Short Communication

Nuclear introns help unravel the diversification history of the Australo-Pacific Petroica robins

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ABSTRACT
Australo-Pacific Petroica robins are known for their striking variability in sexual plumage coloration. Molecular studies in recent years have revised the taxonomy of species and subspecies boundaries across the southwest Pacific and New Guinea. However, these studies have not been able to resolve phylogenetic relationships within Petroica owing to limited sampling of the nuclear genome. Here, we sequence five nuclear introns across all species for which fresh tissue was available. Nuclear loci offer support for major geographic lineages that were first inferred from mtDNA. We find almost no shared nuclear alleles between currently recognized species within the New Zealand and Australian lineages, whereas the Pacific robin radiation has many shared alleles. Multlocus coalescent species trees based on nuclear loci support a sister relationship between the Australian lineage and the Pacific robin radiation—a node that is poorly supported by mtDNA. We also find discordance in support for a sister relationship between the similarly plumaged Rose Robin (P. rosea) and Pink Robin (P. rodinogaster). Our nuclear data complement previous mtDNA studies in suggesting that the phenotypically cryptic eastern and western populations of Australia’s Scarlet Robin (P. boodang) are genetically distinct lineages at the early stages of divergence and speciation.

1. Introduction

Petroica red-robins (Petroicidae) have colonized most major landmasses in the Australo-Pacific region. Five species are present on mainland Australia, two species are restricted to the high-elevation mountains of New Guinea, two species have radiated across the southwest Pacific archipelagos and four species are found on New Zealand’s large North and South Islands and several smaller off-shore islands (Boles, 2007). The origins of the diversity of sexual plumage coloration displayed in Petroica has long intrigued biologists and was central to the development of ideas about the role of geography in speciation and the development of the Biological Species Concept (Mayr, 1934, 1942). Species with striking sexual dichromatism in carotenoid plumage dominate the phylogeny, however, many species and subspecies (especially those that occur on islands) have sexually monochromatic plumage (Boles, 2007). Of particular note is the Pacific robin radiation (P. multicolor, P. pusilla (Vanuatu/Fiji/Samoa), P. pusilla (Solomon Islands), P. goodenovii), which displays a complex geographic mosaic of sexually dichromatic, elaborate monochromatic and dull monochromatic taxa across its range (Miller and Lambert, 2006; Lounes et al., 2009; Christidis et al., 2011; Kearns et al., 2015, 2016, 2018). Critically, attempts to resolve the speciation history of Petroica and understand the origins of the diversity in sexual plumage coloration have long been hindered by uncertain taxonomic boundaries and a lack of a robust phylogenetic hypothesis for Petroica (Miller and Lambert, 2006; Lounes et al., 2009; Christidis et al., 2011; Kearns et al., 2016, 2018, submitted for publication).

Overall, the mtDNA and nuclear datasets examined to date offer the most support for the recognition of four key lineages in Petroica—New Zealand (four species), Australia (four species), New Guinea (two species) and Pacific/Australia (the “Pacific robin radiation” with four species) (see Fig. 2 for details of species and geographic ranges) (Christidis et al., 2011; Kearns et al., 2016, 2018). However, there is no strong consensus on the relationships among these four lineages owing...
to weak support and discordance across datasets at the key nodes uniting the major lineages (Miller and Lambert, 2006; Loynes et al., 2009; Christidis et al., 2011; Kearns et al., 2016, 2018, submitted for publication). There is also mixed support for a sister relationship between the phenotypically similar Rose Robin (P. rosea) and Pink Robin (P. rodingoaster). Finally, there are uncertain relationships among species within the Pacific robin radiation (see Kearns et al., 2016, 2018, submitted for publication) and among the four species from New Zealand (Miller and Lambert, 2006). Most surprising, recent molecular studies have shown that the phenotypically divergent Red-capped Robin (P. goodenovii) from Australia is a member of the Pacific robin radiation despite morphology-based taxonomy long treating the Pacific robins and Australia’s Scarlet Robin (P. boodang) as a single species (P. multicolor sens. lat. Gmelin, 1789) on the basis of their similar plumage (Mayr, 1934; Schodde and Mason, 1999; Kearns et al., 2016) (see Fig. 2). Furthermore, the Pacific robin radiation is comprised of three or four distinct species (P. multicolor from Norfolk Island, P. goodenovii from Australia, and P. pusilla, which itself might be divided into two species—one from Vanuatu, Fiji and Samoa (the name P. pusilla Peale, 1848 has priority), and another from the Solomon Islands (the name P. polynympha Mayr, 1934 has priority) (Kearns et al., 2016, submitted for publication). Resolving the phylogenetic relationships of Petroica is a priority, as the current uncertainty hinders our ability to test hypotheses about the biogeographic history and plumage evolution in this genus (Miller and Lambert, 2006; Loynes et al., 2009; Christidis et al., 2011; Kearns et al., 2016, 2018, submitted for publication).

