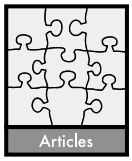

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A tangled tale of two teal: population history of the grey *Anas gracilis* and chestnut teal *A. castanea* of Australia

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Two Australian species of teal (Anseriformes: Anatidae: *Anas*), the grey teal *Anas gracilis* and the chestnut teal *A. castanea*, are remarkable for the zero or near-zero divergence recorded between them in earlier surveys of mitochondrial DNA (mtDNA) diversity. We confirmed this result through wider geographical and population sampling as well as nucleotide sampling in the more rapidly evolving mtDNA control region. Any data set where two species share polymorphism as is the case here can be explained by a model of gene flow through hybridization on one hand or by incomplete lineage sorting on the other hand. Ideally, analysis of such shared polymorphism would simultaneously estimate the likelihood of both phenomena. To do this, we used the underlying principle of the IMa package to explore ramifications to understanding population histories of *A. gracilis* and *A. castanea*. We cannot reject that hybridization occurs between the two species but an equally or more plausible finding for their nearly zero divergence is incomplete sorting following very recent divergence between the two, probably in the mid-late Pleistocene. Our data add to studies that explore intermediate stages in the evolution of reciprocal monophyly and paraphyletic or polyphyletic relationships in mtDNA diversity among widespread Australian birds.

Current interest in population genetics of waterfowl (Anseriformes), especially ducks (Anatidae), arises on several fronts. Firstly, anseriform birds are the principle natural reservoir of avian influenza (AI) viruses (Stallknecht and Shane 1988, Hanson 2003, Stallknecht and Brown 2008). The likelihood of spread of AI by avian vectors can be inferred if contemporary migratory connectivity within a potential host species and its phylogeographic structure are known (Winker et al. 2007, McCallum et al. 2008). Among Australian waterbirds, this places interest squarely in ducks with widespread distributions that extend beyond Australia into New Guinea and the island archipelago of Indonesia (Tracey et al. 2004). Interest in phylogeographic structure and migratory connectivity is heightened in this region because the highly pathogenic H5N1 strain of AI has recently been recorded in poultry in three villages in West Papua (OIE 2006). Second, substantial frequencies of natural hybridization have been recorded among many wild populations of ducks (McCarthy 2006). This can provide a challenging context against which to interpret molecular population genetic data, especially when species are very similar and may have been misidentified when sampled. Third, understanding the links between population structure and environmental constraints on bird movements is critical to successful conservation and management of species in any environment. This is especially so in environments with highly variable climates and patterns of resource availability, such as in much of Australia (see Stafford-Smith and Morton 1990, Roshier et al. 2001). Under such variability, it is even more instructive to test for links between contemporary patterns of gene flow and population discontinuities on the one hand with geographical variability in climate and environment on the other (Martinez-Meyer et al. 2004, Seavy et al. 2008).

Two species of Australian waterfowl the phylogeography and population genetics of which are of interest with regard to these inter-related issues are the grey teal *Anas gracilis* and chestnut teal *A. castanea*. *A. gracilis* occurs over most of Australia and through New Guinea and Indonesia west to the island of Java (Marchant and Higgins 1990, Kear and Hulme 2005). In contrast, *A. castanea* is more restricted and occurs primarily in temperate freshwater environments of south-eastern and south-western Australia (Frith 1982, Marchant and Higgins 1990; Fig. 1). No subspecies are currently recognized in either *A. gracilis* or *A. castanea*. Data

