two species. Only two of the bellbird reads mapped to the available bellbird mitochondria sequence or to the full tui mitochondrial genome (a close relative). A large number of reads assembled into contigs that matched bacteria when run through BLAST2go against GenBank (Figure 13). And with the browncreeper reads only around 250 reads mapped to passerine sequence, again a large proportion of the reads assembled into contigs that matched bacteria (Figure 13). Both of these results indicate that the DNA samples used for Illumina sequencing contained only a very small amount of bird DNA, and a large amount of contaminant DNA. The possible reasons for this I will discuss further.

When the DNA was extracted from the tissue samples both the browncreeper (11.8 ng/µl) and bellbird (20.2 ng/µl) had very low concentrations (Table 3), but the pipit DNA (10.7 ng/µl) was also low though the full genome was assembled using the reads produced from its DNA sample. The DNA in the browncreeper and the bellbird were also degraded, the DNA is visible as smears in the gel electrophoresis photo (Figure 4). The browncreeper DNA was extracted from an old museum sample provided by Te Papa, collected in 1983, it had been preserved in ethanol since then. While the bellbird was extracted from blood collected in 2006 and had been stored chilled (but not frozen) for six years. The long-time stored in ethanol or as chilled blood may be the reason why these two samples had very low DNA concentrations and were degraded. There would also have been considerable opportunity for the samples to become contaminated with bacteria during this time.

But at this stage there was still passerine DNA in the samples, PCR was used to amplify a small region of the mitochondrial genome and the products were capillary sequenced by the MGS. Only primer combinations which produced products around 1000bp worked for the bellbird DNA sample, but for the browncreeper I was only able to produce even smaller products (~400bp). When the sequence from these PCR

products was run on BLAST against GenBank they both matched passerines (Table 3). So there was definitely passerine DNA in the samples, but the question still remains how much was bird DNA and how much was bacterial contaminant DNA?

Because the DNA was low concentration and degraded, REPLI-g® (Qiagen®) was used on both the browncreeper and bellbird samples. This amplifies the DNA producing long concatenated DNA products. REPLI-g® works well on samples with low DNA concentrations, but DNA fragments greater than 2-3kb (10kb is optimal) are preferred for amplification (89, 90). As both DNA samples were degraded (average fragment length under 1kb), especially the browncreeper, it is possible contaminant DNA from bacteria was targeted by the REPLI-g® enzyme for amplification instead of the passerine DNA. Previous studies have shown that when REPLI-g® amplifies a sample of DNA it does so in a biased manner (89, 90); sequences with lower GC content and higher numbers of repeats are amplified preferentially. The bacterial DNA may have had a lower GC content than the mitochondrial genomes and had repeats, and was therefore amplified by the REPLI-g® enzyme instead. After REPLI-g® the DNA concentration had increased and the samples were now a higher molecular weight (Table 5 and Figure 4).

When the quality of the Illumina sequencing reads were checked using Fast QC both the browncreeper and bellbird reads were given warnings or failed a number of categories (Table 7). But they did not appear to be any worse quality then both the waxeye and tomtit reads, from which both their mitochondrial genomes were successfully assembled. Fast Q screen was then run on the bellbird and browncreeper reads to check for any possible signs of contamination by comparing the reads to *E. coli*, yeast, PhiX, adapters and vectors. No matches were seen (Figure 5), suggesting the reads were free of contamination. But when run on BWA out of the 2.5 million bellbird Illumina sequencing reads only two were passerine, the majority were bacterial. BWA did not work very well for the browncreeper as well, out of 1.9 million reads only 121 mapped to the reference genome. Bowtie 2 was then tried which maps the reads locally (Material and Methods section 2.5.3), and another 132 reads mapped to the reference genome. Often these reads were partially browncreeper sequence and part pFossil-cloning vector sequence (GenBank number: JX069762 and JX069764). Perhaps this pFossil-cloning vector sequence was added to reads during the REPLI-g® process? In Geneious the pFossil sequence was then trimmed from the reads. Using this method

only small regions of the browncreeper's mitochondrial genome were recovered (253 reads, 2.2 mean coverage of 10555 bases).

While the use of tissue from museum samples is not ideal because they are often old, the DNA is degraded, and are more likely to be contaminated compared to fresh samples. But museum samples are still very useful for improving phylogenetic studies, and in some occasions they are the only option. Many of the species which are useful for understanding phylogeny are endangered, extinct, under restrictions for the taking of live samples, or in geographically isolated locations, this makes the use of fresh tissue samples difficult (91).

Nevertheless it is a recommendation from the present work that New Zealand establishes a bank of good tissue/DNA samples that are available for use in research such as sequencing. The Australian National Wildlife Collection (managed by CSIRO) is a very good example. They have a large and extensive collection of Australia's land vertebrates, which are available for use in research and includes a bank of frozen tissue from Australasian birds (92). During this study it was difficult to find good quality tissue samples for many of New Zealand's native species, and often all that was available were old museum samples. It would be useful if there was a national database where different museums, government departments, universities and research institutes could document what native animal and plant samples they have, how they are stored (tissue or DNA), and if the samples have been used previously for research. Then anyone who is conducting research could access this database, find samples of the species they are interested in and organise the use of these samples.

4.2 Phylogeny

Many of the results here are not novel but are instead confirmation of results seen in previous studies on passerine phylogeny. All the New Zealand taxa have branched off the phylogenetic tree in the group they were expected to be placed in, but there is evidence to suggest some lineages may need reclassification. I will now go through the different parts of the passerine tree discussing certain aspects of the tree including interesting placements of taxa.

The order of divergence of the three suborders, the Acanthisitti, suboscines and oscines, is unresolved in both the maximum likelihood and Bayesian phylogenetic trees (Figure 15 and Figure 16). But the placement of the Acanthisitti suborder as sister to the

other passerines, with the oscines and suboscines splitting later has been well established by many studies (1, 2, 5, 13). In this study the only out-group taxa used were the parrots, in other studies (5, 13) they have used representatives from a number of avian orders, and there has been full support for the rifleman at the base of the passerines. One reason why the rifleman may have grouped with the suboscines in a large proportion of the trees produced is because of long branch attraction. The rifleman has been separated from all other birds in this study for possibly around 80 million years (1, 17), that means a large amount of changes to the mitochondrial genome sequence would have accumulated. And as there are many oscines but only three suboscine genomes there could be some attraction. A possible solution to this problem would be to sequence extra mitochondrial genomes from both the New Zealand wrens (only other extant species is the rock wren *Xenicus gilviventris*) and other suboscines. These extra genomes may break up the long branches, thus preventing long branch attraction. There was also full support for the monophyly of both the suboscines and oscines. The phylogeny of the suboscines has not been studied in any detail using mitochondrial genomes (5); the sequencing of extra genomes from these may also provide support for hypotheses made about their evolutionary history.

The lyrebird (*Menura novahollandiae*) is sister to the other oscines, this confirms the results from other studies (1, 13, 15) that the Menuridae is the most basal oscine lineage. The next lineage to diverge from the main oscine branch was the three Meliphagoidea species the grey warbler (*Gerygone igata*), tui (*Prosthemadera novaeseelandiae*) and bellbird (*Anthornis melanura*). This again confirms what has been seen in other studies (26, 28, 29), that they form a monophyletic group that branches of basally to the large Core Corvoidea and Passerida split. The sequencing of a mitochondrial genome from a representative of the other two families within the Meliphagoidea (Pardalotidae and Maluridae), along with other deep oscine lineages such as the Climacteridae (tree creepers), Ptilonorhynchidae (bowerbirds), Pomatostomidae (Australian babbler), and Orthonychidae (logrunners), would be very useful for studying the deep phylogeny of the oscines.

All of the Core Corvoidea species have grouped together with full bootstrap support from both the maximum likelihood and Bayesian analysis phylogenetic trees (Figure 15 and Figure 16). This confirms the monophyly of the group. The relationships within the Core Corvoidea are poorly resolved (52). Multiple studies have found a polytomy at the base of the Core Corvoidea (15, 32), with as many as five different lineages more or less

branching from the same point. Previous studies using full mitochondrial genome data have only used species from the true crow family (Corvidae); only the rook (5), the rook and American crow (2), or the full genome of the rook with a few mitochondrial genes of other Core Corvoidea (15). This is the first study to use non Corvidae: the full mitochondrial genome of the fantail (*Rhipidura fuliginosa*) and the partial genome of the browncreeper (*Mohoua novaeseelandiae*), and we can therefore begin to break up the polytomy. The fantail had previously been placed with the crows (31, 33), or with piopios and vireos or at the polytomy (15). While the *Mohoua* has been grouped with the whistlers (28), or the crows (15), or branching of at the polytomy (32). We now have very strong support for the placement of the *Mohoua* as sister to the fantail and crows. The mitochondrial genome sequence of the other groups within the Core Corvoidea superfamily are now needed; including a vireo (Vireonidae), whistler (Pachycephalidae), bird-of-paradise (Paradisaeidae). These could allow a full resolution of the Core Corvoidea polytomy. The browncreeper mitochondrial genome also needs to be completed.

At the crown of Core Corvoidea the six true crow (Corvidae) species have all grouped together with full support (Figure 15 and Figure 16). But it is unresolved how they have all split apart, with little support for any of the divergence events apart from the pairing of the two *Corvus* species together (Supplementary Figure 3).The Core Corvoidea originated in Australasia (32), and both the *Mohoua* (34) and the Rhipiduridae (33) have their centre of diversity here. This suggests these two lineages diverged away from the Corvidae while the Core Corvoidea was still only in Australasia. Jonsson et al. (32) suggests that the formation of the Papuan islands around 34 mya selected for greater flying in the Core Corvoidea and provided the group a passage to reach the rest of the world from Australia. Once the group reached Asia they underwent many rapid radiations filling the many different ecological niches present in the rest of the world (32). As the six crows used here are all found in either Eurasia or the Americas, they may represent the radiations that rapidly expanded after the Corvoidea escaped Australasia. This would explain why resolving this part of the tree is very difficult as many divergence events would have occurred in a short space of time, allowing very few molecular sequence changes to accumulate between each divergence event.

The three families (Petroicidae, Callaeidae and Notiomystidae) that make up the basal Passerida have all been placed in different positions on the tree by earlier studies

including in the Core Corvoidea and the Meliphagoidea (1, 10, 27, 30, 33). However more recent studies agree and have now placed them at the base of the Passerida (15, 21, 22, 28, 31). The present study is the first that uses full mitochondrial genome sequence from at least one species of each of the three families. There is full support for the placement of these three families branching off at the base of the Passerida (Figure 15 and Figure 16), confirming the above studies which focused on the families individually. This suggests the species that make up these three families should be formally reclassified as Passerida. Whether these three families all group together forming their own monophyletic group is still unresolved although there is support for this (Figure 15, Figure 16 and Supplementary Figure 5). The three basal Passerida families may only be grouping together due to long branch attraction as they are all relatively long branches and the three families have been separated for around 30 million years (21, 22). The sequencing of the Kokako (*Callaeas cinereus*) and extra Petroicidae would help break these long branches up, providing better resolution of the phylogeny of the basal Passerida.