Much of the conflict and uncertainty across datasets examined to date stems from limited nuclear species/lineage sampling across Petroica. For example, Loynes et al. (2009) used mtDNA (ND2) and three nuclear loci (c-myc and introns BA20454 and BA23989) to estimate relationships across the Petroiidae, but only the five species of Petroica in Australia were sampled. Christidis et al. (2011) and Kearns et al. (2016) used more comprehensive species sampling of mtDNA (CO1 and ND2) and a few nuclear loci (ACO1 and CLOCK, Kearns et al., 2016; beta-fibrinogen intron 5, Christidis et al., 2011), however, only one species from New Zealand was included for each locus in both studies. The only two studies to sample all four New Zealand species thus far have sampled a single mtDNA locus (cytochrome h, Miller and Lambert, 2006; ND2, Kearns et al., 2018). Neither study found strong support for the reciprocal monophyly of all four New Zealand species (Miller and Lambert, 2006; Kearns et al., 2018). Finally, the most comprehensive sampling of nuclear loci thus far focused exclusively on species boundaries and relationships within the Pacific robin radiation (only endangered P. multicolor was omitted owing to a lack of fresh tissues) using five nuclear loci and a multilocus coalescent approach (Kearns et al., submitted for publication). The resulting nuclear species tree offered strong support for key nodes within the Pacific robin radiation that were poorly resolved in previous mtDNA only phylogenies (Kearns et al., submitted for publication). Critically, Kearns et al. (submitted for publication) focused solely on relationships among the Pacific robins, and did not include any other members of the Australo-Pacific robins. Thus, inferences about species boundaries and phylogenetic relationships of Petroica that so far have been based on morphology, mtDNA and sparse nuclear sampling still require testing with a nuclear dataset with dense locus and taxon sampling.

Here we sequence five nuclear loci across all Australo-Pacific robin species for which fresh tissue are available—resulting in eleven of fourteen species of Petroica sampled and 248 new sequences generated. We explore nuclear support for mtDNA- and morphology-based species boundaries, and use a multilocus coalescent approach to infer the first nuclear species tree for the genus. Our study represents the most comprehensively sampled nuclear phylogeny to date for Petroica, and offers support for some uncertain relationships that have long hindered inferences about the speciation history and origins of variable sexual plumage coloration in Petroica.

2. Methods

2.1. Taxon sampling

We follow the current taxonomy supported by the IOC (IOC World Bird List version 8.1, updated 25 January 2018), which accepts the recent split of Norfolk Robin (P. multicolor) from the rest of the robin populations in the southwest Pacific (currently P. pusilla) (Kearns et al., 2016), and splits North Island Robin (P. longipes) and South Island Robin (P. australis) (Miller and Lambert, 2006; Kearns et al., 2018). Additionally, we divide samples of the southwest Pacific robin P. pusilla by its Solomon Islands and Vanuatu/Fiji/Samoa lineages, which have been argued to represent two distinct species (Kearns et al., submitted for publication). We also divide samples of the Scarlet Robin (P. boodang) by its western (subspecies: campbelli) and eastern (subspecies: leggii and boodang) range to test the suggestion based on mtDNA ND2 that these disjunct populations could represent two phenotypically cryptic but genetically distinct species (Dolman and Joseph, 2012). In both cases we evaluate species distinctiveness under the diagnostic criteria of the Evolutionary, Phylogenetic and General Lineage Species Concepts (reviewed in Wheeler and Meier (2000); de Queiroz (2005); Gill (2014); see also Tobias et al., 2010), as well as the Biological Species Concept under the alternative null hypothesis of Gill (2014).