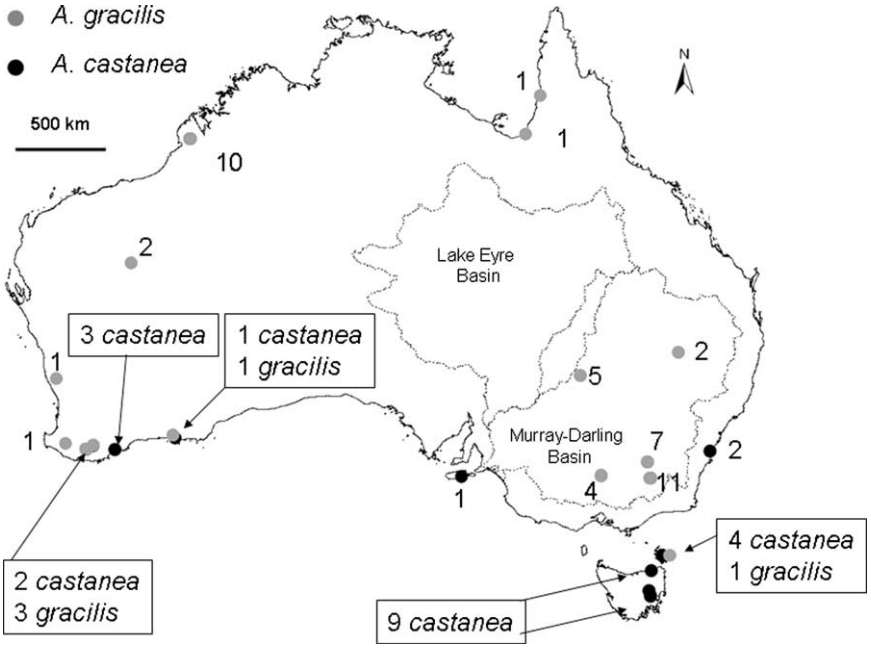

Figure 1. Localities of specimens sampled. Numbers indicate sample sizes. The Lake Eyre and Murray-Darling drainage basins are shown in outline by dotted lines.

from satellite-tracked *A. gracilis* showed very limited overlap in distribution between birds tagged in the Lake Eyre and Murray-Darling basins (Fig. 1) and tracked for up to two years (Roshier et al. 2006, 2008a). The satellite data also confirm that *A. gracilis* can quickly travel hundreds of kilometres between catchments in response to distant rainfall and flooding events (Roshier et al. 2008b). The observed variation in movement among individuals trapped at the same time and location raises the question of how different responses to environmental stimuli impact population genetic structure.

Estimates of mitochondrial DNA (mtDNA) divergence between *A. gracilis* and *A. castanea* observed in earlier work have been equal to or barely above zero (Johnson and Sorenson 1988, Sraml et al. 1996, Young et al. 1997, Kennedy and Spencer 2000), but such low sequence divergence in mtDNA between two species clearly suggests them to be very closely related. Typically, it is due to hybridization or recent speciation with the sharing of variation in daughter species retained from a recent common ancestor (Avise 2000, Joseph et al. 2006). Hybridization between *A. gracilis* and *A. castanea* is a plausible explanation of the earlier finding of low divergence. However, the single substantiated report of such hybridization was based on captive birds in England (Phillips 1923). Moreover, the only reference to hybridization in the wild has no supporting data (Marchant and Higgins 1990). Nonetheless, a substantial frequency of natural hybridization could be easily overlooked by field observers and specimen collectors because females and basic plumaged-males of both species are very similar and notoriously difficult to separate (Pizzey and Knight 1997). Against that, however, male *A. castanea* in alternate (nuptial) plumage resemble no other Australian duck. Male hybrids might be expected to be easier to recognize but have never been described.

This paper has two aims. The first is to test whether previous findings of zero and near-zero mtDNA diversity between the two species, which were based on just a few individuals and a few hundred base pairs of sequence from the cytochrome b and ND2 genes, were robust to more extensive sampling. We applied more extensive geographical sampling and used the more rapidly evolving control region. The second aim is to test whether hybridization or incomplete sorting of ancestral polymorphism can be discriminated as explanations of the low divergence. A key part of our approach has been to use the IMa package (Hey and Nielsen 2007). IMa ("isolation with migration") uses the observation that shared polymorphism can result from incomplete lineage sorting as well as hybridization and the resultant gene flow. Because either model can be fitted to a given data set, IMa's underlying principle is that it is more realistic to simultaneously estimate the likelihood of both. IMa does this by simultaneously evaluating divergence between two populations and the rate of gene flow between them. A key advantage of this and other coalescent methods is that they incorporate the stochasticity of mutation and genetic drift when calculating parameters.