Previous studies have used the presence of duplicated control regions to resolve phylogeny (64, 65). The saddleback has only one control region between tGlu and tPhe, this is the gene arrangement that is seen in most passerines and presumably the common ancestor of all three basal Passerida lineages. The Petroicidae have a partial duplication of the control region, their first control region is located between tThr and tPro and is large (1095-1097 bp), while the second control region is found between tGlu and tPhe and is relatively small (190-246 bp). This suggests there has been a duplication of its control region into the 1st control region position (Figure 9), followed by considerable reduction of the original control region (2nd control region position). The hihi has a duplicated tPro, ND6, tGlu, and control region, with the second copy following directly after the first, this is not seen anywhere else in the passerines. These duplicated genes in the hihi are all the same length and identical sequence, and the two control regions are almost identical (the second has around 100bp extra on the 3' end), the lack of nucleotide changes in these genes/regions suggests the duplication event has occurred relatively recently. So overall these duplication events have not provided any insight into how the three families have split apart. These three families are all found only in Australasia so dating when they broke from the main Passerida branch will provide an upper limit for the time when the Passerida dispersed out into the rest of the world.

The three superfamilies of the Passerida (Sylvioidea, Passeroidea and Muscicapoidea) have all grouped together with full support, and there is full support for the monophyly of each of the three superfamilies, but their order of divergence still remains unresolved. There is weak support (56% and 0.74) for the grouping of the Passeroidea with the Muscicapoidea (Figure 15 and Figure 16). This is the branching order seen in other studies using nuclear data (1, 41), and the latest study using full mitochondrial genomes from 33 passerines (52), but disagrees with other studies using smaller amounts of mitochondrial data (2, 40) and Hackett et al. (13). This uncertainty is likely due to the rapid radiation of the Passerida where the three superfamilies all split apart within a short time period; this has meant there are only short interior nodes between the three superfamilies. Overall the question of which of the three superfamilies diverged first from the Passerida is still not resolved, a new approach is needed to solve it. The use of more molecular data in the form of mitochondrial genomes from multiple species of each superfamily has not improved the resolution. In the molecular data section of the discussion below (section 4.3) I will discuss some possibilities for improving the resolution of some of these sites on the phylogenetic tree that remain unresolved.

The Paridae are a family that are traditionally classified in the superfamily Sylvioidea, but some have suggested they are their own superfamily (2, 40, 41). There are two species classified within the Paridae with mitochondrial genome sequence available, the Hume's ground tit (*Pseudopodoces humilis*) and the blue tit (*Parus caeruleus*). *Pseudopodoces humilis* was placed within the Muscicapoidea with full support (Figure 15 and Figure 16), while *Parus caeruleus* branched off at the base of the Sylvioidea. The placement of these two Paridae species in different parts of the tree suggests the current Paridae family is not a monophyletic group. The Hume's ground tit (*Pseudopodoces humilis*) was originally placed within the Core Corvoidea, but James et al. (93) suggested that it should be instead placed within the Paridae. Our placement of the species within the Muscicapoidea was also seen in the recent study by Marshall et al. (52), who found *Pseudopodoces humilis* paired with *Luscinia calliope*. Both the maximum likelihood and Bayesian phylogenetic trees (Figure 15 and Figure 16) have strong support (80% and 0.97) for this pairing. Therefore, we suggest *Pseudopodoces humilis* needs to be reclassified as part of Muscicapoidea, possibly in the same family as *Luscinia calliope*. The other Paridae *Parus caeruleus* branched off at the base of the Sylvioidea, as this is the superfamily where the Paridae were originally classified I find

little support for the suggestion that they are their own superfamily. Before a decision can be made mitochondrial genome sequence from other Paridae needs to be collected, which can then be used to test whether the Paridae (minus *Pseudopodoces humilis*) is a natural monophyletic group. The position where *Parus caeruleus* diverged from the main Sylvioidea branch is very deep; this suggests the Paridae family has been separated from the other species in the superfamily for a considerable amount of time.

The rest of the Sylvioidea have grouped together with complete bootstrap support (Figure 15 and Figure 16), but the splitting within Sylvioidea is still unresolved (Supplementary Figure 9). A number of my findings for the Sylvioidea support the results of Jonsson and Fjeldsa (33) who looked at the relationships of different families with the superfamily. They separated the different families into 13 clades. I have the native waxeye *Zosterops lateralis* and *Leiothrix argentauris* paired together (representing clade 12), these have grouped with the two *Sylvia* species (clade 13), confirming previous studies results (17, 33). The polytomy seen in Figure 15 and Figure 16, is also seen in other studies (8, 33), where seven clades diverge at the same point on the tree, my study does not improve the resolution of this site with five different lineages diverging from this point (Supplementary Figure 9). Jonsson and Fjeldsa (33) placed the fernbird's genus (*Bowdleria*) in a clade with the genus *Acrocephalus*, but I have found no support for this pairing. Both the Bayesian analysis (Figure 16) and maximum likelihood consensus network (Supplementary Figure 10) provides strong support for the branching pattern seen Cerasale et al. (44) who examined the phylogeny of the *Tachycineta*.

The majority of divergence events in the Muscicapoidea had strong support and the superfamily is relatively well resolved (Figure 15 and Figure 16). My results confirmed the branching pattern seen in previous studies on the Muscicapoidea (33, 41, 52). Jonsson and Fjeldsa (33) divided the Muscicapoidea into six clades, clade four contained the *Sturnus, Acridotheres,* and *Gracula*, clade five is represented here by *Turdus*, and the other five species all belong to clade six. Clades five and six are expected to pair together with four branching off basal to this pair (33), this is confirmed by both trees (Figure 15 and Figure 16). One part of the Muscicapoidea is still unresolved; how the *Sturnus* and *Acridotheres* species diverge. Just over half of the trees produced in the maximum likelihood analysis supported splitting the foursome into two pairs (Supplementary Figure 11), with the *Acridotheres* pairing with *Sturnus tristis*, but around 40% of the other trees had *Sturnus sericeus* as the sister taxa of this

pair. What is fully supported is the pairing of *Acridotheres cristatellus* with *Sturnus sericus*, and the placement of *Acridotheres* within the Genus *Sturnus*, questioning the monophyly of *Sturnus*. Previous studies have also found *Sturnus* to be paraphyletic (33, 91, 94), due to a number of other genera being found within this genus, including the *Acridotheres*. *Sturnus tristis* has sometimes been classified as *Acridotheres tristis*, so it would be expected that it groups with *Acridotheres cristatellus* (91, 94). Also the other two *Sturnus, S. sericeus* and *S. cieraceus*, have been shown to be sister taxa and this pairing has strong support (91, 94). The pair have also been renamed as *Poiopsar* by some studies (91) making a genus comprising only these two species. The 26 species of European starlings that traditionally made up the paraphyletic genus *Sturnus* have been classified into as many as 10 different genera (91).

Most divergence events within the Passeroidea are fully supported by both the maximum likelihood and Bayesian analyses (Figure 15 and Figure 16), the results here also confirm much of what was given by Jonsson and Fjeldsa (33). The pair *Taeniopygia guttata* and *Vidua chalybeata* branch off at the base of the superfamily, they represent clade 7. In the study by Jonsson and Fjeldsa (33) there was a polytomy seen with clades 8, 9, 10 ,11-21 all branching from the same point. This is now partially resolved with strong support for clade 9 (the New Zealand native *Anthus novaeseelandiae*) branching off basally to clades 10 (*Carduelis*), 20 (*Emberiza*) and 21 (The large Icteridae family containing the remaining 22 species). The large new world blackbird family Icteridae was studied by Powell et al. (55) using full mitochondrial genomes. Overall the maximum likelihood and Bayesian analyses phylogenetic trees (Figure 15 and Figure 16) confirm the results in Powell et al. (55). Some minor disagreements were found, but these occurred at the sites were the bootstrap support was very weak (Supplementary Figure 12).

Some recommendations for the conservation of New Zealand's native passerines can be made based on the phylogenetic results produced in this study (Figure 15 and Figure 16) and other studies. There are a number of passerine lineages that are native to New Zealand that are genetically very diverse from other passerines found anywhere else in the world. First, the rifleman and the rock wren make up their own suborder the Acanthisitti, which have been separated from all other passerines for potentially around 80 million years (17) and are possibly the only passerines that have inhabited New Zealand since it split from Gondwana, so are phylogenetically distinctive. The rifleman is at risk of extinction with both of its populations declining (Table 2), while the rock

wren is nationally vulnerable to extinction, the preservation of these two species should be one of the highest priorities for DoC. The three families that make up the basal Passerida should also be a high priority for DoC. The three families are: the Callaeidae, containing the kokako (nationally vulnerable) and the saddleback (at risk), the Notiomystidae, containing only the hihi (nationally endangered), and the Petroicidae, containing four native species (46 species worldwide). These are also an important part of New Zealand's biodiversity, and they have been separated from the Passerida for possibly around 30 million years (21, 22). All three Meliphagoidea species (tui, bellbird and grey warbler) are not threatened with extinction, although a number of their populations are at risk or threatened which may have unforeseeable implications in the future. The four native lineages within the crown Passerida (*Zosterops, Bowdleria, Anthus and Hirundo*) all have close relatives found elsewhere in the world. Too much uncertainty surrounds the subdivisions within the Core Corvoidea to make an informed decision about how genetically diverse the *Mohoua* or *Rhipidura* really are.

4.3 Molecular data

As mentioned above the use of mitochondrial genomes as a molecular marker for the understanding of the phylogeny of the passerines has been very useful, and has produced phylogenetic trees (Figure 15 and Figure 16) that have strong support for the majority of branching events. But a number of subdivisions remain unresolved (see phylogeny section of discussion above). The use of many mitochondrial genomes from these lineages has not improved the resolution of the branching pattern, because it is likely that there was only a very short time between each divergence event, which meant very few changes in the nucleotide sequence were able to accumulate. So what are the options for improving the resolution? First, even more nuclear molecular data from each species in the study could be collected and compared. Second, the number of taxa with sequenced mitochondrial genomes could be increased. Third, we could use the data we have now more effectively.

Studies using both mitochondrial and nuclear DNA markers have been found to be the most informative for resolving phylogeny in the passerines (94). When introgression or incomplete lineage sorting occurs within the mitochondrial genome nuclear markers will be needed to completely resolve the phylogeny of the lineage (50, 51), introgression may have occurred at some subdivisions within the passerines. The results from this study will also need to be tested by comparing them to other molecular

markers, such as nuclear genes, introns, intragenic regions, microsatellites, deletions and transposons. Perhaps much of the nuclear gene data available from the 79 passerine species analysed here could be added to the mitochondrial genome nucleotide alignment and the phylogenetic trees reproduced. Hackett et al. (13) used 19 nuclear intron loci from 80 different birds including 24 passerines, Treplin et al. (41) used four nuclear genes from 80 passerines (26 families), and many other studies that have used nuclear data to focus on specific lineages within the passerines (17, 26, 28, 34).