We sampled at least three individuals from each species for which fresh tissue was available and aimed for a broad geographic sample including multiple subspecies where possible. We were unable to obtain fresh tissue from three species (P. multicolor, P. archboldi, P. bivittata) owing to their rarity and geographic remoteness, however, all were sampled for mtDNA ND2 in the previous study of Kearns et al. (2018). Our final nuclear sampling consisted of eleven of fourteen species of Petroica—14 samples were from the New Zealand lineage (australis n = 3; longipes n = 4; traversi n = 4; macrocephala n = 3), 33 samples were from the Australian lineage (rodingoaster n = 9; rosea n = 5; phoenicea n = 4; boodang (EAST) n = 10; boodang (WEST) n = 5), and 23 were samples from the Pacific/Australian lineage (pusilla (Solomon Islands) n = 7; pusilla (Vanuatu/Fiji/Samoa) n = 7; goodenovii n = 9) (see Appendix 1 for details). We also included two samples from Eurygomea rubra, five samples from Amalocichila incerta, and five samples from Pachycephalopsis poliosoma to act as outgroups in all phylogenetic analyses following Christidis et al. (2011).

2.2. DNA amplification and sequencing

We sequenced five nuclear introns—two from the Z (sex) chromosome (ACO1, BRM) and three from autosomal chromosomes (CLOCK, GAPDH, PCBD). DNA extraction, amplification and sequencing following the protocol described in Kearns et al. (2016) using primers and annealing temperatures from previous studies (Borge et al., 2005; Kimball et al., 2009). In total, we collected the following new nuclear sequences for this study: ACO1 n = 42, BRM n = 54, CLOCK n = 45, GAPDH n = 56, PCBD n = 51 (see Appendix 1 for sample details). Additionally, we obtained from GenBank 27 individuals from eight species sequenced for ACO1 and CLOCK (2 macrocephala, 4 boodang, 1 phoenicea, 2 rosea, 2 rodingoaster, 5 goodenovii, 7 pusilla Solomon Islands/Bougainville lineage, 7 pusilla Vanuatu/Fiji/Samoa lineage; GenBank accessions: KT372722-KT372779; Kearns et al., 2016), and 23 individuals from the Pacific/Australia lineage sequenced for BRM, GAPDH and PCBD (GenBank accessions: BRM: MK121750-MK121772; GAPDH: MK127556-MK127575; PCBD: MK127576-MK127598; Kearns et al., submitted for publication) (see Appendix 1 for sample details). Owing to differences in DNA and sequence quality not all individuals were included in analyses for each nuclear locus (maximum missing per locus = 8 out of 70 sampled Petroica individuals; maximum missing per individual = 2 out of 5 introns; see Appendix 1 for details of per locus sampling). In order to augment the sparse species sampling previously presented for mtDNA COI (Christidis et al., 2011; Kearns et al., 2016),
we sequenced COI for an additional 50 individuals following the protocol presented in Kearns et al. (2016) and included an additional 21 individual sequences obtained from GenBank. Our COI dataset sampled the same eleven species of Petroica as the nuclear datasets (see Appendix 1 for sample details). All new mtDNA (COI) and nuclear intron (ACO1, BRM, CLOCK, GAPDH, PCBD) sequences were deposited in GenBank (accessions: COI: MH258001–MH258073; ACO1: MK127599–MK127640; BRM: MK127641–MK127694; GAPDH: MK127695–MK127750; PCBD: MK127751–MK127801; CLOCK: MK127802–MK127846).

The genotype of heterozygous individuals for each nuclear intron was determined using the ‘subtraction method’ (Dolman and Moritz, 2006) for individuals with length polymorphisms and the program PHASE v2.1 (Stephens and Donnelly, 2003) for those without length polymorphisms. Phased alleles inferred from individuals with length polymorphism were used as alleles of ‘known phase’ in PHASE, which was run under the following settings—5 independent runs, final run repeated 10 times. When the genotype of heterozygous individuals was uncertain (< 70% probability threshold) we coded the uncertain heterozygous sites with the appropriate IUPAC ambiguity code in order to reduce the amount of missing data. Indels in nuclear introns were treated as missing data in all analyses. We tested for signals of recombination using the difference of sums-of-squares method implemented (sliding window: 100 bp, step size: 10 bp) in TOPALi v1 (Milne et al., 2004). No significant signals of recombination were detected.