Materials and methods

Sample collection and identification

We obtained 72 samples from throughout the Australian range of *A. gracilis* (n = 50) and *A. castanea* (n = 22) sourced through field surveys (blood stored in 70% or higher ethanol) or vouchered, cryofrozen tissue samples held by the Australian Natl. Wildl. Coll. (ANWC; Appendix; Fig. 1). Sampling was designed to span western and eastern extremes of mainland Australia and the island of Tasmania. To address the likelihoods of sample misidentification (see Introduction) and of whether interpretation of

molecular data from the two species might be compromised, LJ and KEO examined all ANWC voucher skins of samples used. Notwithstanding the possibility that some may have been backcross progeny descended from a past hybridization event, all but two of the *A. castanea* were males (Appendix 1). They were particularly easy to identify by plumage features typical of male *A. castanea* and showed no hint of hybridization. LJ and KEO then confirmed the identification of the only two female *A. castanea*, which were significantly darker in many plumage characters than any of the female or male *A. gracilis* in the study and showed diagnostic traits listed in Marchant and Higgins (1990), Pizzey and Knight (1997). In addition, we presented a third unbiased observer (see Acknowledgements) with study skins of fourteen of the female grey teal and the two female chestnut teal in the study. The skins were randomly intermixed without labels showing, and we provided no information on how many of the two species were present. The observer immediately discerned the only two female *A. castanea*, thus confirming the original plumage-based identifications. Finally, LJ and KEO examined two captive-bred specimens (again at ANWC) and identified as hybrids of *A. gracilis* and *A. castanea*. Both are males with obvious intermixing of the basic *A. gracilis* plumage with the alternate male *A. castanea* plumage. None of the ANWC voucher specimens of mtDNA samples that we used resembled these two hybrids. Although it would have been difficult for us to identify other partial hybrids (e.g., female F2 backcrosses), there was no hint from any of the voucher skins that any of the study birds were hybrids or in any way cause for mistaken species names. Thus, close examination of the voucher skins provided no reason to question the original identifications.

Molecular methods

DNA was extracted from blood and tissue samples using the DNAeasy extraction kit (Qiagen, Valencia, California) following the manufacturer's methods. Mitochondrial control region DNA was amplified using primers L78 (Sorenson and Fleischer 1996) and H774 (Sorenson et al 1999), the numbering referring to the position on the chicken mtDNA genome (Desjardins and Morais 1990). The following reaction conditions were used: approximately100 ng of DNA, 0.5U of Taq DNA polymerase, 2.5 mM $MgCl_2$, 1 × reaction buffer and dNTPs (200 μM), all supplied by Promega, and 200 nM of each primer in a 20 μl reaction. Amplification began with one cycle at 94° C at 90 s, followed by 38 cycles at 94° C for 20 s, 52° C for 20 s and 73° C for 90 s. PCR products were precipitated and resuspended then sequenced on both strands using each of the original primers and the BigDye terminator sequencing kit (Applied Biosystems) according to the manufacturers instructions. The sequence reactions were then resolved on an ABI3100 automated sequencer.

Data analysis

Geneious v3.7 (Drummond et al. 2007, Biomatters Ltd) was used to align and edit sequences from each strand for each individual and to align sequences of all individuals. Aligned sequences were used for analyses of molecular variance (AMOVA, Excoffier et al. 1992) as implemented in GenAlex (Peakall and Smouse 2006). Genetic structure was examined by testing the partitioning of variance within each species and between species using geographically defined groups of individuals. A statistical parsimony network of haplotypes was derived with TCS 1.21 (Clement et al. 2000). Nucleotide diversity statistics, tests for population stability vs increase or decline (Tajima's 1989 D, Fu's 1997 Fs and Ramos-Onsin and Rozas's 2002 R_2), and mismatch analyses were all generated in DnaSP 4.10.2 (Rozas et al. 2003). These are tests for reductions of old mutations and excesses of many low frequency younger alleles expected under a scenario of population growth (Fu 1997). Tests were done for Tajima's (1989) D, Fu's (1997) Fs, and Ramos-Onsin and Rozas's (2002) R_2).

Isolation with migration coalescent analyses

We used IMa (Hey and Nielsen 2007) and its model of isolation (i.e., sorting of divergent lineages) with migration (i.e., gene flow through hybridization) to simultaneously determine time since divergence of *A. gracilis* and *A. castanea* (t), effective population sizes of each species ($\theta_{gracilis}$ for *A. gracilis* and $\theta_{castanea}$ for *A. castanea*), the ancestral population size (θ_A), and immigration rates (m_1 and m_2) from one species into the other (m_1 = rate of migration from *A. gracilis* into *A. castanea* and vc for m_2, details below). IMa implements the same Isolation with Migration model as IM (Hey and Nielsen 2004), but estimates the joint posterior probability density of the model parameters, and allows log likelihood ratio tests of nested demographic models.