The genes on the mitochondrial genome are known to be under selection (51, 52, 95), under both purifying selection to remove any mutations that have a negative effect on the functioning of the mitochondria, and positive selection where mutations that increase the efficiency of respiration in certain environments will be selected for. Dowling et al. (95) states that because there is a large amount of interactions between mitochondrial and nuclear genes and their products, when there is selection on nuclear genes there will likely be some selection pressure placed on the connected mitochondrial genes. Also Marshall et al. (52) suggested that because the passerines are the largest avian order and there is huge diversity in their habitats (climate and altitude) and diets (type, calorie content and distribution), it is understandable that different species face different selection pressures on their mitochondrial genes. These selection pressures would lead to variation in the efficiency of the core process the mitochondria performs for the cell, respiration. This means we cannot rule out that the mitochondrial genomes of different lineages have convergently evolved; which would influence the phylogenetic analyses of these genomes. Therefore the mitochondrial genome should be used as one of many molecular markers of phylogeny (55).

As mentioned above there are situations where extra sequenced mitochondrial genomes would be useful for improving resolution in the phylogeny. These include when lineages are not represented at all by a species with a sequenced mitochondria (such as some of the groups in the Core Corvoidea that branch off at the polytomy and some of the deep Australian oscines), or where representation is low (New Zealand wrens, suboscines and basal Passerida). Pacheco et al. (5), states that by adding extra mitochondrial genomes from critical groups the stability of a number of phylogenetic hypothesises have increased when looking at the base of the neoaves phylogeny. This should also work with adding more mitochondrial genomes from certain groups within then passerines. But multiple genomes have not improved resolution in number of

already highly represented lineages such as the three Passerida superfamilies, the true crows (Corvidae) and the Sylvioidea.

Perhaps in these lineages where multiple genomes have already been sequenced the molecular data needs to be used more effectively. One way to do this is use different partitioning of the nucleotide sequence. Partitioning has been shown to give different results when resolving phylogeny: In this study the mitochondrial genome was split into five partitions: 1st, 2nd and 3rd (RY) codons, RNA stems and loops. This has been used in other studies (5, 7, 65), and has been shown to have high resolving power. Another possible partitioning scheme divides the protein coding genes into three groups based on the mutation rate, with the tRNAs, the 12S and the 16S genes making another three partitions, this has been used in a number of studies of avian phylogeny (5, 96). Pacheco et al. (5) used a third scheme with 15 different partitions, the 12 protein coding genes separately, 12S, 16S, and the tRNAs combined. The study found this partitioning worked better than the other two partitioning schemes mentioned above as it allowed the parameters of each protein coding gene to be optimized separately, which allowed their variable evolutionary rates to be taken into account. Perhaps this partitioning scheme could be tested using the 79 passerine mitochondrial genomes to compare if it improves the resolution of any sites compared to the five partitions.

The use of different genes or regions of the mitochondrial genome sometimes produces slightly different phylogenetic tree topologies, often due to different substitution rates (50). Single mitochondrial genes have been shown to have insufficient variation in them to be informative for phylogeny (50), but the full mitochondrial genome may not be required for all phylogenetic studies. Duchene et al. (50) found that a subset of mitochondrial genes can be just as informative (by producing the same results) as the full mitochondrial genome. When studying two different lineages within the cetaceans only around 25% of the genome was needed to reproduce the full mitochondrial genome results (50), the only problem was the most informative (highest resolving power) gene subset was different for the two lineages and finding out these genes required analysing the full mitochondrial genome! This may sound unhelpful but if the mitochondrial genomes of the current 79 passerines are analysed we may be able to identify which genes are the most informative. Then only these subsets of genes would need to be collected from other passerine species for future phylogenetic studies.

What subset of genes is the most informative for studying passerine phylogeny? When looking at 33 species of passerines Marshall et al. (52) found the most variable sites in the mitochondrial genome were the intragenic regions, the ND6 gene and the control region while the two rRNA coding genes (12S and 16S) were the least variable. Each protein coding gene also had different rates of synonymous substitutions (change in nucleotide sequence that does not change the amino acid translated) and non-synonymous substitutions (mutation in the nucleotide sequence that causes a change in the amino acid sequence when translated). The Cox3 gene had the lowest synonymous substitution rate, ND6 had the highest (52). While ND5 had the highest non-synonymous rate, and four genes (Cox1, Cox3, ATP8 and ND3) had no non-synonymous substitutions (52). Overall it was observed the NADH dehydrogenase genes (ND) have higher variation than the other protein coding genes (Cox, ATP, CytB) (52).

Synonymous substitutions are assumed to be neutral (they should not be selected for or against) as there will be no effect on how the resulting protein functions, but non-synonymous can be positively selected for or negatively selected against as they may change how the protein functions. Therefore the rate of synonymous substitutions (dS) can be higher than the rate of non-synonymous substitutions (dN). The ratio of non-synonymous substitutions to synonymous substitutions is known as the dN/dS ratio. When Marshall et al. (52) compared each genes dN/dS ratio they found it differed at the different levels of taxonomy. This suggested that each gene was evolving independently and is under different selection pressures in different species. They compared the rates at three levels of taxonomy: between four subspecies of chaffinch (*Fringilla coelebs*), the Fringillidae family (*Fringilla coelebs, Fringilla teydea, Carduelis sinica* and *Carduelis spinus*), and all passerine species available. At the highest taxonomic level the synonymous substitution rate was found to be saturated, with variation mainly relying on the non-synonymous substitution rate (52). Marshall et al., (52) found that ND5 had the highest dN/dS ratio at the four subspecies level, suggesting it may not be informative when studying phylogeny at higher taxonomic levels. The three genes that had the highest dN/dS ratio at the family and order taxonomic levels were ATP8, ND6 and ND2 indicating these genes may have greater phylogenetic resolving power at higher taxonomic levels.

Pacheco et al. (5) calculated the average substitution rate for each gene in the mitochondrial genome for the passerines and a number of other avian orders. They

found that all of the passerine protein coding genes and the tRNAs had a higher substitution rate then the avian average (5), which is consistent with the mitochondrial genes are evolving faster in the passerines than in the other birds, which has been well known since Harrison et al. (16). The rRNAs, tRNAs and Cox1 had the lowest substitution rates of the passerine mitochondrial genes, with around 0.003 substitutions per site per million years. The genes with the highest substitution rate was ND2 (0.009) followed by ATP6 and ND3 (~0.007). This is consistent with what is seen in the study by Marshall et al. (52), who found the rRNAs and the Cox genes have the lowest variation (therefore substitution rate), while the ND genes have the highest variation.

As most of the deeper divergence events focused on in my study on passerine phylogeny occurred at least 30 million years ago, some of these fast evolving genes may not be suitable for resolving phylogeny. These fast evolving genes may have reached saturation of synonymous substitution and there is a large chance the same change in the nucleotide sequence could have occurred independently on multiple occasions. For example the ND2 gene has an estimated substitution rate of 0.009 substitutions per site per million years. If this rate is extrapolated out to 30 million yeas (0.009 x 30 = 0.27) there is around a 30% chance the each nucleotide has changed in this time. As some nucleotides are constrained by selection to remain the same, the substitution rate of the other sites must be higher. This means there is a very high chance that two independent lineages could have independently substituted the same nucleotide complicating the phylogenetic analysis. But these fast evolving genes may also be the only way to resolve polytomies. Polytomies occur when more than two divergence events happen within a very short time frame, meaning very few nucleotide changes have occurred between each divergence event. ND2 with its substitution rate of 0.009 per site per million years is around 1000bp long, this means nine substitutions occur on average in the gene every million years. With many of the polytomies such as the three Passerida superfamilies diverging within what is likely to be only a few million years this gene would be able to provide some evidence about the correct order of branching. The key is to use these different evolving genes in a way that allows us to use the valuable rare changes in the fast evolving genes without getting false signals due to chance independent substitutions in different lineages.

At the highest taxonomic level (the whole order of passerines) synonymous substitution rates appear to become saturated and overall rate variation seems to be mainly driven by the non-synonymous rate (52). RY coding of the third codon tries to

account for much of this, by removing some of the noise caused by this saturation of synonymous substitutions (16). One possible solution would be to assess the different taxonomic levels separately. The RY coding of the third codon could be removed when the synonymous substitution rate has not become saturated, such as at lower levels of taxonomy such as within a family. Or perhaps it may be beneficial to remove the third codon data all together when looking at higher taxonomy levels such as the passerine whole order. Saturation of nucleotide variation should also be taken into account when choosing genes for gene subsets or removing uninformative data from mitochondrial genome (50).

4.4 Phylogeny an insight into physical characteristics

One must be careful in using physical characteristics for resolving phylogeny, as a number of traits have been shown to have evolved on an number of independent occasions by convergent evolution (91), such as skull morphological changes in response to prying open soil to grab food, or migratory behaviour (both in the starlings). As the phylogeny of the Passerines is slowly becoming better resolved the can we use it to tell us something else about the evolution of the group. Does it give any insight into what the common ancestor of certain groups were like? Looking at the more basal species of each lineage do we see certain physical/ecological characteristics of the common ancestor? Does it tell us anything about the biogeography of the group?

Can the passerine phylogeny tell us anything about the evolution of flight? There are two main hypothesis of how birds evolved flight. The first was the top down: This is where birds were originally climbers of trees, they then evolved the ability to fly by first being able to parachute or glide down from the tree to the ground (97), in much the same way as the flying squirrels do today. The other possible hypothesis is the bottom up option: This is where birds have evolved flight by first evolving wings for aiding in running, either along the ground (98) or up an inclined surface (99), and were then adapted for taking flight. The ancestors of birds have developed the physical characteristics (feathers, wings, light body) essential for flight, for some other reason and then borrowed these for flight (98, 100). Feathers are now essential for flight in birds, but the presence of feathers in fossils predates the assumed ability of the species to fly (100). On some dinosaur fossils they have been found where there shape would not have allowed flight. They would have evolved earlier where they possibly had a function in firstly insulation for thermo-regulation and then sexual attraction (being

colourful) (100). They were then later adapted for use in powered flight. Can the avian phylogeny provide an insight into which of these hypotheses is more likely?

