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Uncovering cryptic evolutionary diversity in extant and extinct populations of the southern Australian arid zone Western and Thick-billed Grasswrens (Passeriformes: Maluridae: *Amytornis*).

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Abstract

The Western and Thick-billed Grasswrens (Aves: Passeriformes: Maluridae:*Amytornis textilis* and *A. modestus*, respectively) exemplify issues surrounding the evolution, biogeography and conservation of Australia's arid and semi-arid zone fauna. The two species together have historically occurred across much of southern Australia. They showed high intraspecific taxonomic diversity and short range endemism but suffered high rates of recent anthropogenic extinction. Of 11 named and one un-named subspecies, five are extinct and three are vulnerable or critically endangered. To clarify taxonomic issues, and to understand their pre-extinction phylogeography and identify extant populations and taxa of conservation value, we sequenced ~1000 bp of the mtDNA ND2 gene from all extant populations and all but one extinct population. We confirmed reciprocal monophyly of *A. modestus* and *A. textilis* and identified strong phylogeographic structure associated with morphological divergence within each species. Populations of *A. t. myall* at the western edge of their range in South Australia may preserve “ghost” lineages of extinct subspecies from Western Australia as a result of ancient gene flow. Our results support recent taxonomic revisions, and highlight the critical importance of including samples of extirpated populations and extinct species to fully understand and interpret extant diversity. Conservation and management

plans should recognise and seek to preserve the unique evolutionary diversity present in surviving populations.

Introduction

Interest in how DNA sequence data aid in understanding evolutionary history and conservation of southern Australia's arid and semi-arid zones has been growing. Research within individual bird and mammal species across their geographical ranges has provided evidence of whether patterns of spatial structure in genetic and morphological diversity are concordant; further, it has addressed the long-term environmental drivers that may have shaped concordance or discordance in these patterns (Joseph *et al.* 2006; Donnellan *et al.* 2009; Kearns *et al.* 2009; Guay *et al.* 2010; Toon *et al.* 2010, 2012; Dolman and Joseph 2012; Neaves *et al.* 2009, 2012). Conservation of the southern Australian biota can only be enhanced if patterns of spatial structure in genetic and morphological diversity and their drivers are clearly understood.

Byrne *et al.* (2008) reviewed the evolution of the Australian arid zone biota in general and framed hypotheses and questions still useful in guiding further study. An example is whether phylogeographic structure is inversely proportional to body size and vagility. That is, small species having low vagility and modest spatial requirements (e.g. many plants, insects, reptiles, some terrestrial birds) are expected to show marked structure; highly mobile larger species capable of maintaining genetic connectivity across habitat patches (e.g., kangaroos, many birds) should have less marked structure. Comparative studies across multiple species also demonstrate the potential for sharpening our understanding of when particular events occurred, especially in the Pleistocene, to shape patterns of morphological and genetic diversity (Dolman and Joseph 2012). Critical here has been the role of Pleistocene glacial cycles, which are thought to have peaked in severity at the Last Glacial Maximum (~20,000 years ago) (reviewed in Byrne *et al*. 2008).

Amytornis grasswrens (Passeriformes: Maluridae, fairy-wrens and allies) are an ideal group with which to expand our understanding of these broad evolutionary questions in southern Australia as well as how they can guide conservation and management. The 11 *Amytornis* species currently recognised (Black *et al*. 2010; Garnett *et al.* 2011) occur mostly across Australia's arid zone and monsoon tropics (Schodde 1982a; Rowley and Russell; Christidis *et al.* 2010; Joseph *et al.* in press). Christidis *et al.* (2010) have shown that in *Amytornis* "plumage differentiation between discrete populations is taxonomically significant and not as greatly influenced by ecophenotypic variation as previously thought." *Amytornis* generally is thus a rare example among birds of high taxonomic diversity combined with

restricted geographic distributions (short range endemism) more typical of poorly dispersing organisms.

Here we focus on the Western Grasswren *A. textilis* and Thick-billed Grasswren *A. modestus* of southern Australia (*sensu* Black 2011a; Black *et al.* 2010; Garnett *et al.* 2011) and which have been shown to be sister taxa (Christidis *et al*. 2010). Found today mainly in chenopod shrublands, fossil and historical evidence shows that this group has declined in range and habitat diversity from its former, broad distribution across arid and semiarid southern and central Australia (Baird 1990; Black 2004, 2011b; 2012). It suffered major population declines and extirpations at the end of the 19th century as a result of habitat loss due to overstocking, feral herbivores and drought (McAllan 1987; Garnett *et al.* 2011). Consequently, relatively few specimens were collected and knowledge of original distributions of individual populations and taxa is sparse. When coupled with the birds' extreme morphological crypsis, it is little surprise that the taxonomy of this group was long chaotic although relative order has now been achieved (for reviews of taxonomy and distribution see Parker 1972; Black 2004, 2011a,b; Black *et al.* 2009, 2010, 2011). We now appreciate, therefore, that several populations and subspecies are threatened or endangered and are the subject of recovery plans (Garnett *et al*. 2011). Conservation action should be directed at appropriate taxonomic/evolutionary groups, so identifying these has significant conservation significance. That, in turn, requires that diversity in extinct as well as extant populations is understood and this paper seeks to sharpen that understanding.

A brief review of this group's current taxonomy and distribution will help develop this study's aims (Figure 1). Recognition of *A. textilis* (including *A. t. myall*) and *A. modestus* as separate species has been supported by morphological (Black *et al.* 2010) and molecular data (Christidis *et al*. 2010). Within *A. textilis*, Black (2011b) concluded that *A. t. textilis* of inland, mostly drier parts of south-western Western Australia is polytypic, showing morphological diversity among four or five separate populations. He recognised two of its now extinct populations, which inhabited eucalypt-dominated habitats, as *A. t. macrourus*; three more northern populations had also been regarded previously as separate taxa but evidence was inconclusive for maintaining separate subspecific identities. They are populations from Shark Bay (*A. t. textilis*; still extant), Dirk Hartog Island, (*A. t. carteri*; extinct) both of which generally occupy *Acacia* spp. communities, and lastly, those in arid inland chenopod communities, (*A. t. giganturus*; extinct). Within *A. modestus*, the possibility of phylogeographic structure has been noted (Black *et al*. 2010): a western phylogroup would comprise two subspecies *A. m. modestus* and *A. m. indulkanna* and an eastern phylogroup

would comprise five or six allopatric populations. These include two named subspecies in New South Wales *A. m. obscurior* and *A. m. inexpectatus*, as well as *A. m. raglessi* from the northern Flinders Ranges periphery, *A. m. curnamona* from the southern Lake Frome basin and a little sampled population from northeast of Lake Eyre that is un-named and here referred to as "eyre" (Black 2011a).

The broad aim of the present study was to document mitochondrial DNA (mtDNA) diversity within and between *A. textilis* and *A. modestus* based on all extant populations and all but one extinct population. Specifically, two further aims were to (1) test the two species' taxonomic limits based on almost complete population-level sampling across both, and (2) develop an initial molecular phylogeography within the two species to guide later, multilocus work as well as conservation and management priorities.

Materials and Methods

Samples

We sampled seventy-five contemporary and historical museum samples from all known populations and thus of the 11 named and one un-named subspecies of *A. textilis* and *A. modestus* (Figure 1). This represented 65% and 52% of the 115 and 145 specimens we have identified to subspecific level in Australia (n =115) and worldwide (n =145), respectively (Table 1); we are aware of a few other specimens not yet identified to subspecies level. Samples were either toe pads taken from museum specimens up to 100 years old, frozen tissue from vouchered museum specimens, or plucked feathers from live birds (Supplementary Table 1).