For optimization of the runs, wide prior distributions were assigned to parameters. This served to find a range of prior distributions that are fully contained within the bounds of the prior distribution. Flat prior distributions were obtained for all parameters except for current population sizes of *A. gracilis* where posterior distributions contained a distinct peak but the tail of which did not approach zero. In these cases, priors were set to encompass the peak, but the tail was cut near the point of flattening as suggested by Won and Hey (2005), and Kondo et al. (2008). To achieve convergence, final analyses consisted of a burn-in of 800,000 updates, 2,000,000 steps and a two-step linear heating scheme with five chains. Three runs with different random seed numbers resulted in similar parameter estimates and 95% HPDs with effective sample sizes (ESS) exceeding 120 for each parameter. A longer run of 1,000,000 updates and 10,000,000 steps also resulted in similar parameter estimates, however ESSs exceeded at least 500 for each parameter, so we only report results from the long run.

To convert IMa parameter estimates to biologically meaningful values, parameters were scaled to a substitution rate (μ) calculated previously for the mtDNA control region of wood ducks *Aix sponsa* (4.8×10^{-8} substitutions per site per year (s/s/y)). This substitution rate was determined based on the percentage sequence divergence of four pairs of closely related ducks (Peters et al. 2005). We also used a lower and upper limit to this estimation (3.1×10^{-8} and

6.9×10^{-8} s/s/y; Peters et al. 2005, 2008) when calculating biologically meaningful values from parameter estimates. The HKY model of mutation was used as in other studies using the same locus in birds (Kondo et al. 2008, Peters et al. 2008). An inheritance scalar was defined in the input file (0.25 for maternally inherited DNA) and this was corrected for using haploid mtDNA. Population size estimates from IMa are therefore comparable to that of a diploid autosomal locus. For calculations of effective population sizes ($\theta = 4N_e\mu$), where N_e is the effective population size and θ is the population size scaled to the substitution rate per generation. To estimate generation time (G) we used the equation $G = \alpha + (s/(1-s))$, where α is the age of maturity and s is the expected adult survival rate (Sæther et al. 2005) as two years for teal using data in Braithwaite and Norman (1974). Threfore, μ was multiplied by a generation time of 2 years to calculate effective population sizes. For migration estimates m_1 and m_2: $m_1 = m_1/\mu$, where m_1 is the parameter estimate in IMa and μ is the substitution rate per locus per generation, and similarly for m_2. Estimates of t was converted to real time (t) using $t = t\mu$.

Results

Partial control region I sequences (609 base pairs) were obtained for 50 grey teal and 22 chestnut teal and have been deposited in Genbank (accession numbers EU846117to EU846188). Haplotype diversities were high whether in the total, *A. gracilis* or *A. castanea* samples (0.99, 0.99 and 0.97, respectively). Nucleotide diversities within *A. gracilis* and *A. castanea* were 1.37% and 1.25%, respectively, and net divergence between them, Da, was 0.02%. Haplotype diversity is reported in Table 1 and is explored further in an unrooted network of the sequences (Fig. 2). Inspection of the network readily shows the high nucleotide diversity. Further, the sequences do not fall into two groups corresponding with species identifications, which we have earlier shown are reliable. The total number of haplotypes was 53. Ten haplotypes were shared by up to five individuals and four of these haplotypes were found in both species, sometimes from widely scattered localities. For example, of three *A. gracilis* and one *A. castanea* that shared a haplotype, one *A. gracilis* (ANWC 50757) was collected at Broome in north-western Australia, whereas the *A. castanea* (ANWC 29938) was collected on the opposite side of the continent south of Sydney in coastal mainland south-eastern Australia. Similarly, two other haplotypes were also shared by *A. gracilis* from southeastern (ANWC 50097, 51290) and southwestern Australia (ANWC 50318), and *A. castanea* from Tasmania (DPIW CT132). Nonetheless, the sequences of *A. castanea* are mostly in two discrete parts of the network.

Partitioning the diversity by species in AMOVA reiterated the finding of low net divergence between the two species. Zero of the variance in the network (Fig. 2) is attributable to between-species variation ($P = 0.43$). In contrast, analyses within the two species show that 16% of the variance in *A. castanea* is attributable to between-population variation ($P < 0.01$) whereas in *A. gracilis* 0% of the variance is due to its among-population variation ($P = 0.81$). Mismatch curves (Fig. 3) generated from either species alone or from both species pooled are unimodal. Fu's Fs was significant but Fu and Li's F* and D* were not; Ramos-Onsins and Rozas's R2 and Tajima's D were not significant (Table 1).