The most basal lineage in the Aves are the Palaeognaths (101), containing the ratites and tinamous. Flight has been shown to have been lost on multiple independent occasions in the group (101), and the majority now cannot fly, with only the tinamous still being able to fly. The Palaeognaths are all ground foragers and the common ancestor of all was assumed to be a relatively poor flier. The next group to branch from the main avian branch are the combined group of the galliformes and anseriformes (5), these are also ground/water foragers and the majority are relatively poor fliers, in the sense they cannot fly between branches in a tree. Also the basal species within many of the other avian orders are also ground foragers and poor fliers; this suggests that the common ancestor of all birds was a ground forager. This would provide evidence for the hypotheses that flight evolved from the ground up (98, 99), not from the trees down. Within the passerines the basal group is the New Zealand wrens which are basically flightless capable of only hopping around the ground. Also many of the deep oscines are poor fliers; it is not until the tips of the Passerida, Core Corvoidea and Meliphagoidea that we see very strong fliers that spend the majority of their time in trees. This would also support the ground up hypotheses.

How then have many of the passerines evolved the ability to be such strong fliers, capable of difficult feats such as catching insects in the air? The Oscines originated in Australia (1, 15, 22), and to stay here would not have required any great ability for flight. But lineages that were good fliers were able to disperse further out into the world, to locations where there were unoccupied niches such as flying insectivores or honeyeaters. A number of studies have provided support for this: Jonsson et al. (32) suggested the adaption of the Core Corvoidea to flying over water in the Papuan Islands selected for lineages that were strong fliers, which allowed them to radiate out to the rest of the world. This is probably also true for the Passerida which originated in Australia as well, and the task of flying to PNG would have placed a selection pressure on flight ability, and because of this the majority of the Passerida are now very strong fliers. A number of groups within the passerines have very high speciation rates these are the Zosteropidae (insectivore or fruit and nectar), the *Turdus* (insectivore or omnivore), the Pachycephalidae (insectivore) and the *Myzomela* (honeyeater) (43), they are all insectivores or honeyeaters which involves flight. The honeyeaters of the Meliphagidae also have radiated out into one of the most diverse Australasian passerine

family (26), their flying ability would have allowed them to be such successful speciators. The Petroicidae originated in Australia and PNG before dispersing to the Pacific Islands. But only lineages with a flycatcher body form have dispersed into these smaller islands of the Pacific (37), this suggests the unoccupied ecological niches on these islands were only available for lineages that were strong fliers. Most bird speciation events are caused by geographical isolation (allopatric) as opposed to ecological isolation (sympatric) (37), this suggests that the reason the passerines have become so diverse and numerous is partially due to their ability to disperse to different locations. While the Passerines were probably originally poor fliers, the selection for the characteristics needed for flight has allowed the order as a whole to be one of the most successful vertebrate orders, with ~6000 species occupying many different ecological niches worldwide.

Avian genomes are relatively compact and streamlined, with fewer repeats and non-coding DNA than in other vertebrates (102). This was assumed to be an adaptation which allowed flight in birds, and is supported by the fact that flightless birds have larger genomes than flying birds (102). Organ et al. (102), looked at the relationship between the size of the genome and the size of the osteocyte (bone cell) and found that in extant tetrapods 59% of the variation seen in genome size was predicted by the size of the osteocyte. They then used this to predict the size of the genomes of 31 extinct dinosaurs (102). They calculated that the small genome evolved before the evolution of the avians, and before flight, after the split of the two main dinosaur lineages, Ornithischiums and Theropods (which birds belong to) (102). From this study they inferred that genome size reduction was not an adaption that evolved to allow flight. The study also estimated that the genome size of avian lineages has not change in the last 230 million years (102), but that flight and genome size may be functionally related.

4.5 Conclusions

I have successfully sequenced the full mitochondrial genome sequence of the fernbird, tomtit, pipit and waxeye, and have also sequenced partial mitochondrial genomes for the browncreeper and bellbird. This means there is now at least a partial reference mitochondrial genome for each family of the native New Zealand passerines, apparently a first for any country. These genomes were aligned and analysed along with seven previously unpublished genomes from New Zealand passerines, four mitochondrial transcriptomes and all available passerine genomes on GenBank (63

species), producing maximum likelihood and Bayesian analyses phylogenetic trees. A number of exciting results have been seen, and each New Zealand species has been branched off where expected. In particular there is strong support of the placement of the Petroicidae, Callaeidae and Notiomystidae at the base of the Passerida in the phylogenetic tree. There is evidence to suggest that these should be formally classified as Passerida, although whether the three families form a monophyletic group is unresolved. The polytomy at the base of the Core Corvoidea is starting to be resolved, but sequenced mitochondrial genomes from representatives of some lineages are still needed. Our results support an Australasian origin for the oscines. We recommend a greater availability of tissue or DNA specimens of New Zealand native species for use in research. I have identified a need to sequence deep oscines from Australasia. Perhaps the New Zealand passerines could also have their mitochondrial genomes sequenced down to the generic level, this would require the completion of the browncreeper and bellbird genomes along with the sequencing of the kokako, rock wren and welcome swallow.

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6 Appendices

6.1 Supplementary tables

Supplementary Table 1: List of all primers used during study. Name of primers, nucleotide sequence of the primer and a description of what the primers were used for.

Name	Sequence	Used For
Av438dloopF	TCACGWGAAATSAGCW ACTC	Short range PCR and sequencing: waxeye
Av492dloopR	GGGTKTAGGGGGAAAG AAT	Short range PCR and sequencing: waxeye
Av510dloopF	ATTCTTTCCCCCTAMAC C	Short range PCR and sequencing: fernbird, bellbird, and NZ robin
Av1249tPheR	TGGCATCTTCAGTGYCA TGCTT	Short range PCR and sequencing: waxeye and NZ robin
Av1753F12S	AAACTGGGATTAGATAC CCCACTAT	Long range PCR: all species Short range PCR and sequencing: all species
Av1806R12S	AGTTTTAAGCGTTTGTG CTCGTA	Short range PCR and sequencing: NZ robin
Av2150R12S	GAGGGTGACGGGCGGTR TGTAC	Long range PCR: waxeye and NZ robin Short range PCR and sequencing: all species
Av2246F12S	GAGGTAAGTGGTAACAA GGTAAG	Short range PCR and sequencing: bellbird
Av2703F16S	GACTTGTTAGTAGAGGT GAAAAGCC	Short range PCR and sequencing: bellbird
Av2901R16S	GCACTCTTTGTTGRTGG CTGCTT	Long range PCR: fernbird Short range PCR and sequencing: all species
Av3782R16S	CGGTCTGAACTCAGATC ACGTA	Short range PCR and sequencing: bellbird
Av3787F16S	CGATTAACAGTCCTACG TG	Short range PCR and sequencing: bellbird
Av4165ND1F	CGAAAARTCCTAAGCTA CATRCA	Short range PCR and sequencing: waxeye
Av4747ND1F	CCATTCGCCCTATTCTTC CTAGC	Short range PCR and sequencing: bellbird
Av4921ND1F	CCCACGATTTCGMTAYG ACCA	Short range PCR and sequencing: bellbird
Av5201tMetR	CCATCATTTTCGGGGTA TGG	Long range PCR: all species Short range PCR and sequencing: all species
Av5583ND2R	CCTTGGAGGACTTCTGG GAA	Short range PCR and sequencing: bellbird
Av6335tTrpF	GGCCTTCAAAGCCTTAA ATAAGAG	Short range PCR and sequencing: bellbird
Av7138CoIF	ACATTCTTTGAYCCWGC RGGAGG	Long range PCR: waxeye
Av7195CoIR	GGTAATTAGGACGGATC AGACAA	Short range PCR and sequencing: bellbird
Av7546CoIF	GTCGGAATAGACGTAGA CAC	Short range PCR and sequencing: bellbird
Av7662CoIR	AGGAAGATGAAGCCYA GAGCTCA	Short range PCR and sequencing: bellbird