Precautions against contamination

Contamination of historic museum samples with contemporary DNA and previously amplified mtDNA PCR products of the ND2 gene was controlled by conducting all pre-PCR work at dedicated ancient DNA facilities at Museum Victoria and the Australian Centre for Ancient DNA, University of Adelaide. No contemporary bird samples or DNA had ever been present in either of the pre-PCR laboratories. The museum toe pad samples were extracted in a pre-PCR laboratory physically separate from post-PCR laboratories and included the use of dead-air glove boxes fitted with internal UV lights for DNA extraction and PCR set-up, regular decontamination of all work areas and equipment with sodium hypochlorite, PPE including disposable laboratory gown, face mask, face shield, shoe covers and double-

gloving and strict one-way movement of personnel (shower > freshly laundered clothes > pre-PCR laboratory > post-PCR laboratory).

DNA Extractions: Historical museum specimens

Sampling restrictions and methodology aimed at minimizing destructive sampling of museum specimens limited toe pad sample size to enough for just a single DNA extraction attempt for all historical museum skins. Tissue samples (~3mm^3 of toe pad) were rehydrated in 1 mL of 10mM Tris (pH 8.0) for 24 hr and chopped finely using a sterile scalpel blade. DNA was subsequently extracted using the DNeasy Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions with the following modifications: (i) double volumes of ATL buffer, proteinase K, AL buffer and ethanol, (ii) 2 uL of carrier RNA was added to each sample following proteinase K digestion, and (iii) DNA was eluted twice from the silica spin columns with 100 uL of buffer AE for a final volume of 200 uL. A negative extraction control was included with every set of DNA extractions and all extractions were carried out in small sets (seven individuals or less).

DNA Extractions: Contemporary tissues and feathers

DNA extractions on contemporary (feathers and frozen tissue) were carried out in a separate pre-PCR laboratory. DNA was extracted from tissue samples (~3mm^3 of frozen muscle) and feathers (single feather tip) using a salting-out method (Nicholls *et al.* 2000). A negative extraction control was included with every set of DNA extractions.

PCR Amplification and DNA Sequencing

Contemporary samples were amplified using existing primers (L5216 and H6313, Sorenson et al 1999) that flank the mtDNA ND2 gene. PCR reactions were performed in 25 µl volumes containing 2 uL of extracted DNA, 1x HotMaster Buffer (Eppendorf), 200 µM of each dNTP, 200 µM of each primer (GeneWorks) and 0.5 units of HotMaster *Taq* DNA Polymerase (Eppendorf). Thermocycling conditions were: denaturation at 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 20 s, annealing at 55 °C for 10 s and extension at 65 °C for 60 s, with a final extension step at 65 °C for 10 minutes. All PCR attempts included a PCR no template control and the relevant negative extraction control (s) for the samples being amplified.

Historical museum samples were amplified using six sets of primers targeting ~250 base pairs (bp) overlapping fragments. Primer combinations were: fragment 1 (L5216/5'-GGCTGAAGCRGATGCTTGAA-3'), fragment 2 (5'-CGRGCAATTGAAGCAGCAAC-3'/5'-AGTGTTGACAGRAGGAGGCT-3'), fragment 3 (5'-ACTAGGTCTAGCCCCRTTCCA-3'/5'-AGGTGAGAGATGGAGGAGAAGG-3'), fragment 4 (5'-ACTCACTCAACCCAACACTAC-3'/5'-AGTGTGGAGAGTTTTAGGACTT-3'), fragment 5 (5'-ACCTGTACAGCCTAATAACAGC-3'/5'-GGCTGAGTAGGGACAGGAGG-3'), fragment 6 (5'-AAGCAAGAAATGGCTCCGGC-3'/H6313). PCRs were performed in 25 µl volumes containing 2 uL of extracted DNA, 1x High Fidelity PCR Buffer (Invitrogen, California, USA), 200 µM of each dNTP, 400 µM of each primer (Geneworks), 2 mM $MgSO_4$ 1 µg/µl RSA (Sigma) and 0.5 units of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, California, USA). Thermocycling conditions were: denaturation at 94 °C for 2 minutes, followed by 40 cycles of 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 68 °C for 30 seconds, with a final extension step at 68 °C for 10 minutes. All PCR attempts included a PCR no template control and the relevant negative extraction control (s) for the samples being amplified.

PCR products were visualized under UV light on 2-3.5% agarose gels stained with ethidium bromide. Successful amplifications were purified using Ampure (Agencourt) according to manufacturer's instructions and sequenced directly using Big Dye v3.1 chemistry and an ABI 3130XL Genetic Analyzer (Applied Biosystems). Sequence chromatograms were edited and assembled using Sequencher Software (GeneCodes).

Phylogenetic Analyses

DNA sequences were aligned using Geneious (Biomatters) and included homologous sequence from four outgroup species *A. ballarae, A. purnelli, A. goyderi, A. housei* (Christidis *et al.* 2010, GenBank Accession: HQ118180, HQ118184-5, HQ118187). We did not include three sequences from Christidis *et al.* (2010) for *A. t. textilis* (HQ118192), *A.t. myall* (HQ118191) and *A. modestus* (HQ118190) because preliminary analyses revealed that these sequences were chimeric in origin. For example, the sequence for HQ118190 (*modestus*) matches other *A. modestus* sequences for the first 300 bp, but after a series of 36 N's the remaining 670 bp matches *A. textilis* sequences. Christidis *et al*. (2010) state that "all gene regions were amplified and sequenced as single fragments except ND2 which was amplified as two non-overlapping fragments". A laboratory mix-up could explain the

chimeric origin for these three sequences.

Phylogenetic analyses were carried out using a Bayesian approach in the program MrBayes 3.2 (Ronquist *et al.* 2012). We conducted two runs, each starting from a random tree, for 10 million generations, sampling every 1000 generations and using the "mixed" substitution model which incorporates Bayesian model jumping during the MCMC simulation to sample across all time-reversible rate matrices. Convergence of runs was assessed using the average standard deviation of split frequencies, which should approach zero as runs converge. Tree topology and posterior probability support for individual nodes was generated after discarding the first 25% of values as burn-in.

We estimated approximate divergence times within and between *A. textilis* and *A. modestus* using a strict-clock approach in BEAST v.1.7.5 (Drummond *et al.* 2012). An appropriate evolutionary model was selected using jModelTest 2.1.3 (Darriba *et al.* 2012) and we ran two different BEAST analyses to account for the intra-specific (coalescent) and inter-specific (speciation) nature of the dataset. BEAST XML files were generated using BEAUti v. 1.7.5 using the TN93+I substitution model with empirical base frequencies applying a lineage substitution rate of 0.029 substitutions per site per million years with a normal prior distribution and a standard deviation of 0.005. This rate was estimated for the mtDNA ND2 gene in birds by Lerner *et al.* (2011). The first analysis (coalescent) included all 74 ingroup and four outgroup sequences and implemented a coalescent constant population size tree prior. The second analysis (speciation) included only two sequences from each species (one from each of two haplogroups) and the four outgroups with a Yule process speciation tree prior. For both analyses two separate MCMC analyses were run for 10 million generations with parameters sampled every 1000 steps. Individual runs were checked for convergence and to ensure the effective sample size (ESS) was >200 using Tracer v1.5 (Rambaut and Drummond 2007). The two separate MCMC runs for each analysis were combined using LogCombiner v1.7.5 (Drummond *et al.* 2012) with 10% burn-in removed. Tracer v 1.5 was used to calculate the mean and upper and lowed bounds of the 95% highest posterior probability density interval (95% HPD) for the divergence between *A. modestus* and *A. textilis*, the two haplogroups within *A. modestus*, the two haplogroups within *A. textilis*, and the time to most recent common ancestor (TMRCA) for sampled diversity within each of the two major haplogroups within each species. Tree topologies were assessed using TreeAnnotator v 1.7.5 (Drummond *et al.* 2012) and FigTree v 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

A haplotype network showing genealogical relationships between all *Amytornis* sequences was generated using statistical parsimony implemented in TCS v1.17 (Clement *et al.* 2000). Nucleotide and haplotype diversity and net nucleotide divergence was calculated using DnaSP v5.10.01 (Librado and Rozas 2009).