In the IMa analysis, unimodal distribution of parameter estimates was achieved (Fig. 4) except for $\theta_{gracilis}$ where both extent and magnitude of the range of values were large and the tail did not approach zero (Fig. 4a). Setting larger priors for this parameter did not improve its convergence, nor did it change the peak position. Although effective population sizes did increase with larger priors, estimates were always higher for $\theta_{gracilis}$ than for $\theta_{castanea}$, therefore we only report conservative measures with a prior encompassing the peak but not the entire tail for this parameter. The posterior distribution of $\theta_{castanea}$ peaked at 77.0 (95% HPD = 20.6 to 276.6), $\theta_{gracilis}$ peaked at 248.6 (95% HPDs not shown because the tails of the distributions did not reach zero), and $\theta_{ancestral}$ peaked at 21.8 (95% HPD = 2.8 to 61.1). The estimated posterior distribution for $\theta_{gracilis}$ is therefore approximately 3.2 times higher than for $\theta_{castanea}$ and 11.4 times higher than for $\theta_{ancestral}$. Converting these densities into biologically meaningful values by using the geometric mean of the mutation rates (2.9×10^{-5} substitutions per locus per year) and generation time of 2 y, we estimated effective population sizes of *A. castanea* to be 330,900 (95% HPD = 88,600 to 1,188,600), relatively small compared to that of *A. gracilis*, which peaked at 1,068,300 individuals. Estimated effective populations size of the ancestral population was small, 93,800 (95% HPD = 12,000 to 262,500) individuals, which suggests substantial population expansion after the populations diverged. Coalescent analyses suggest that *A. gracilis* and *A. castanea* most likely diverged about 103,000 years before present (ybp), with a possible range of 70,000 to 165,000 ybp.

The analyses showed non-zero peaks for both directions of gene flow. This could reflect multiple gene flow events between *A. gracilis* and *A. castanea,* however, high posterior probabilities at the lower limit of m_1 and m_2, indicate that

Table 1. Diversity statistics and tests of population expansion versus stability. *$P < 0.05$; NS - not significant.

	Haplotype diversity	Nucleotide diversity	Fs	R_2	F*	D*	Tajima's D
Grey	0.99	0.014	−26.59 (−6.88, 7.62)*	0.07 (0.05, 0.17) NS	−1.33 (−2.23, 1.64)	−1.20 (−2.35, 1.29)	−0.97 (−1.67, 1.70)
Chestnut	0.97	0.013	−5.37 (−5.22, 5.03)*	0.11 (0.08, 0.19) NS	−1.04 (−2.47, 1.53)	−1.00 (−2.41, 1.28)	−0.64 (−1.73, 1.66)
All	0.986	0.014	−41.34 (−8.05, 9.21)*	0.07 (0.05, 0.17) NS	−1.63 (−2.30, 1.69)	−1.56 (−2.55, 1.38)	−1.06 (−1.77, 1.99)

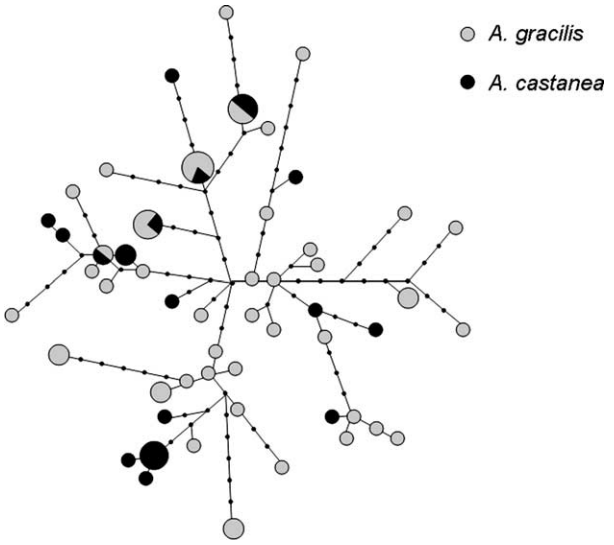

Figure 2. Unrooted statistical parsimony network of control region sequences for *A. gracilis* and *A. castanea*. Unsampled haplotypes are shown as small circles.

zero gene flow is nearly as well supported by the data as are nonzero gene flow levels (Figure 4e).