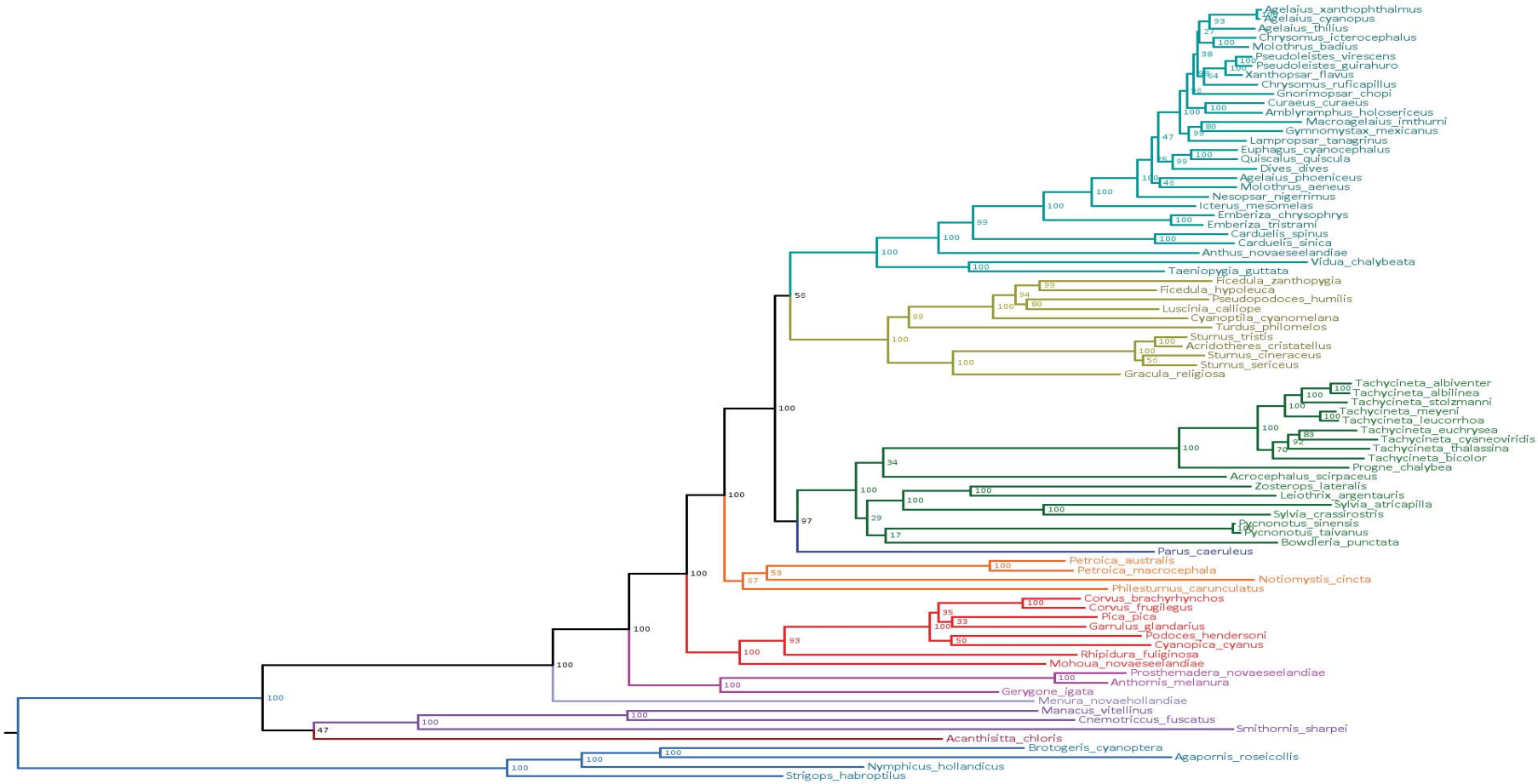
Av8381CoIIF	GACGCCTCATCTCCTATCATA	Short range PCR and sequencing: bellbird
Av8508CoIR	GGATGGTTCAGATTAGTTCGAC	Short range PCR and sequencing: bellbird
Av8872CoIIF	ATCCCAGGACGACTAAAYCAAAC	Short range PCR and sequencing: bellbird
Av8936CoIIR	GTTGGCTCCACAGATTTCTGAGC	Short range PCR and sequencing: bellbird
Av9043tLysF	GCTATGGAACAGCACTAGC	Short range PCR and sequencing: waxeye
Av9341Atp6R	TGGTCGAAGAAGCTTAGGTTCA	Short range PCR and sequencing: bellbird
Av9942CoIIIF	ATGGCHCACCAAGCACACTC	Long range PCR: waxeye Short range PCR and sequencing: waxeye
Av10088CoIIIR	CGTACGATGTCTCGTCATCATTG	Short range PCR and sequencing: waxeye
Av10116CoIIIR-LR	GGGGTGTGGTGGCCCTGGAAGGTGC	Long range PCR: waxeye
Av10307CoIIIF	CCGTCACATGAGCCCACCACA	Short range PCR and sequencing: waxeye and pipit
Av10647CoIIIF	TTTGAAGCAGCAGCCTGATAYTG	Long range PCR: waxeye Short range PCR and sequencing: waxeye and pipit
Av10884ND3R	GGGTCRAAGCCRCATTCGTAGGG	Short range PCR and sequencing: pipit
Av11168tArgF	AGACAGTTGRTTTCGRCTCAACA	Short range PCR and sequencing: waxeye and bellbird
Av11492ND4F	AACYTNAATCTHCTACAATGCTAA	Short range PCR and sequencing: waxeye and NZ robin
Av12138ND4R	ATTGGAGCTTCTACGTGGGCTT	Short range PCR and sequencing: bellbird
Av12217ND4F	CTAGGMGGMTATGGCATTATACG	Short range PCR and sequencing: NZ robin
Av12788ND4F	CTCAAACACACGAGAACACC	Short range PCR and sequencing: waxeye
Av12912tHisR	CGGCAGGTAAGAAGAGTCTAAC	Short range PCR and sequencing: NZ robin
Av12955tSerR	GGCTCAGATGCAAGAATTAGCAGTTC	Short range PCR and sequencing: waxeye bellbird and NZ robin
Av12976tSerF	CAAGAACTGCTAATTCCTGCATCTG	Short range PCR and sequencing: waxeye and bellbird
Av13563ND5F	GATGACACGGACGAGCAGAAG	Long range PCR: fernbird Short range PCR and sequencing: fernbird
Av13734ND5R	AGGCCAAATTGRGCTGATTTTCC	Short range PCR and sequencing: waxeye
Av13840ND5F	AGCACHATAGTHGTAGCCGGAA	Short range PCR and sequencing: bellbird
Av13853ND5F	AACACCTGAGAAATCCAAC	Short range PCR and sequencing: waxeye
Av14050ND5R	GTTGAGATATGGAGGAAGGC	Short range PCR and sequencing: bellbird
Av14208ND5R	GCTAGGAACGGGGTTCCTATTAG	Short range PCR and sequencing: bellbird
Av15107CytBF	CATCCGTTGCCCACACATGYCG	Short range PCR and sequencing: waxeye

Av15266CytbR	TATCCTACGAAGGCAGTTGCTA	Short range PCR and sequencing: bellbird
Av15425CytBR	GGAAGTGAAGGGCGAAGAATC	Short range PCR and sequencing: waxeye
Av15671CytbF	CCCAGAAAACTTCACACCAGC	Long range PCR: waxeye Short range PCR and sequencing: fernbird, pipit and bellbird
Av15951CytbF	CCCTTCATCATCATTGGCCAA	Long range PCR: NZ robin Short range PCR and sequencing: waxeye
Av16137tProR	ARAATRCCAGCTTTGGGAGTTGG	Long range PCR: fernbird and waxeye Short range PCR and sequencing: fernbird, pipit, waxeye and NZ robin
Av16152tProF	CTCCAGCTCCCAAAGCTG	Short range PCR and sequencing: waxeye
Av16531ND6F	ACCACCARCATHCCCCCYAAATA	Short range PCR and sequencing: waxeye and NZ robin
Av16653ND6F	GGAGAAGGATTGGATGCCACTG	Short range PCR and sequencing: pipit
Av16728tGluR	GGYTTTTCAGGCCGTAGRTCTTGG	Short range PCR and sequencing: waxeye
Av16758tGluF	TRTGGCYTGAAAARCCRTCGTTG	Short range PCR and sequencing: waxeye, pipit and NZ robin
PipitND6F	AGCCGCTACCACCAACCCCA	Short range PCR and sequencing: pipit
PipitcontrolR	GCCTGGTGGGATGTCTGTCCG	Short range PCR and sequencing: pipit
AvtGluRobR	CTCCGGGATCCGTGGCCTGA	Short range PCR and sequencing: NZ robin
AvND6RobF	TTAAAAGCAGCCCCGCCCCC	Short range PCR and sequencing: NZ robin
WeND5F	ACCCGCCTAGCTCTAGGTAGCA	Short range PCR and sequencing: waxeye
WeCytBF	TCCACATCGGCCGAGGCTTT	Short range PCR and sequencing: waxeye
WeCytbR	AGCCTCGGCCGATGTGGAAGT	Short range PCR and sequencing: waxeye
WeCR1F	GCCCCCGCGCCTTTTACCTT	Short range PCR and sequencing: waxeye
WeCR1R	TCGTGTGACGCGTGTGTTGGG	Short range PCR and sequencing: waxeye
WeND6R	CGGTGTCTTTGGCGGCGGAT	Short range PCR and sequencing: waxeye

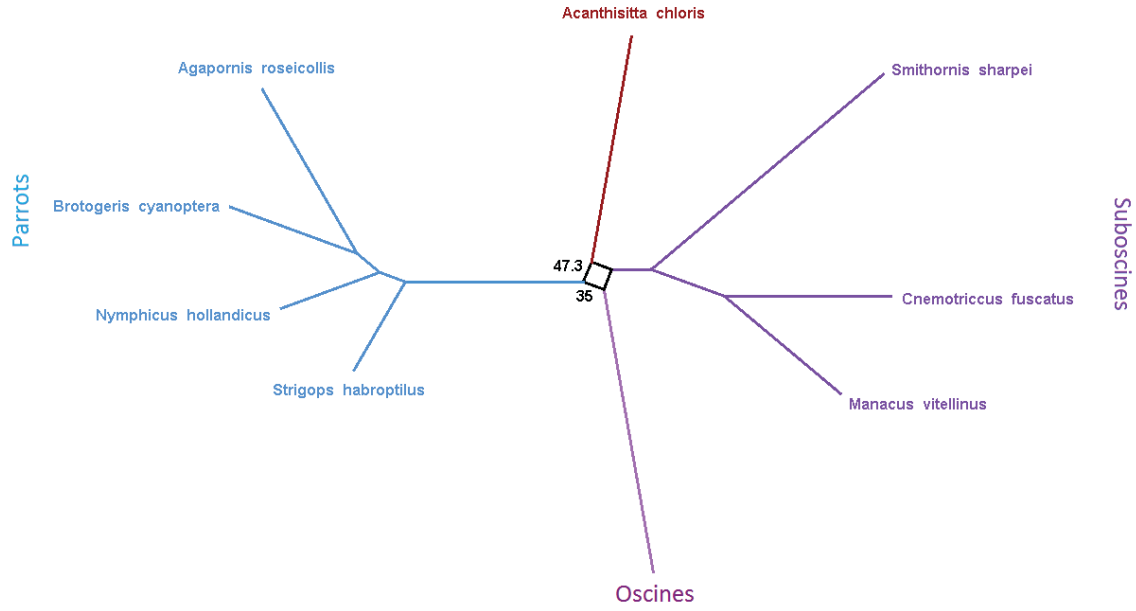
Supplementary Table 2: Estimation of DNA concentration using Nanodrop for the long range PCR products of the waxeye. After clean up using the Zymoclean™ Gel DNA Recovery Kit.

LR PCR product	DNA Conc. (ng/µl)
Av1753F12S-Av5201tmetR	26.5
Av4165nd1F-Av7662co1R	32.2
Av7318co1F-Av10116co3R	43.8
Av9942co3F-Av16137tproR	28.2
Av15671cytbF-Av2150R12S	32.4