Results

We obtained 1016 bp of mtDNA ND2 sequence from 74 of 75 specimens of *A. textilis* and *A. modestus*. The only available museum specimen for *A. m. inexpectatus* failed to yield amplifiable DNA but we obtained sequence from 3-18 specimens for the remaining 10 named and one unnamed subspecies. Forty-two unique haplotypes were identified, each represented by 1-10 specimens (Figures 1, 2, 3). All named subspecies except *A. t. textilis* and *A. m. modestus*, and the one unnamed subspecies, showed moderate haplotype and nucleotide diversity (Hd= 0-1, π= 0-0.0045, Table 2). The Bayesian phylogeny (Figure 2) revealed strong support for a sister-species relationship between *A. textilis* and *A. modestus* and the net nucleotide divergence between them was 3.3%. Within each species, a number of haplogroups were well resolved. Within *A. textilis* two haplogroups were identified (net nucleotide divergence 1.2%). The first contained all of the Western Australian samples from the subspecies (epithets only for brevity) *textilis, carteri*, *macrourus* and *giganturus*. This first haplogroup also contained the two westernmost samples that we sequenced of the eastern subspecies, *myall*, one sequenced from fresh tissue collected in 2006 near Lake Gairdner north of the Gawler Ranges and the second sequenced from a skin collected in 1909 near Barton, Yellabinna Region *ca* 400 km to the west (Black 2004) (Figures 1, 2); these localities are highlighted as squares in Figure 3. The second haplogroup had all of the remaining *myall* samples. Within *A. modestus* two haplogroups were also identified (net nucleotide divergence 1.7%). The first had subspecies *modestus* from the Northern Territory and *indulkanna* from north-western South Australia. The second had all of the more eastern samples of that species from its subspecies *curnamona*, *raglessi*, 'eyre', and *obscurior*. Within the two species, five of the named subspecies (*textilis, carteri*, *macrourus*, *modestus* and *indulkanna*) were monophyletic, albeit with limited Bayesian support. The samples of *giganturus* and *macrourus* and the two westernmost samples of *myall* formed a monophyletic group and those of *textilis* and *carteri* formed another. Samples of *curnamona*, *obscurior*, *raglessi* and 'eyre' formed a haplogroup within which there was no clear phylogenetic or

taxonomic structure. The haplotype networks provided some additional resolution as well as support for the overall phylogenetic relationships identified in the Bayesian analysis (Figure 2).

Molecular dating estimates using either a speciation or coalescent tree prior produced congruent results suggesting that *A. modestus* and *A. textilis* diverged ~0.87 Mya (speciation: 0.85 Mya, 95% HPD: 0.51-1.27 Mya; coalescent: 0.90 Mya, 95% HPD: 0.52-1.32 Mya, Figure 4). Within *A. textilis* the two major haplogroups (1. western *textilis, carteri, macrourus* and *giganturus* and two samples of *myall;* 2. eastern *myall*) diverged ~0.33 Mya (speciation: 0.34 Mya, 95% HPD: 0.17-0.54 Mya; coalescent: 0.32 Mya, 95% HPD: 0.17-0.50 Mya, Figure 4). The time to most recent common ancestor (TMRCA) for each of the western and eastern haplogroups was very similar, 0.13 Mya and 0.12 Mya, respectively (95% HPD: 0.06-0.21 Mya and 0.05-0.21 Mya, respectively). Within *A. modestus* the two major haplogroups (1. *modestus* and *indulkanna*; 2. *curnamona, obscurior*, *raglessi*, "*eyre*") diverged ~0.36 Mya (speciation: 0.34 Mya, 95% HPD: 0.16-0.54 Mya; coalescent: 0.38 Mya, 95% HPD: 0.21-0.59 Mya, Figure 4). The TMRCA for each of the two haplogroups was 0.11 and 0.11 Mya, respectively (95% HPD: 0.05-0.19 Mya and 0.06-0.18 Mya, respectively).

Discussion

We surveyed mtDNA diversity in extant and extinct populations of the Thick-billed Grasswren *A. modestus* and Western Grasswren *A. textilis* based on the most extensive sampling of these birds to date. We aimed to test current understanding of their species limits, provide an initial survey of phylogeographic diversity within the group, and help guide their conservation and management. Whilst acknowledging the limitations of relying solely on mtDNA for avian phylogeographic and taxonomic studies (Edwards and Bensch 2009) we argue that our data establish an important baseline for future multi-locus nuclear analyses. Reciprocal monophyly of *A. modestus* and *A. textilis* with respect to each other and net DNA divergence between them of *ca* 4% affirmed Christidis *et al*.'s (2010) findings based on limited sampling. This supports current taxonomic recognition of the two as species. Based on current understanding of rates of mtDNA evolution from Lerner *et al*. (2011) and recognising that gene-divergence may pre-date population-divergence, we suggest that *A. modestus* and *A. textilis* diverged from a common ancestor in the mid-Pleistocene ~0.9 million years ago.. The pronounced phylogeographic structure we have documented within

both *A. modestus* and *A. textilis* ought to be maintained and accommodated in conservation and management plans, whether developed for entire communities or for the individual species. We will argue below that had extinct populations not been included, some of our findings concerning extant diversity would remain puzzling. We now more closely examine these findings and place diversity within and between the two species in the context of southern Australian biogeography generally.

Biogeography and taxonomy

The sister species relationship and level of divergence between *A. modestus* and *A. textilis* are together similar to other sister species divergences attributed to Plio-Pleistocene vicariance across the Eyrean barrier (essentially the Lake Eyre Basin *sensu* Serventy 1972; Schodde 1982b) and its fringing xeric habitats, e.g. White-naped and Western Honeyeaters *Melithreptus lunatus* and *M. chloropsis*, respectively (Toon *et al*. 2010), and Eastern and Western Ground Parrot (*Pezoporus wallicus, P. flaviventris*; Murphy *et al*. 2011). In part because of long-standing confusion about species limits in these birds relative to the Dusky Grasswren *A. purnelli* but which is now resolved (see Parker 1972; Schodde 1982a; Black 2004; Christidis *et al*. 2010), the geography and ecology of speciation of *A. modestus* and *A. textilis* have been little discussed (Keast 1958, 1961; Ford 1974, 1987; Christidis 1999; Schodde 1982b; Christidis *et al*. 2010). Vicariance across the Lake Eyre Basin (i.e., Eyrean Barrier) has been hypothesized as causing their divergence (Schodde 1982a; Schodde and Mason 1999). Similarly, later westwards dispersal of *A. modestus* across that region has also been proposed (Schodde 1982a) because records of *A. modestus* do span the Lake Eyre Basin (Figure 1; Black 2012; Black *et al*. 2010).

Our data are not inconsistent with much of this scenario but the two haplogroups we recorded within *A. modestus* are not readily predicted by it. Certainly, speciation of *A. modestus* and *A. textilis* and later divergence within *A. modestus* could both have been caused by vicariance across the Lake Eyre Basin at different times. Dolman and Joseph (2012), for example, showed that at least three temporally separated divergence events were involved in explaining the distributions of southern Australian birds. In the present case, secondary expansions after each of two vicariance events would be needed to explain present-day distributions. Ribeiro *et al*. (2011, 2012) illustrate an approach that further study of the present case could take in discriminating among alternative hypotheses of vicariance and dispersal and when either may have operated. Certainly, the problem of what caused the speciation of *A. modestus* and *A. textilis*, specifically its geography and ecology, remains.