Discussion

We set out to test and refine an earlier finding of extraordinarily low divergence between *A. gracilis* and *A. castanea* (Sraml et al. 1996, Kennedy and Spencer 2000). We used an expanded data set based on 72 individuals (*A. gracilis*, n = 52, *A. castanea* n = 20) from across mainland Australia and Tasmania and examined 609 base pairs of the mtDNA control region. We confirmed very low net divergence in mtDNA (0.02%) between the two species despite high diversities within them. Geographical structure was evident in AMOVA between the isolated populations of *A. castanea* in south-eastern and south-western Australia (16% o variance, $P < 0.01$). Within both species and for the pooled samples, we found generally close fit with expectations of geographically unstructured diversity being due to population expansions (mismatch analyses, Fig. 3) and not selection (pattern of significance and non-significance of test statistics in Table 1, see Fu 1997). We hypothesize that this equates with range expansions after the Last Glacial Maximum at 18–20,000 years ago (ybp). With the earlier result of near-zero divergence between the two species confirmed, we then used IMa (Hey and Nielsen 2007) to estimate divergence times and to evaluate whether low divergence between the two species could be ascribed to hybridization or incomplete sorting of ancestral polymorphism. We simultaneously estimated the likelihoods of the low net divergence between the two species being due to hybridization or incomplete sorting of the polymorphism of a common ancestor. The two species appear to have diverged in the late Pleistocene within a range from 70,000 to 165,000 ybp. Despite some ambiguity in the IMa analysis, its key results are the high posterior probabilities at the lower limit of m_1 and m_2. These are the two rates of gene flow from one species into the other. The low or zero gene flow levels suggests that incomplete lineage sorting is the most likely explanation for shared haplotypes between the two species, and that it is not due to hybridization. We have already noted that the scant evidence for hybridization between these two species in the wild is limited to personally communicated sight records (see Introduction). Further to the high posterior probabilities at the lower limits of migration estimates, there are two inter-related factors suggesting that incomplete sorting of ancestral polymorphism is an equally if not more plausible explanation of our findings. First is that we have estimated a very recent range of time in which the two species appear to have diverged from a common ancestor (from 70,000 to 165,000 ybp). Second is the expectation that the number of generations that mtDNA takes to sort to reciprocal monophyly is a function of population size (Neigel and Avise 1986). We have estimated the current effective population size of *A. gracilis* as nearly one million. Together, these two points suggest that we would not yet expect the process of lineage sorting to have reached reciprocal monophyly.

We also make a comparative comment on the effective population sizes of *A. gracilis* and *A. castanea*. The key finding was different orders of magnitude in the estimates

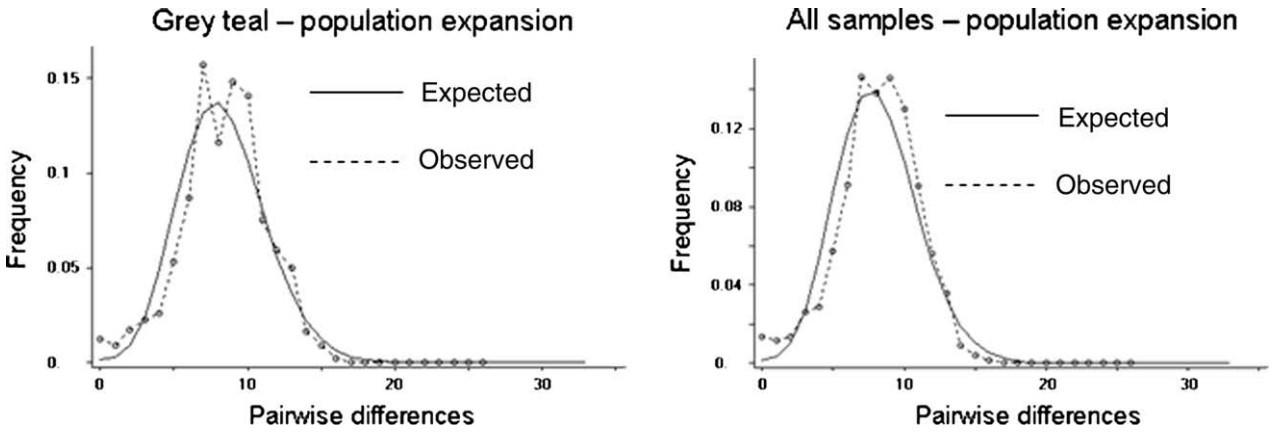

Figure 3. Mismatch analysis curves for *A. gracilis* and for both species combined under conditions of population growth. Expected and observed curves are shown as continuous and dotted lines, respectively.