2.3. Single locus phylogenetic analyses

mtDNA—A complete species-level phylogeny was recently published for Petroica based on the single ND2 mtDNA locus (Kearns et al., 2018). To better explore previous observations of possible discordance between mtDNA ND2 and COI we expanded species sampling for the mtDNA “barcode” COI and estimated a phylogeny using RAxML (Stamatakis, 2006) and MrBayes (Huelsenbeck and Ronquist, 2001). RAxML was run using a GTRGAMMA model, 1000 bootstrap iterations and using codons as partitions. MrBayes was run twice for 3 × 10^9 generations, sampling every 100,000 generations and using the most appropriate substitution models for each codon partition as determined by PartitionFinder v2 (Lanfear et al., 2017)—codon position 1: GTR + I + G, codon position 2: HKY + I, codon position 3: GTR + G. We ensured good mixing and stationarity for both runs and excluded a 25% burnin before calculating a consensus tree from both runs.

Nuclear introns—Initial tests using RAxML showed that nuclear loci in Petroica are not tree-like owing most likely to slow mutation rates, recent divergence and incomplete lineage sorting. Thus, standard phylogenetic approaches using single locus gene trees and concatenation are not appropriate for Petroica, and instead unrooted networks and species trees (see below) are the more appropriate way to explore our datasets. We estimated unrooted allele networks in PopART (Leigh and Bryant, 2015) for each nuclear locus using the TCS method (Clement et al., 2000) calculated using default settings.

2.4. Multilocus species tree analyses

We performed multilocus species tree analyses using `BEAST (Heled and Drummond, 2010) implemented in BEAST v2.4.8 (Bouckaert et al., 2014) on two datasets—one using phased alleles from all five nuclear loci (“nuclear only”), and the other combining all five nuclear loci with the two mtDNA genes ND2 and COI (“mtDNA + nuclear”). For both, we used species boundaries as our a priori taxonomic boundaries in `BEAST following confirmation of the distinctiveness of each currently recognized species based on limited sharing of alleles in each nuclear network (see Fig. 1). We also treated the putatively distinct Solomon Islands and Vanuatu/Fiji/Samoa lineages of P. pusilla (Kearns et al., submitted for publication), and western and eastern lineages of P. boodang (Dolman and Joseph, 2012) as separate a priori “species” in `BEAST analyses. Initial trials confirmed that a strict clock was most appropriate for all loci in both datasets (the uclst.stdev parameter and coefficient of variation both overlapped with zero; Drummond et al., 2007). We applied a HKY + I + G substitution model to all loci with empirical base frequencies and estimated values for kappa, gamma, shape and proportion of invariants. For the nuclear only dataset, we ran two independent runs of 1 × 10^8 generations with samples taken every 5000 generations using a Yule speciation prior, a lognormal prior on birthrate (M = 4.0, S = 1.25) and population mean (M = 5.0, S = 1.2) and a strict clock on all introns applying an exponential prior on the clock rate parameter. For the combined mtDNA and nuclear dataset, we estimated divergence times using a strict clock with a lognormal prior reflecting the mean substitution rates estimated by Lerner et al. (2011) for ND2
Among a polytomy of \( P. \) pusilla \( P. \) boodang \( mtDNA \) in \( mtDNA \) and nuclear loci suggest that western and eastern \( P. \) boodang haplotypes from two other lineages that appear to contain distinctive, reciprocally could also arise due to incomplete lineage sorting and/or from rooting flow (Omland et al., 2006). The lack of reciprocal monophyly of wes- originate from incomplete lineages sorting of ancestral alleles than the gene tree places eastern and western \( P. \) boodang \( ND2 \) weakly supported a sister relationship between the Australian and New Zealand lineages (pp/Maximum likelihood bootstrap (bs) = 0.87/ 55) (Kearns et al., 2018). Our combined \( mtDNA \) and nuclear species tree reflects this uncertainty in the phylogenetic relationships and sequence of divergence of the Australia, Pacific/Australia and New Zealand lineages (Fig. 2). The MCC species tree shows a sister relationship between the Pacific/Australia lineages and the Australia lineage, however, this relationships does not receive support (pp = 0.54; Fig. 2). In contrast with \( mtDNA \) CO1 and \( ND2 \) single locus phylogenies, the nuclear only species tree and the combined nuclear and \( mtDNA \) species tree both strongly supported the monophyly of the New Zealand lineage (Figs. 1 and 2). Notably, the same paraplethric relationship for the New Zealand lineage observed in \( mtDNA \) CO1 (Supplementary Fig. 1) was also re- covered in previous maximum likelihood (but not parsimony) analyses of \( mtDNA \) cyt \( \text{c} \) (Miller and Lambert, 2006).