If indeed the speciation of *A. modestus* and *A. textilis* was caused by broad-scale vicariance and not more subtle smaller scale features of substrate type (see Ford, 1987 his Table 1; Schodde 1982b), then it may be worth asking whether the Nullarbor Plain and its fringing sand dunes and stony areas, may also have contributed. This is despite general acceptance that the Lake Eyre Basin has likely been the more relevant barrier to speciation in southern Australia (e.g. Schodde 1982). *A. modestus and A. textilis* are known to have approached each other geographically in and about the Yellabinna dunes (Black 2004; Figure 3) so in the past may not have been wholly isolated from each other there. Black (2004) suggested that the Great Victoria Desert may have been an isolating mechanism. It is notable in this context that today the two species are separated by a narrow region west and south-west of Lake Torrens some 140 x 60 kilometres in extent. Neither species has been recorded here at all (Figure 3 and Atlas of Living Australia http://www.ala.org.au; accessed 10 May 2013). Black *et al.* (2011) searched unsuccessfully for the two species there and noted that suitable habitat is either absent or present only in small isolated patches. Multilocus data may refine estimates of the timing of their divergence but, again, the geography and mode of their speciation provide scope for further work. This should integrate the age of landscape features such as dunes and stony environments with their roles as potential barriers.

Phylogeography of *A. modestus*

Phylogeographic structure within *A. modestus* is similar to that reported within other southern Australian birds thought to have been separated well into the Pleistocene, and again by the Eyrean Barrier and other xeric landscapes across the region. Such structure is currently recognized at the subspecific level and examples are the Australian Ringneck *Barnardius zonarius* (Joseph and Wilke 2006), Scarlet Robin *Petroica boodang* (Dolman and Joseph 2012), Splendid Fairy-wren *Malurus splendens* (Kearns et al 2009). A further species having a similarly recognized divergence at this longitude where the two haplotype groups of *A. modestus* approach each other today is the Short-tailed Grasswren *Amytornis merrotsyi* (Christidis *et al* 2008) (but see also geography of *A. modestus* vs. *A. t. myall*). Our data can certainly support at least subspecific recognition of the two haplogroups within *A. modestus* which diverged ~ 0.36 Mya.

Within the western haplogroup, two monophyletic subgroups comprise samples of either *modestus* or *indulkanna* (*ca.* 0.5% divergence), whereas within the eastern haplogroup there is incomplete separation of lineages among the four represented populations/subspecies. Each of three centrally placed haplotypes in the eastern haplogroup is common to two of the

subspecies. In contrast, peripheral haplotypes are not shared among subspecies. Given their geographical distribution (Figure 1, 3), we suggest that this is consistent with early stages of lineage separation in allopatric populations rather than sampling of a panmictic population (Omland *et al.* 2006). The TMRCA for both haplogroups (0.11 Mya) indicates diversification commenced around the end of the last interglacial.

Phylogeography of *A. textilis*

Two haplogroups were also observed in *A. textilis*. Net divergence between the two was lower than that within *A. modestus* (1.2% *cf* 1.7%) but this is again consistent with mid-Pleistocene divergence ~0.33 Mya. It accords well with Dolman and Joseph's (2012) finding of 1.28% net divergence between eastern and western subspecies of New Holland Honeyeater *Phylidonyris novaehollandiae*, which are largely separated by the Nullarbor Plain and the wooded habitats of the Great Victoria Desert that fringe its northern margins. The mainly western haplogroup has two subgroups, one containing *giganturus* and *macrourus* (and the two peripheral *myall*) and the other containing *textilis* and *carteri*. All four have some unique haplotypes and *textilis, carteri* and *macrourus* are monophyletic. This complexity is comparable to that found in the western subspecies of *Malurus splendens* (Kearns *et al*. 2009). Similar to *A. modestus*, the two haplogroups have TMRCA's around the end of the last interglacial.

Although the distributions of the two haplogroups are today isolated from each other by the Nullarbor Plain and its fringing habitats, two reasons make it difficult to ascribe the divergence of the two haplogroups to a single landscape feature such as the Nullarbor Plain. First, habitat diversity in *A. textilis* including its now extinct populations has spanned chenopod and eucalypt-acacia communities (Schodde 1982b; Black 2004; 2011b; Black *et al.* 2009) and chenopods are the dominant vegetation of the Nullarbor. Second, Baird (1990) recorded subfossil *A. textilis* from the Nullarbor Plain itself. A further key result from our study is pertinent here. One haplogroup in *A. textilis* included all samples from the west of the continent and the two westernmost specimens of the eastern isolate *myall*. The other haplogroup included all other specimens of *myall*. In particular, the mtDNAs of the two westernmost *myall* specimens were more closely related to the now extinct western forms *giganturus* and *macrourus*. This result prompted careful reassessment by ABB and an independent observer of the identity of the two genetically anomalous *myall* specimens (results not shown); they are indeed phenotypically unremarkable specimens of *myall*. The most parsimonious inference, then, is that at some time since divergence of eastern *myall* and

all other, western populations, gene flow from western proto-*giganturus/macrourus* to eastern proto-*myall* has occurred; subsequently, mtDNA more typical of the extinct western forms was retained as "ghost" lineages in peripheral *myall* individuals some 1,000 km distant. An alternative explanation – one of retained ancestral polymorphisms within the two western-most *myall* specimens – appears less likely from the data but cannot be entirely excluded. This is clearly an area where multilocus nuclear data could distinguish between these two alternatives and provide more informed estimates of the timing and direction of gene flow and so inform the origin of the two haplogroups.

In contrast to the concordance we have discussed between molecular and taxonomic diversity, a few instances of discordance warrant comment. Several recent studies in other birds reported substantial intraspecific mtDNA divergences that are likely attributable to factors other than vicariance such as selection (Ribeiro *et al*. 2011; Webb *et al*. 2011). These and other studies caution against uncritical interpretation of mtDNA structure as a signal of vicariance (see also Ballard and Rand 2005). Ribeiro *et al.* (2011), for example, especially noted strong nuclear gene flow in the face of mitochondrial differentiation. They concluded that selective pressures on birds' physiologies, mediated by the mitochondrial genome, may underpin local adaptation to climatic conditions. This in turn may explain some of the mitochondrial-phenotypic discordance we have documented here. It also helps set an agenda for further work on these birds where multilocus data would be of use in discriminating among alternative hypotheses such as selection, ongoing gene flow, and vicariance.

Conclusions

Our data reveal major genetic disjunctions among surviving populations of both *A. modestus* and *A. textilis*. Further, they well illustrate the need, where possible, to include extinct as well as extant populations of threatened species when studying their genetic diversity. Had extinct populations not been included here, the origin of the high diversity within *A. t. myall*, for example, would be enigmatic. Inclusion of extinct populations from western regions of Australia suggests, however, that it is explained by past gene flow and that this can be tested further using nuclear data. This points to the value more generally of maintaining suitable geographical corridors across large geographical scales for arid zone species that still survive. *A. m. inexpectatus* is the single remaining extinct population from this group that remains to be studied. It was the easternmost known population of *A. modestus* and we look forward to analyses of it from museum specimens. The mtDNA phylogeny and phylogeography suggest that three extant taxa – *textilis*, *myall* and *indulkanna*

should be managed and conserved as separate evolutionary lineages. Pending future multi-locus nuclear data, four taxa within *A. modestus – curnamona*, *obscurior*, *raglessi* and "eyre", which together form another independent lineage, may also warrant separate recognition and conservation.