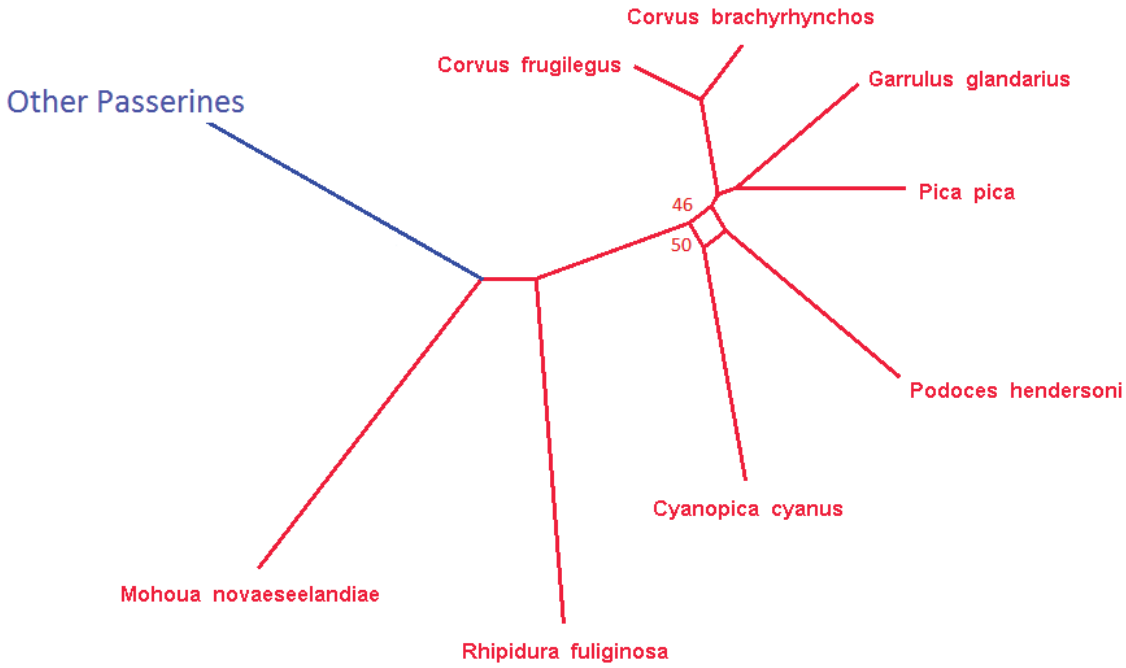
6.2 Supplementary figures



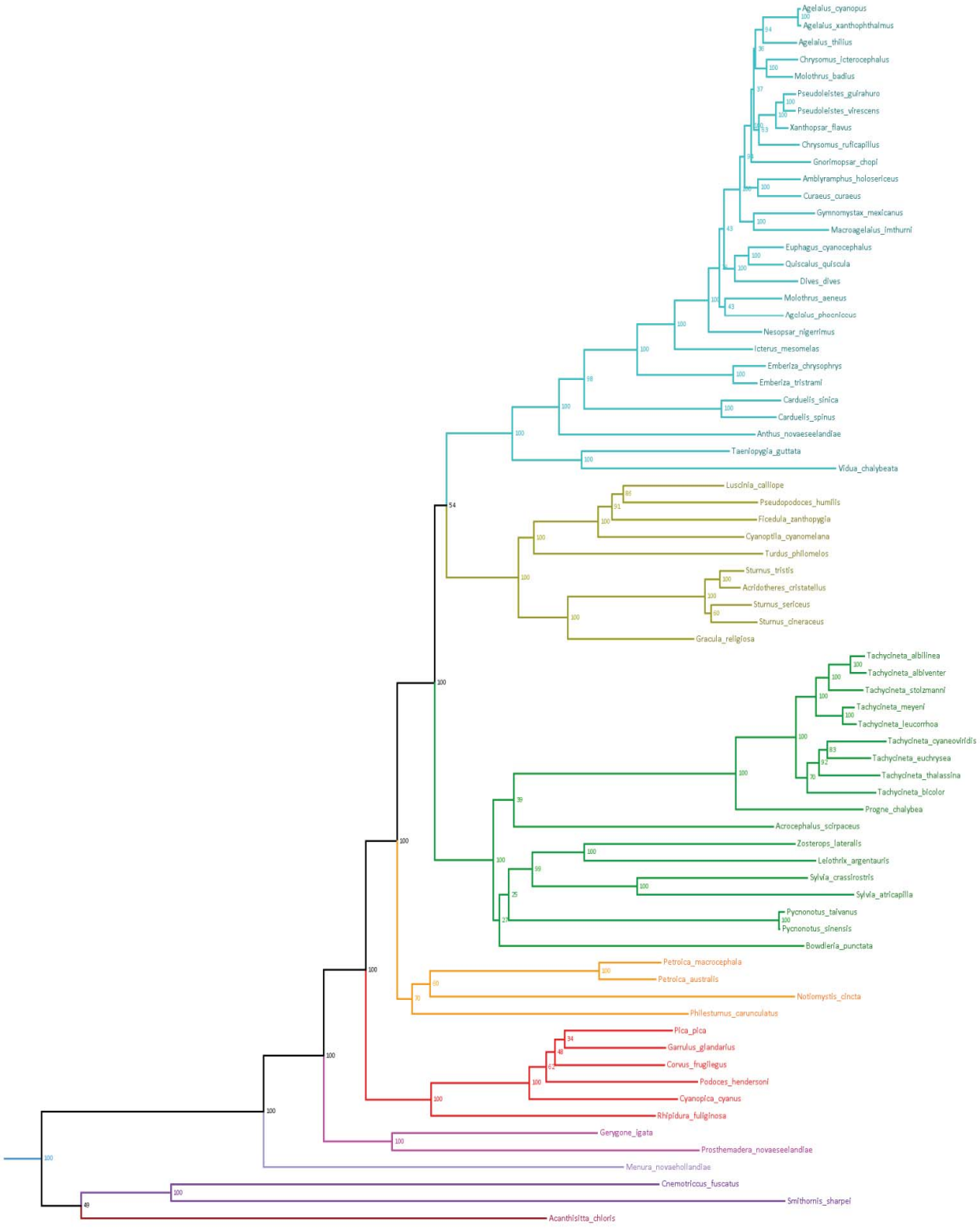
Supplementary Figure 1: Full maximum likelihood tree of 83 species. Includes the four parrot species as the out-group. Produced from maximum likelihood analysis on RaxML, using full mitochondrial genome data (13588bp). Posterior probability values show support for each node. Made in Fig Tree v1.40.



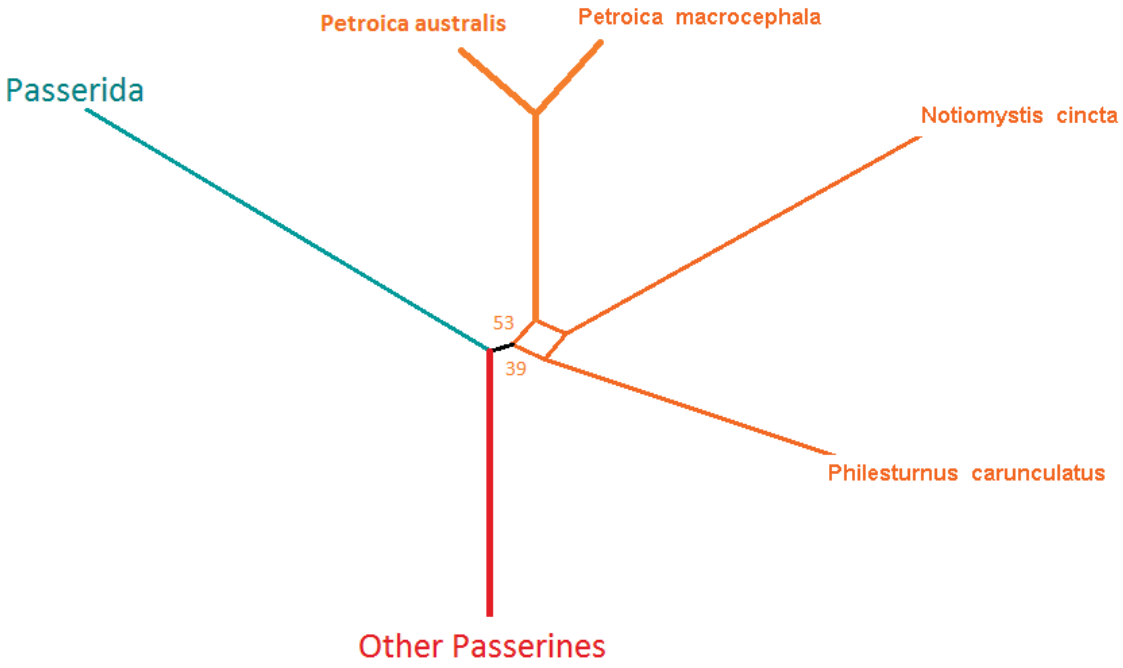
Supplementary Figure 2: Maximum likelihood consensus network showing split of the three suborders of the Passerines. Posterior probability threshold is set at 33%; all other nodes are fully supported (100%) and are not shown. Consensus network made in Splits Tree v4.11.3 using RaxML maximum likelihood data from the full species set (83 species); all oscines were condensed into a single branch.



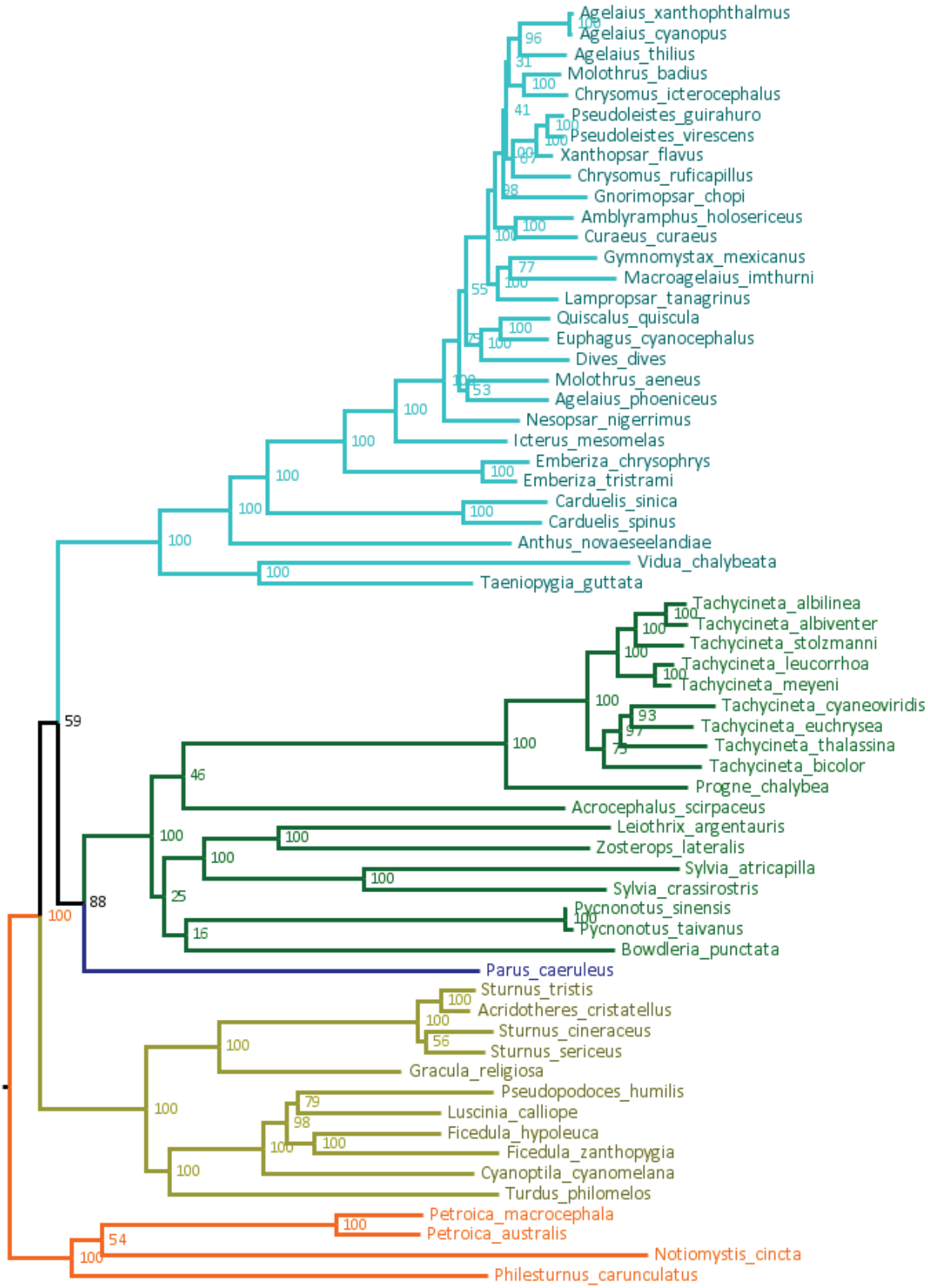
Supplementary Figure 3: Maximum likelihood consensus network of the Core Corvoidea. Posterior probability threshold is set at 33%. Consensus network made in Splits Tree v4.11.3 using RaxML maximum likelihood analysis data from the full species set (83 species), all other passerines were condensed into a single branch.