In contrast with single locus \( mtDNA \) phylogenies (Supplementary Fig. 1; Kearns et al., 2016, 2018), both the nuclear only and combined \( mtDNA \) and nuclear species trees offer strong support for a sister relationship between southwest Pacific \( P. \) pusilla lineages from the Solom- on Islands and from Vanuatu/Fiji/Samoa (see Kearns et al., sub- mitted for publication for further discussion and analyses), as well as between \( P. \) traversi and \( P. \) macrocephala (Figs. 1 and 2). However, nei- ther species tree offered strong support for the placement of Australia’s \( P. \) rosea and \( P. \) rodinogaster. Our combined \( mtDNA \) and nuclear species tree places \( P. \) rosea and \( P. \) rodinogaster as sisters, but with such poor support (pp = 0.55) that it should be considered a polytomy (Fig. 2). The nuclear species tree places \( P. \) rodinogaster sister to the rest of the Australian species, but again with no/weak support (pp = 0.5; Fig. 1). \( MtDNA \) ND2 supports a sister relationship (pp(bs) = 0.93/81; Kearns et al., 2018), while \( mtDNA \) CO1 supports \( P. \) rosea sister to the rest of the Australian species (pp(bs) = 0.99/71; Supplementary Fig. 1; also see Kearns et al., 2016). The uncertainty present in the nuclear species tree likely stems from the variable position of \( P. \) rosea and \( P. \) rodinogaster with respect to the other species in the Australian lineage in each nuclear locus (Fig. 2). The top 95% of sampled nuclear species trees had three frequent topologies (rodinogaster (rosea/phiocenia + boodang)) (i.e., topology in Fig. 1a) was present in 37% of sampled species trees, (rodinogaster + rosea)/phiocenia + boodang) was present in 26% of sampled species trees, and (rosea/rodinogaster/phiocenia + boodang) was present in 14% of sampled species trees. Denser sampling of the nuclear genome is clearly necessary to resolve the phylogenetic placement of \( P. \) rosea and \( P. \) rodinogaster, and to more fully infer the speciation history of the Australian lineage. Our data currently suggests that the species in the Australian lineage may have diverged from each other in rapid succession leading to differential retention and sorting of ancestral alleles across the nuclear genome (Fig. 2).
3.3. Origins of sexual dichromatism and history of diversification

The origins of sexual dichromatism and history of diversification of *Petroica* have been extensively discussed elsewhere (Miller and Lambert, 2006; Loynes et al., 2009; Christidis et al., 2011; Kearns et al., 2015, 2016, 2018, submitted for publication). Here we discuss new insights from our multilocus coalescent analysis of nuclear loci (Figs. 1 and 2), which offers the most comprehensive phylogenetic hypothesis for *Petroica* produced to date. Unlike phylogenies based on mtDNA alone, nuclear loci offer most support for a sister relationship between the Australian and Pacific/Australian lineages to the exclusion of the New Zealand lineage. This relationship fits with patterns of plumage coloration in *Petroica* since both the Australian and Pacific/Australian lineages are dominated by forms with similar patterns of sexual dichromatism wherein females are mostly brown and males have bold carotenoid (red/pink) breast plumage and black backs and heads (Fig. 2). In contrast, most members of the New Zealand lineage are either monochromatic (*P. traversi*) or have subtle sexual dichromatism (*P. australis* and *P. longipes*). If the Australian and Pacific/Australian lineages are sisters, that suggests that marked sexual dichromatism could have evolved in the ancestor of the Australian and Pacific/Australian lineages. Conversely, if the Australian and New Zealand lineages are sisters (as per the relationship supported by mtDNA ND2), that would suggest that marked sexual dichromatism either evolved independently in both Australian and Pacific/Australian lineages, or it was present in the ancestor of all of *Petroica* and was subsequently secondarily lost in the New Zealand lineage. Notably, while *P. australis* and *P. longipes* have markedly different plumage compared to the rest of *Petroica*, males of *P. macrocephala* have plumage that