Finally, our study adds to the growing body of data on mtDNA divergences within southern Australian vertebrates and initial estimates of divergence times to explain these observations (Dolman and Joseph 2012; Neaves *et al*. 2012). Together they reveal the complexity in space and time of divergences in the region's biota. This, in turn, frames an interest in better linking improved estimates of divergence times and extents of past gene flow to locations of putative barriers and the origins of these divergences.

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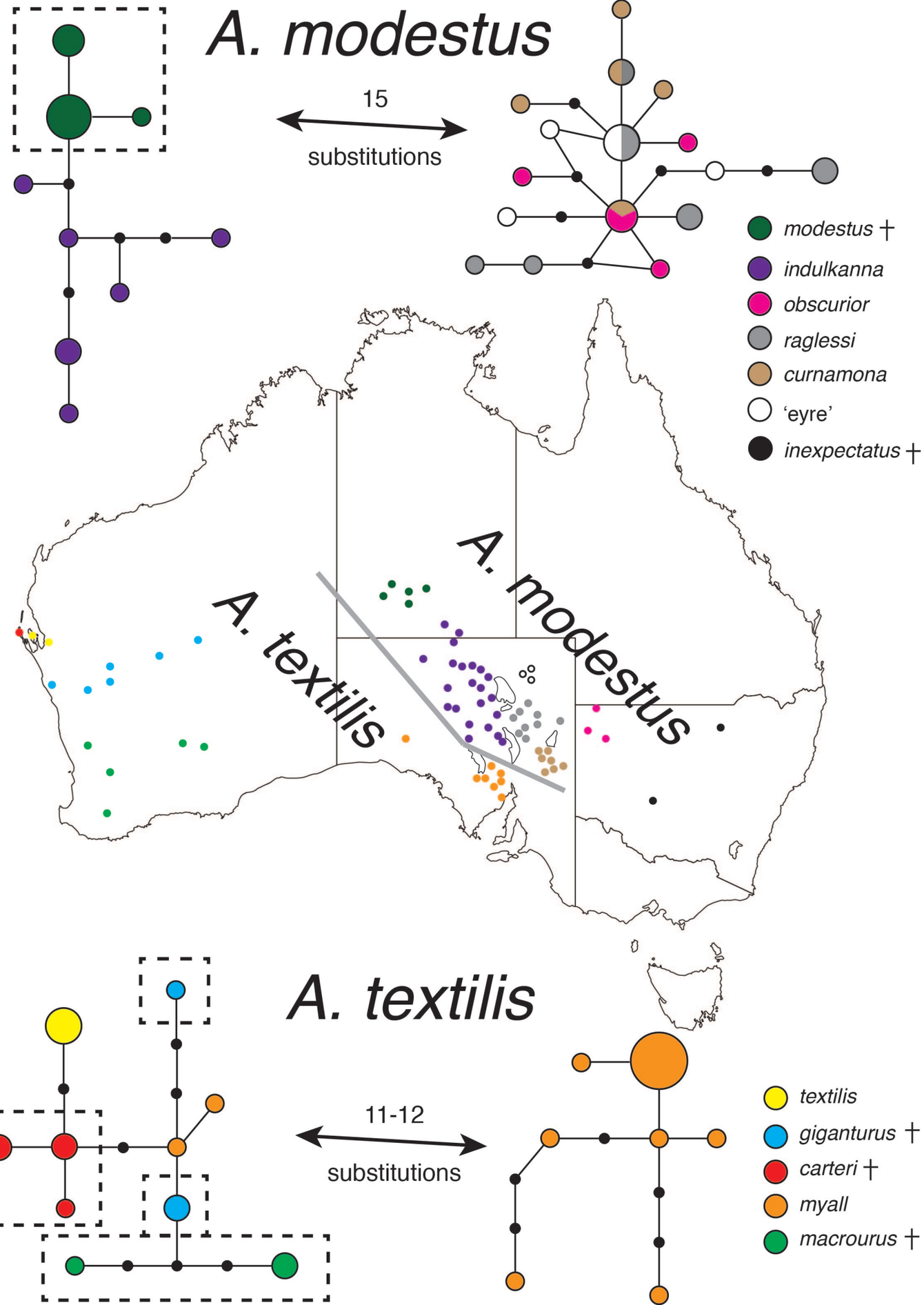
Figure Legends

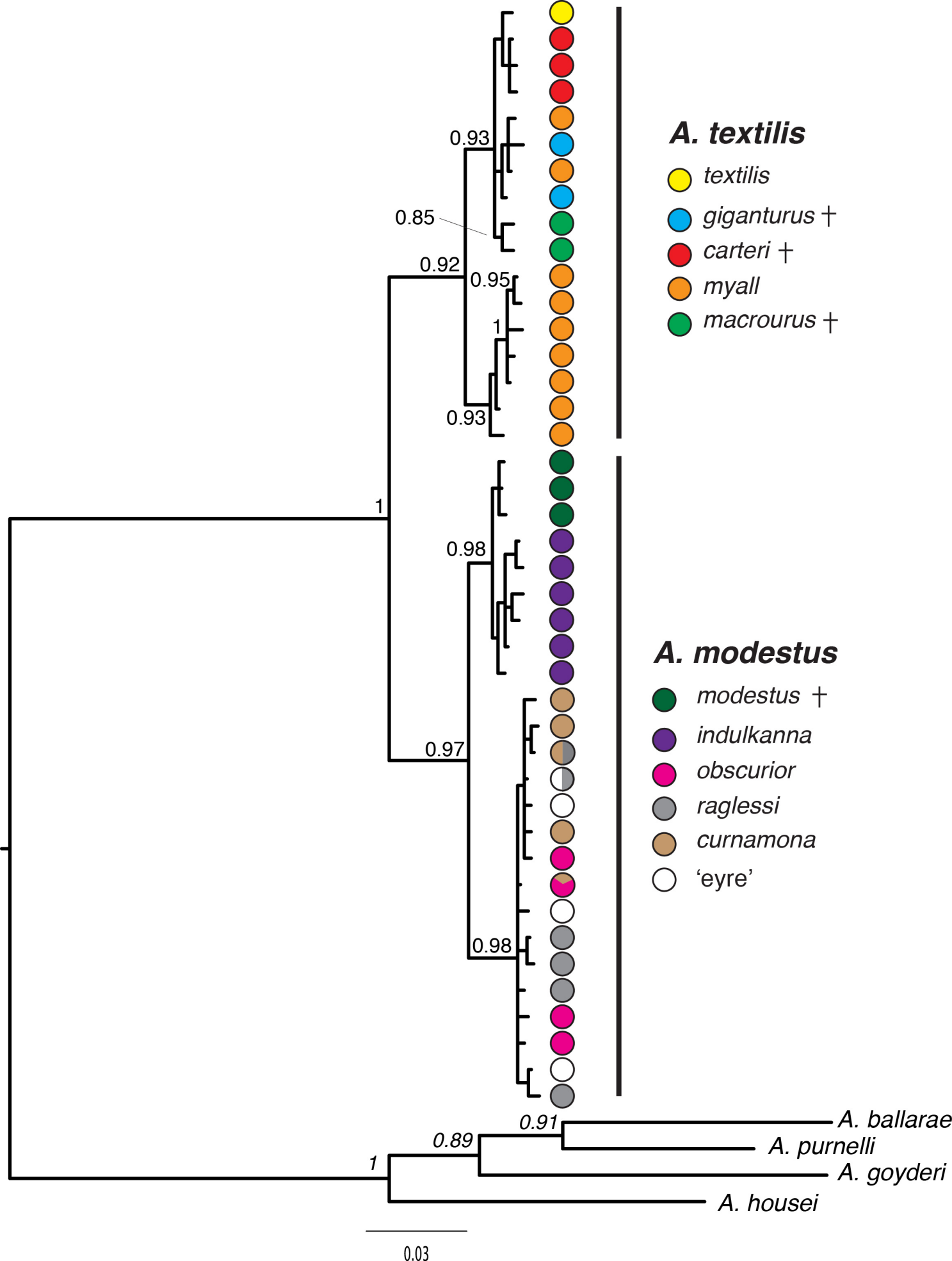
Figure 1. Map of Australia showing the distributions of Thick-billed and Western grasswrens *A. modestus* and *A. textilis*, respectively, the distributions of the specimens sampled, and relationships within and among the sequences derived from these specimens as unrooted networks. Daggers indicate and dotted lines enclose extinct taxa.