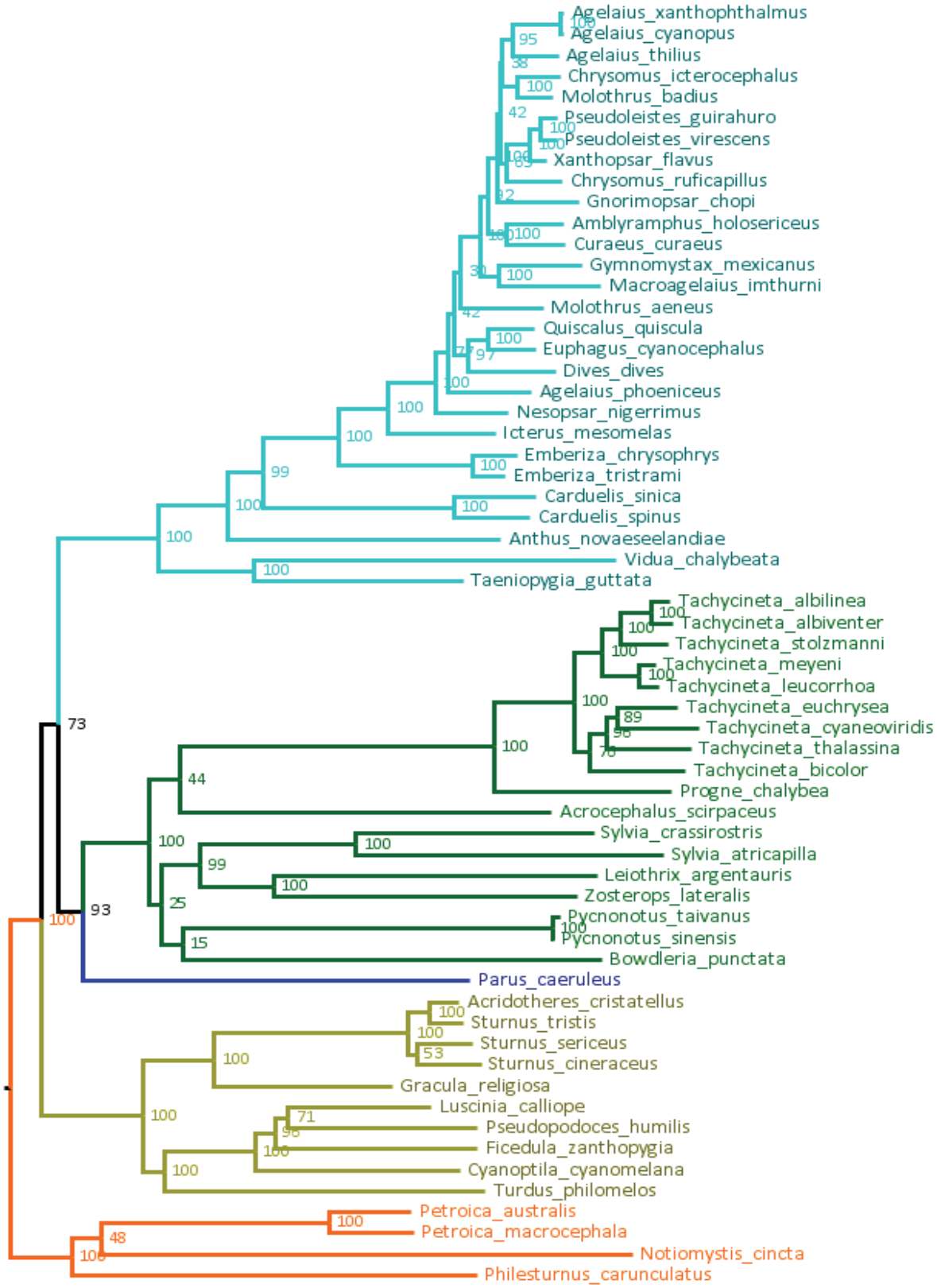
Supplementary Figure 4: Maximum likelihood phylogenetic tree from 76 passerine species. Original species set of 83 had all species removed which had uncalled bases. Produced from maximum likelihood analysis on RaxML, using full mitochondrial genome data (13588bp). Posterior probability values show support for each node. Made in Fig Tree v1.40.



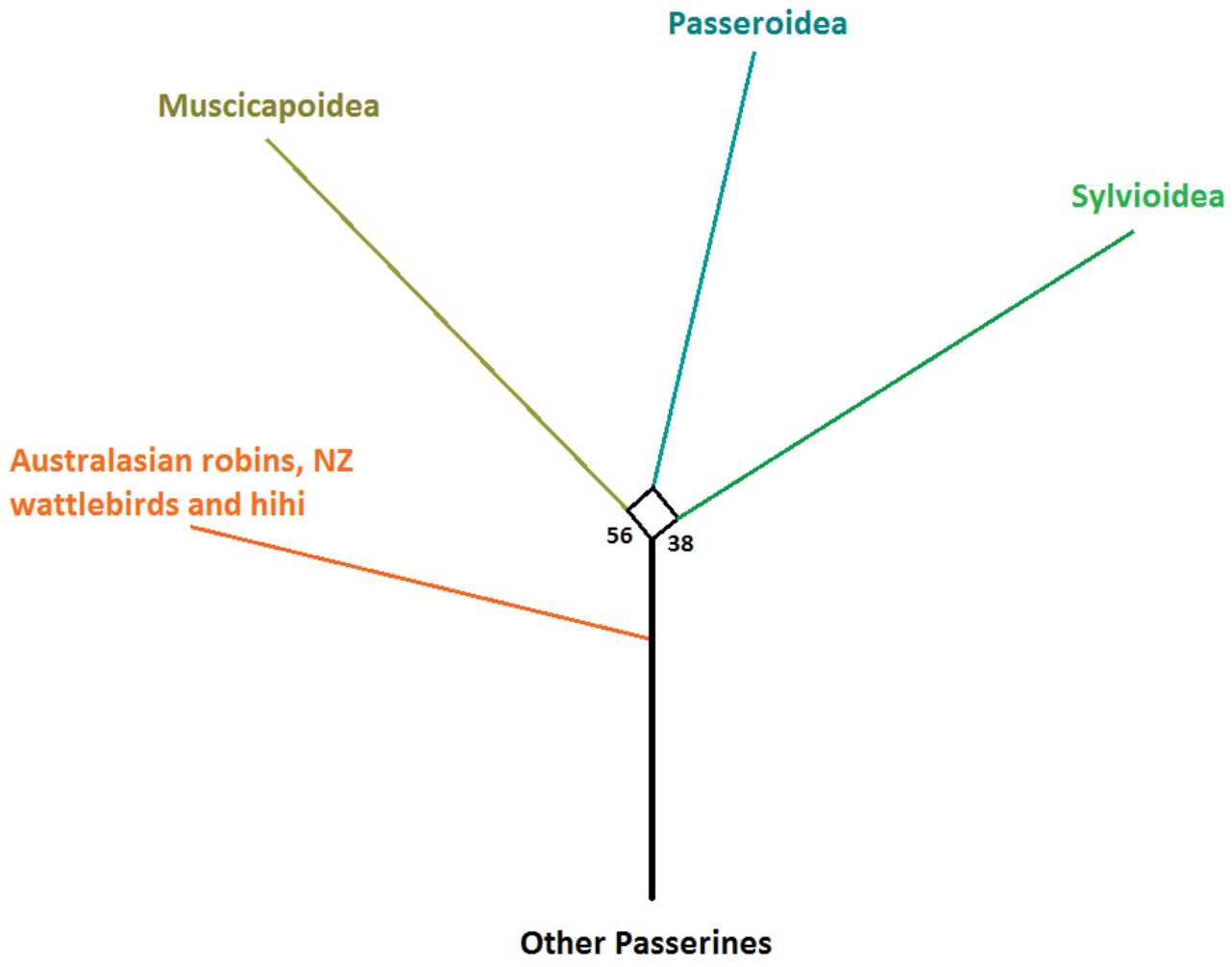
Supplementary Figure 5: Maximum likelihood consensus network showing split of the basal Passerida (Australasian robins, New Zealand wattlebirds and hihi) from the Passerida. Posterior probability threshold is set at 33%. Consensus network made in Splits Tree v4.11.3 using RaxML maximum likelihood analysis data from the full species set (83 species), all other passerines were condensed into a single branch.



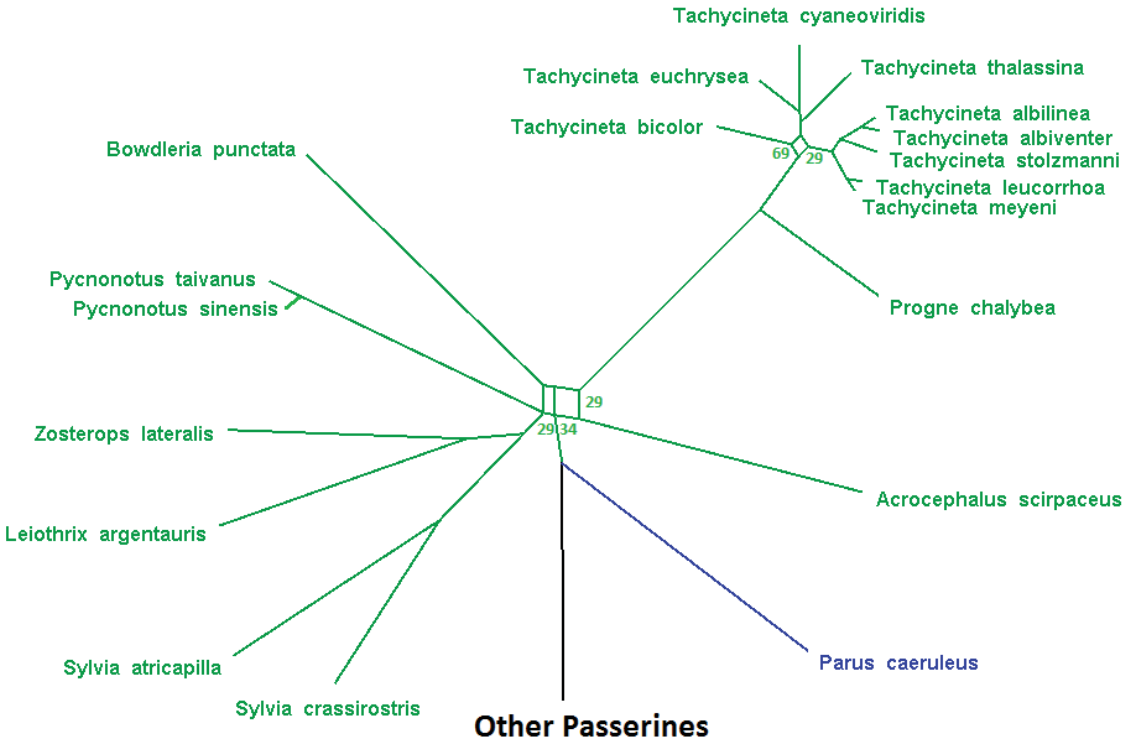
Supplementary Figure 6: Maximum likelihood phylogenetic tree from all 63 Passerida species (including basal Passerida). Produced from maximum likelihood analysis on RaxML, using full mitochondrial genome data (13588bp). Posterior probability values show support for each node. Made in Fig Tree v1.40.