Fig. 2. Phylogenetic relationships and timing of diversification of the Australo-Pacific *Petroica* robins based on combined coalescent analyses of two mtDNA loci and five nuclear introns. Ninety-five percent highest posterior densities (HPD) for divergence time estimates are indicated by grey bars at each node. HPD intervals are also given in parentheses below the branch at each major node. Posterior probabilities are shown above the branch at each node. *Petroica multicolor*, *P. bivittata* and *P. archboldi* were not sampled for the nuclear and mtDNA COI datasets and thus were not included in *BEAST species tree analyses, however, their putative phylogenetic position based on mtDNA ND2 is indicated by grey dashed lines. See Kearns et al. (2018) (ND2) and Supplementary Fig. 1 (COI) for single locus phylogenies for the mtDNA loci. Bird illustrations show the male (left) and female (right) plumage of each species. Three species (*P. macrocephala*, *P. pusilla* (Solomon Islands), and *P. pusilla* (Vanuatu/Fiji/Samoa)) have multiple plumage morphs across their range. The geographic range of each species of *Petroica* is re-drawn from Boles (2007).
is similar to many species in Australia and the Pacific (having males with carotenoid breast plumage and black heads and backs). Thus, even if the Australian and Pacific/Australian lineages are sisters, the plumage of *P. macrocephala* argues that the ancestor of all Petroica likely had already marked sexual dichromatism and carotenoid breast plumage.

Sampling the nuclear genomes of *P. bivittata* and *P. archboldi* from New Guinea is now the priority for future work on Petroica. MtDNA places both species sister to the Pacific/Australian lineage (pp/b = 1.0/94) (Kearns et al., 2018), however, it is uncertain whether this relationship would be supported by nuclear loci given the discordance between mtDNA and the nuclear species tree observed at similarly deep nodes in the phylogeny (Fig. 1). Furthermore, ND2 only weakly supported a sister relationship between the two species (pp/b = 0.73/67) (Kearns et al., 2018). Both species, but especially *P. archboldi*, have highly distinctive plumage for Petroica (Boles, 2007). Thus, any inferences about the evolution of sexual plumage coloration in this group are limited without nuclear sampling for these two species. Our biogeographic inferences about the colonization history of Petroica are also limited by the current nuclear sampling given the important role that New Guinea plays as a ‘stepping-stone’ in the upstream (island to mainland) colonization histories of species across the Australo-Pacific region (Filardi and Moyle, 2005; Schweizer et al., 2011). A next-generation target capture approach tailored for acquiring dense nuclear datasets from degraded historical (“ancient”) museum specimens could offer the best solution for future work (Bi et al., 2013).

### 3.4. Conclusions

The *Petroica* robins contain a diverse array of charismatic, (mostly) brightly plumaged, species that have colonized most major landmasses in the Australo-Pacific region. Despite their notoriety and conspicuousness, details of their speciation history, routes of colonization and drivers of striking variation in sexual plumage coloration remained understudied for a long time. Here we complement recent mtDNA-only studies that used ancient DNA approaches and historical museum specimens to sample mtDNA from some of the most poorly known species of *Petroica* (Kearns et al., 2015, 2016, 2018) with a well-resolved nuclear-based phylogenetic hypothesis for the Australian, New Zealand and Pacific/Australian lineages of *Petroica*. This phylogeny can be used as a framework for exploring long-standing hypotheses about the evolution of carotenoid plumage coloration, sexual dichromatism and the role of islands as a natural laboratory for studying the role of islands in speciation (Mayr, 1942).

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