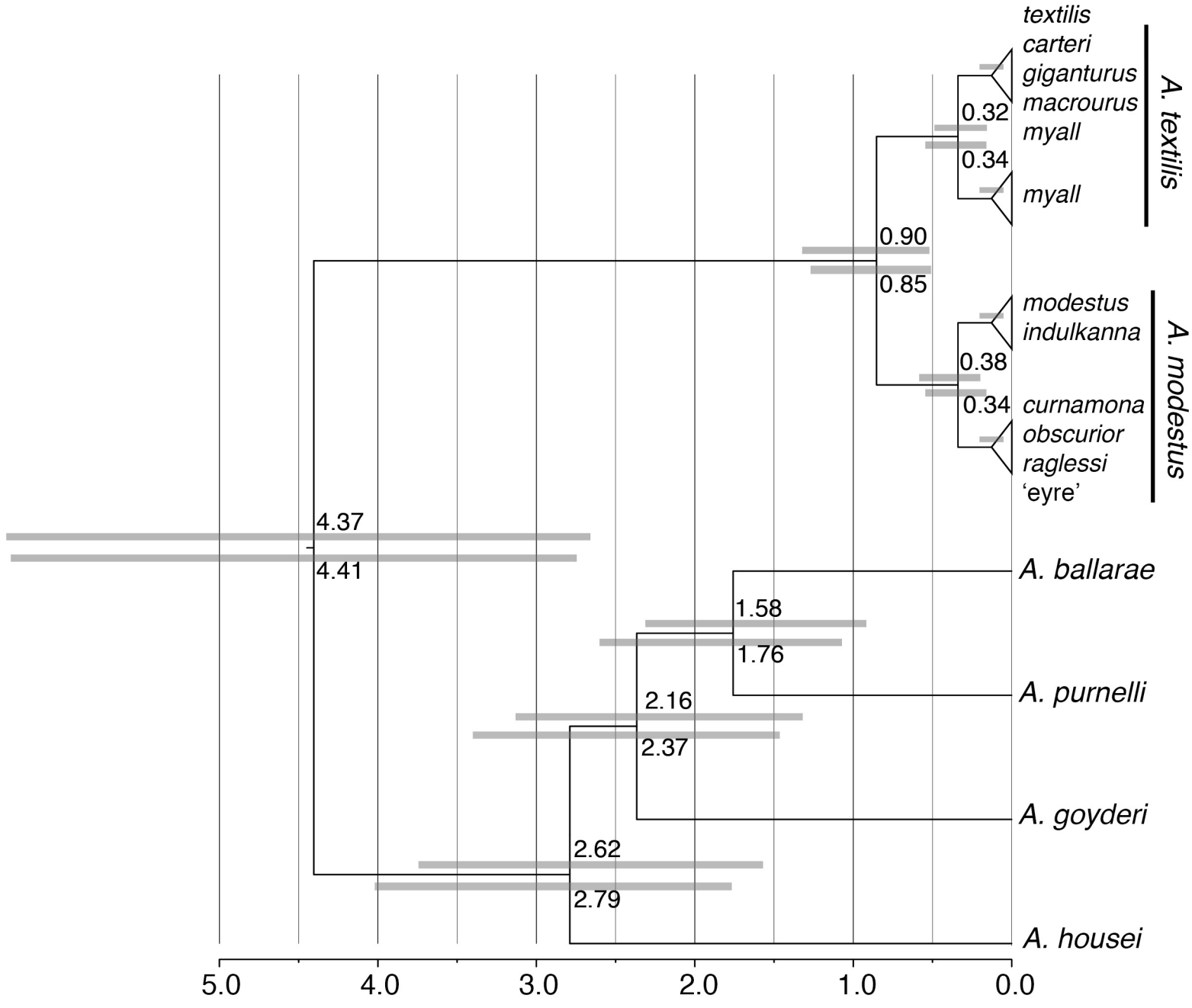
Figure 2. Bayesian phylogeny of relationships among the specimens and taxa studied. Daggers indicate extinct taxa.

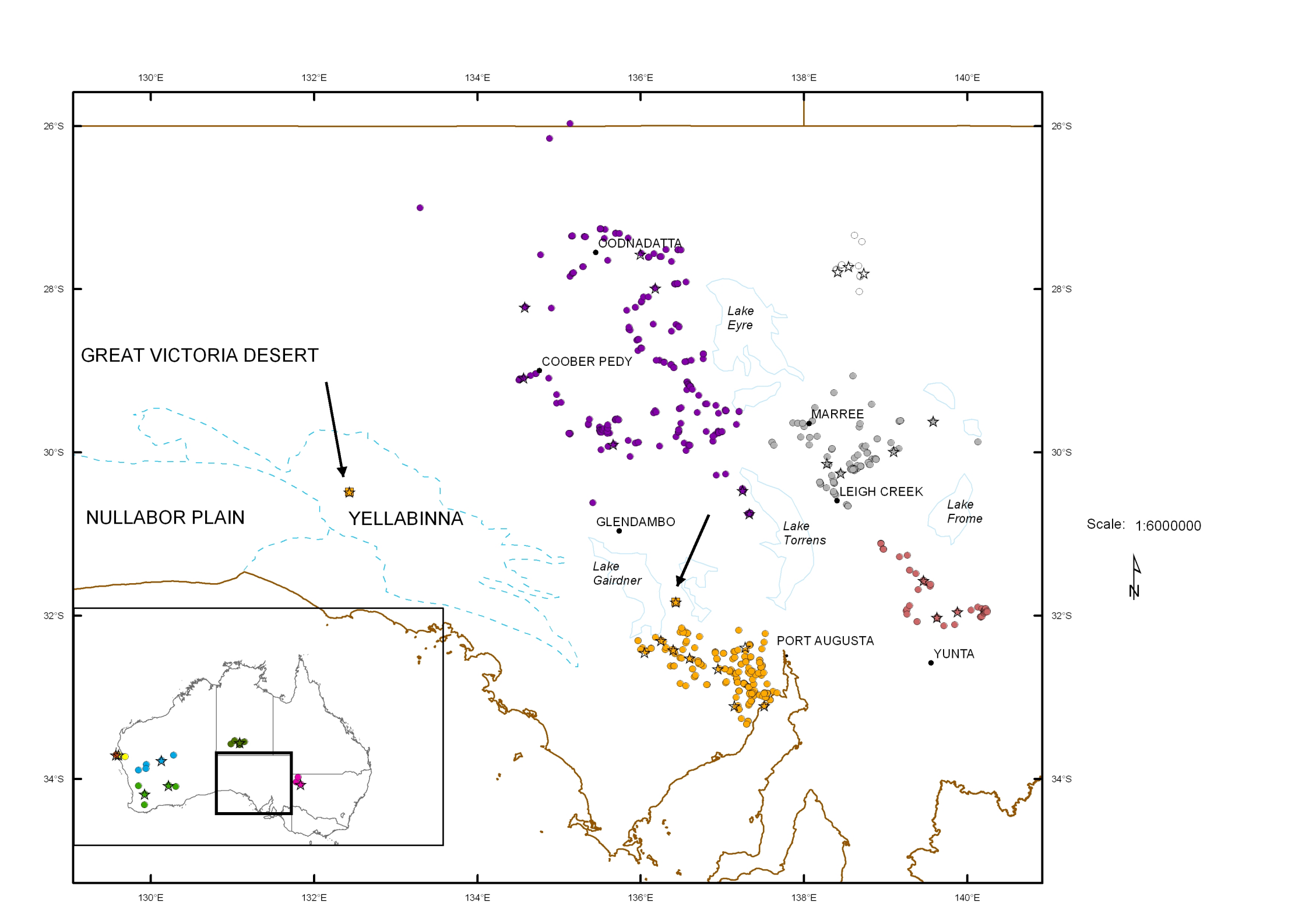
Figure 3. Detail of the total known geographical ranges from specimens sampled (stars) and other records (circles) of *A. textilis myall* (orange symbols) and subspecies of *A. modestus* (all other colours) in South Australia to highlight (a) their proximity to each other in the vicinity of Lakes Torrens and Gairdner and (b) that *A. modestus* occurs on either side of Lake Eyre. Squares (arrowed) depict the locations of two specimens of *A. t. myall* that are paraphyletic with respect to all other specimens of that form (see Figure 2 and text).