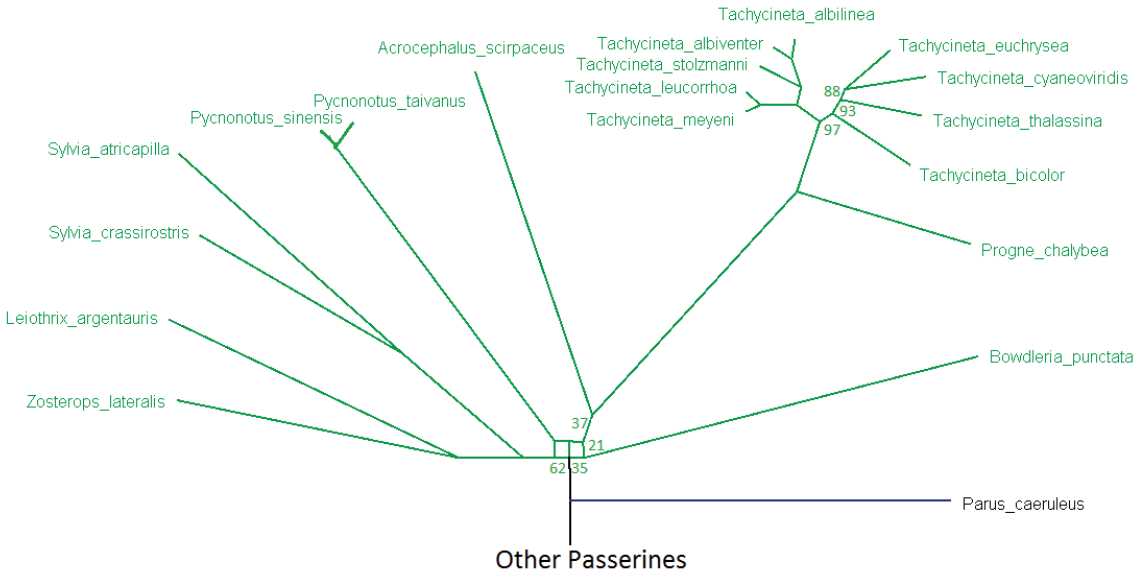
Supplementary Figure 7: Maximum likelihood phylogenetic tree from 61 Passerida species (including basal Passerida). Ficedula hypoleuca and Lampropsar tanagrinus were removed from data set as they had uncalled bases and had relatively close relatives in data set. Produced from maximum likelihood analysis on RaxML, using full mitochondrial genome data (13588bp). Posterior probability values show support for each node. Made in Fig Tree v1.40.



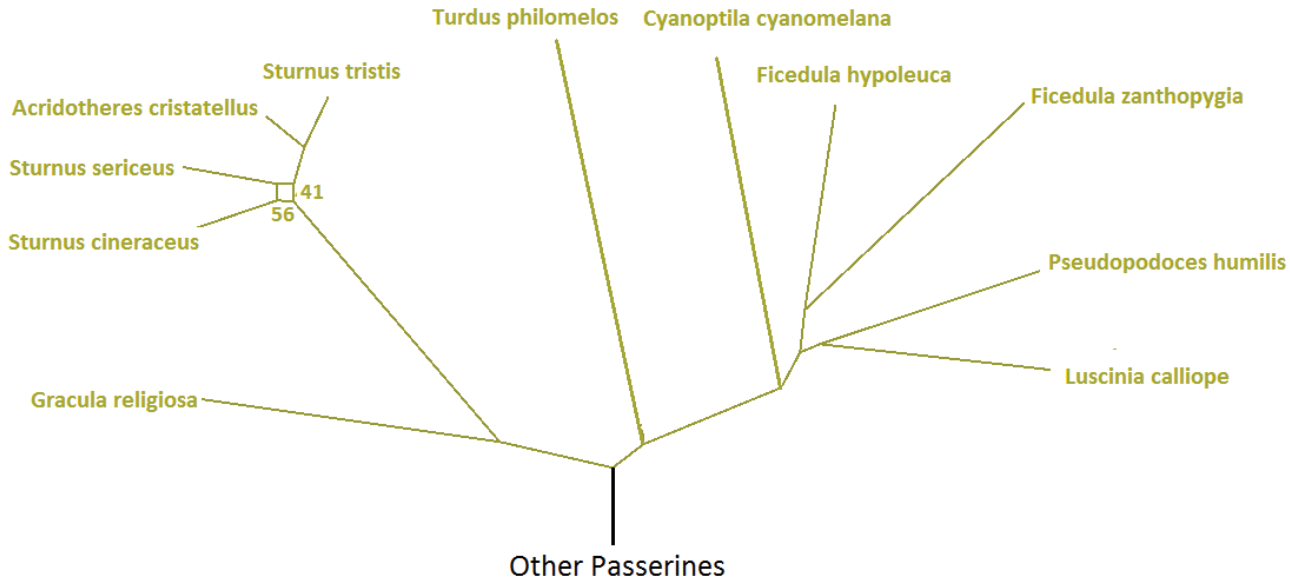
Supplementary Figure 8: Maximum likelihood consensus network of the three superfamilies of the Passerida. Posterior probability threshold is set at 33%. Consensus network made in Splits Tree v4.11.3 using RaxML maximum likelihood analysis data from the full species set (83 species). The species in each group have been condensed into a single branch. Australasian robins, NZ wattlebirds and hihi make up the basal Passerida.



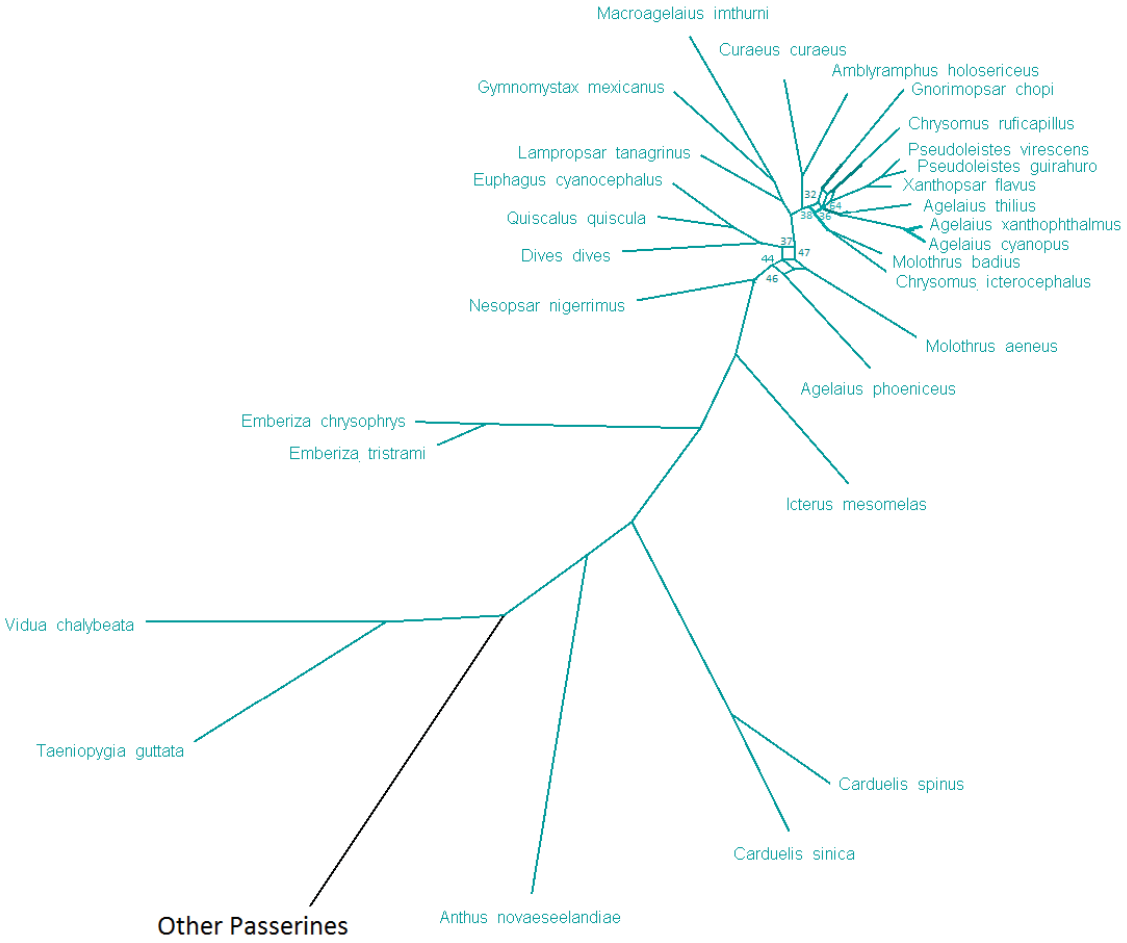
Supplementary Figure 9: Maximum likelihood consensus network of the superfamily Sylvioidea. Posterior probability threshold is set at 28%. Consensus network made in Splits Tree v4.11.3 using RaxML maximum likelihood analysis data from the full species set (83 species), all other passerines were condensed into a single branch.



Supplementary Figure 10: Maximum likelihood consensus network of the superfamily Sylvioidea. Posterior probability threshold is set at 20 Consensus network made in Splits Tree v4.11.3 using RaxML maximum likelihood analysis data from the 19 Sylvioidea species only (83 species).



Supplementary Figure 11: Maximum likelihood consensus network of the superfamily Muscicapoidea. Posterior probability threshold is set at 30%. Made on Splits Tree v4.11.3 using RaxML maximum likelihood analysis data from the full species set (83 species), all other passerines were condensed into a single branch.



Supplementary Figure 12: Maximum likelihood consensus network of the superfamily Passeroidea. Posterior probability threshold is set at 30%. Made on Splits Tree v4.11.3 using RaxML maximum likelihood analysis data from the full species set (83 species), all other passerines were condensed into a single branch.