Figure 4. Molecular dating of divergence events within and between *A. textilis* and *A. modestus*. The horizontal scale bar and node ages are in millions of years. Horizontal grey bars represent the 95% HPD for node ages. The upper and lower numbers and bars are derived from the coalescent tree prior analysis and speciation tree prior analysis, respectively.


A. modestus
15
substitutions
modestus †
indulkanna
obscurior
raglessi
curnamona
'eyre'
inexpectatus †
A. textilis
A. modestus
A. textilis
11-12
substitutions
textilis
giganturus †
carteri †
myall
macrourus †


A. textilis
textilis
giganturus †
carteri †
myall
macrourus †
A. modestus
modestus †
indulkanna
obscurior
raglessi
curnamona
'eyre'
0.93
0.85
0.92
0.95
1
0.93
1
0.98
0.97
0.98
0.91
0.89
1
A. ballarae
A. purnelli
A. goyderi
A. housei
0.03


textilis
carteri
giganturus
macrourus
myall
myall
A. textilis
0.32
0.34
0.90
0.85
modestus
indulkanna
curnamona
obscurior
raglessi
'eyre'
A. modestus
0.38
0.34
4.37
4.41
A. ballarae
1.58
1.76
A. purnelli
2.16
2.37
A. goyderi
2.62
2.79
A. housei
5.0
4.0
3.0
2.0
1.0
0.0


130°E
132°E
134°E
136°E
138°E
140°E
26°S
28°S
30°S
32°S
34°S
GREAT VICTORIA DESERT
NULLABOR PLAIN
YELLABINNA
COOBER PEDY
OODNADATTA
Lake Eyre
MARREE
LEIGH CREEK
Lake Frome
GLENDAMBO
Lake Torrens
Lake Gairdner
PORT AUGUSTA
YUNTA
Scale: 1:6000000
N

Supplementary Table1. Details of specimens examined. Abbreviations in specimen codes: ABBBS – Australian Bird and Bat Banding Scheme; AM – Australian Museum, Sydney; ANWC – Australian National Wildlife Collection, Canberra; MV – Museum Victoria, Melbourne; HLW - H.L. White Collection held at MV; SAMA – South Australian Museum, Adelaide. Abbreviations in localities: SA – South Australia; NSW – New South Wales; WA – Western Australia; NT – Northern Territory; Stn – Station; OS - Outstation; HS - Homestead; Latitudes and longitudes are in degrees and minutes South and East, respectively; Sample type abbreviations: TP - toe pad; CFT - cryofrozen tissue; F - feather from capture-release. Abbreviations for sex: uns – unsexed; M – male; F – female. Months in specimen dates are indicated in roman numerals e.g., 7 ix 2006 is 7 September 2006; bp – base pairs. All locality data should be considered as approximate and further details may be available from the curators of indicated collections.

Taxon, Specimen Number	**Locality**	**Latitude**	**Longitude**	**Sample type**	**Sex**	**Date Collected**	**Sequence length (bp)**	**Genbank Accession Number**
***A. m.* "eyre"**								
MV R8315	Between Cowarie and Mungeranie, SA	27 47	138 24	TP	M	9 vi 1922	1016	KF053456
SAMA B56068	Karakaranthina Creek, Cowarie Stn, SA	27 42	138 26	CFT	M	23 ix 2008	1016	KF053474
SAMA B56066	Karakaranthina Creek, Cowarie Stn, SA	27 42	138 26	CFT	M	23 ix 2008	1016	KF053475
SAMA B56235	S of Mira Mitta Bore, SA	27 48	138 44	CFT	F	26 x 2009	1016	KF053476
SAMA B56067	Karakaranthina Creek, Cowarie Stn, SA	27 42	138 26	CFT	M	23 ix 2008	1016	KF053477
A. m. curnamona								
SAMA B55708	13 km NW Curnamona HS, Siccus River, Curnamona Stn, SA	31 33	139 27	CFT	M	7 ix 2007	1016	KF053470
SAMA B55709	13 km NW Curnamona HS, Curnamona Stn, SA	31 34	139 28	CFT	F	7 ix 2007	1016	KF053471
SAMA B55710	9 km NW Curnamona HS, Curnamona Stn, SA	31 35	139 29	CFT	M	7 ix 2007	1016	KF053472
SAMA B55648	Buckeroo Paddock, 8 km NE of Mt Victoria, Plumbago Stn, SA	31 54	139 56	CFT	F	1 x 2006	1016	KF053487
SAMA B55647	6.4 km N of Mt Victor HS, SA	32 01	139 38	CFT	M	7 ix 2006	1016	KF053488

A. m. indulkanna								
SAMA B55669	18 km WSW of Coober Pedy, SA	29 05	134 34	CFT	M	21 vi 2007	1016	KF053465
SAMA B55670	~ 5 km N of Sloane Hill, Mt Eba Stn, SA	29 54	135 40	CFT	M	22 vi 2007	1016	KF053466
SAMA B55705	~ 5 km N of Wood Duck Bore, Peake Stn, SA	27 59	136 11	CFT	F	22 viii 2007	1016	KF053467
SAMA B55706	~ 8 km SSE of Mt Arthur, Allandale Stn, SA	27 34	136 00	CFT	F	22 viii 2007	1016	KF053468
SAMA B55707	~ 10 km E of Evelyn Downs HS on Mount Barry Stn, SA	28 13	134 35	CFT	F	24 viii 2007	1016	KF053469
SAMA B55668	Trig Creek, 7 km E of Andamooka, SA	30 28	137 15	CFT	F	29 iv 2007	1016	KF053489
SAMA B55667	2.5 km E of Myall Well, Andamooka Stn, SA	30 45	137 20	CFT	M	29 iv 2007	1016	KF053490
A. m. inexpectatus								
AM 0.10581	Yandembah Stn 40 km ESE of Mossgiel, NSW	33 26	144 58	TP	M	?xi 1886	PCRs failed	-
A. m. modestus								
MV HLW 7502	Finke River, James Range, NT	23 56	132 43	TP	F	8 iv 1923	1016	KF053457
MV HLW 7647	Finke River, Hermannsburg, NT	23 56	132 43	TP	M	20 vii 1912	864	KF053528
MV HLW 7648	Missionary Plain, NT	23 53	132 18	TP	M	1 vii 1923	1016	KF053458
MV HLW 7649	Missionary Plain, NT	23 53	132 18	TP	M	30 vii 1923	994	KF053529
MV HLW 7650	Missionary Plain, NT	23 53	132 18	TP	M	23 vi 1923	1016	KF053459
MV HLW 7651	Gilbert Creek, Missionary Plain, NT	23 55	132 29	TP	M	28 viii 1923	1016	KF053460
MV HLW 7652	Missionary Plain, NT	23 53	132 18	TP	F	1923	1016	KF053461
MV HLW 7653	Missionary Plain, NT	23 53	132 18	TP	F	13 vii 1923	1016	KF053462

MV HLW 7654	Missionary Plain, NT	23 53	132 18	TP	F	20 vi 1923	1016	KF053463
MV HLW 7655	Missionary Plain, NT	23 53	132 18	TP	F	23 vi 1923	1016	KF053464
A. m. obscurior								
ABBBS 033-92623	NW of White Cliffs, NSW	~30 32	~142 27	F	M	14 iv 2010	1016	KF053491
ABBBS 033-92624	NW of White Cliffs, NSW	~30 32	~142 27	F	F	14 iv 2010	1016	KF053492
ABBBS 033-92626	NW of White Cliffs, NSW	~30 32	~142 27	F	M	14 iv 2010	1016	KF053493
ABBBS 033-92627	NW of White Cliffs, NSW	~30 32	~142 27	F	M	15 iv 2010	1016	KF053494
ABBBS 033-92628	NW of White Cliffs, NSW	~30 32	~142 27	F	uns pullus	16 iv 2010	1016	KF053495
A. m. raglessi								
ANWC B40176	8 km NE of Lyndhurst, SA	30 15	138 27	CFT	M	13 ix 1985	1016	KF053478
ANWC B40177	8 km NE of Lyndhurst, SA	30 15	138 27	CFT	F	13 ix 1985	1016	KF053479
ANWC B40179	8 km NE of Lyndhurst, SA	30 15	138 27	CFT	F	13 ix 1985	1016	KF053480
ANWC B40180	8 km NE of Lyndhurst, SA	30 15	138 27	CFT	M	13 ix 1985	1016	KF053481
ANWC B40189	17 km N of Lyndhurst, SA	30 08	138 17	CFT	M	14 ix 1985	1016	KF053482
ANWC B40190	17 km N of Lyndhurst, SA	30 08	138 17	CFT	F	14 ix 1985	1016	KF053483
ANWC B40192	17 km N of Lyndhurst, SA	30 08	138 17	CFT	F	14 ix 1985	1016	KF053484
SAMA B55666	2km SE of Calcutta OS, Mt Lyndhurst Stn, SA	29 59	139 06	CFT	M	29 iii 2007	1016	KF053485
SAMA B56154	7 km NW of Petermorra Creek, SA	29 36	139 31	CFT	F	23 v 2009	1016	KF053486
A. t. carteri								
MV HLW 6599	Dirk Hartog Is, WA	25 55	113 07	TP	F	21 v 1916	1016	KF053520

MV HLW 6600	Dirk Hartog Is, WA	25 55	113 07	TP	M	28 x 1916	1016	KF053521
MV HLW 6601	Dirk Hartog Is, WA	25 55	113 07	TP	F	8 v 1916	1016	KF053522
MV HLW 6602	Dirk Hartog Is, WA	25 55	113 07	TP	M	18 v 1916	1016	KF053523
MV HLW 6603	Dirk Hartog Is, WA	25 55	113 07	TP	M	8 v 1916	1016	KF053524
A. t. giganturus								
MV HLW 2759	Lake Way, East Murchison, WA	26 46	120 21	TP	M	15 vii 1909	1016	KF053516
MV HLW 2760	Lake Way, East Murchison, WA	26 46	120 21	TP	M	26 viii 1909	1016	KF053517
MV HLW 2761	Lake Way, East Murchison, WA	26 46	120 21	TP	M	23 viii 1909	1016	KF053518
A. t. macrourus								
SAMA B07358	E of Beverley, WA	32 07	117 40	TP	F	vi 1906	1016	KF053527
MV HLW 2757	Kalgoorlie, WA	30 45	121 28	TP	M	2 xi 1910	1016	KF053514
MV HLW 2758	Kalgoorlie, WA	30 45	121 28	TP	F	2 xi 1910	1016	KF053515
A. t. myall								
ANWC B40314	27 km NW of Iron Knob, SA	32 39	136 57	CFT	M	26 ix 1985	1016	KF053496
ANWC B40330	Sinclair Gap, SA	33 06	137 08	CFT	F	27 ix 1985	1016	KF053497
SAMA B55505	Bluff Dam, Mahanewo Stn, SA	31 50	136 26	CFT	M	13 xii 2006	1016	KF053498
SAMA B55504	North Four Mile Dam, Carriewerloo Stn, SA	32 22	137 17	CFT	M	12 xii 2006	1016	KF053499
SAMA B55503	Gunter's Dam, Cariewerloo Stn, SA	32 21	137 20	CFT	M	2 xii 2006	793	KF053500
SAMA B27933	Eight Mile Creek, S of Whyalla, SA	33 06	137 31	TP	M	26 ii 1972	1016	KF053501
SAMA B27932	Eight Mile Creek, S of Whyalla, SA	33 06	137 31	TP	M	26 ii 1972	1016	KF053502

SAMA B7359	Fly Camp, N of Nonning HS, SA	32 25	136 24	TP	M	6 viii 1902	1016	KF053503
SAMA B52954	Myall Creek, Cariewerloo Stn, SA	32 31	137 19	TP	M	16 ix 1912	1016	KF053504
SAMA B27931	Eight Mile Creek, S of Whyalla, SA	33 06	137 31	TP	F	26 ii 1972	1016	KF053505
SAMA B27934	Eight Mile Creek, S of Whyalla, SA	33 06	137 31	TP	M	26 ii 1972	1016	KF053506
SAMA B57492	Eight Mile Creek, S of Whyalla, SA	33 06	137 31	TP	F	26 ii 1972	1016	KF053507
SAMA B49667	Mt Miccollo, SA	32 31	136 36	TP	M	19 ix 1998	1016	KF053508
SAMA B7360	9 km ESE Mt Ive HS, SA	32 28	136 08	TP	M	10 viii 1902	1016	KF053509
SAMA B39441	3 km E Shinnick's Dam, Kolendo Stn, SA	32 18	136 15	TP	M	20 x 1985	1016	KF053510
SAMA B57493	Eight Mile Creek, S of Whyalla, SA	32 06	137 31	TP	M	26 ii 1972	1016	KF053511
SAMA B52955	Myall Creek, Cariewerloo Stn, SA	32 31	137 19	TP	M	16 ix 1912	1016	KF053512
MV R10002	Between Barton and Bates, Yellabinna Region, SA	30 29	132 26	TP	F	16 i 1909	1016	KF053519
A. t. textilis								
MV HLW 2754	Shark Bay, WA	25 56	113 32	TP	F	8 xi 1918	1016	KF053473
MV HLW 2755	Peron Peninsula, Shark Bay, WA	25 56	113 32	TP	M	28 ix 1920	1016	KF053513
MV HLW 7457	Peron Peninsula, Shark Bay, WA	25 56	113 32	TP	F	4 x 1920	1016	KF053525
MV HLW 7458	Peron Peninsula, Shark Bay, WA	25 56	113 32	TP	M	8 x 1920	1016	KF053526