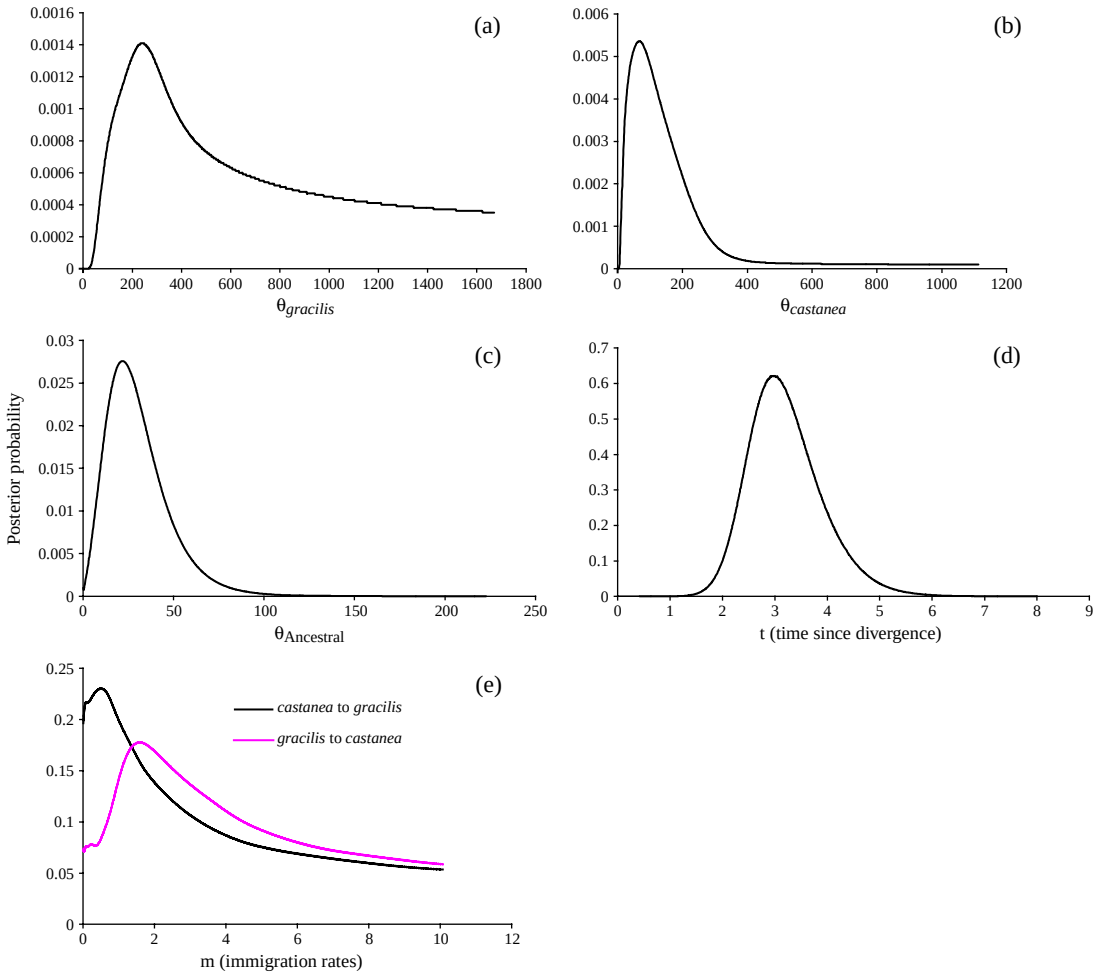

Figure 4. Estimated demographic parameters scaled to the neutral mutation rate of the divergence between *A. gracilis* and *A. castanea* calculated using IMa. (a) current *A. gracilis* effective population size, (b) current *A. castanea* population size, (c) ancestral population size, (d) time since the divergence of *A. castanea* and *A. gracilis*, (e) immigration rates between *A. castanea* and *A. gracilis*. The limits of the x-axis indicate the upper limits of the chosen prior probabilities (see text).

for the two species, the continent-wide *A. gracilis* having a three times larger estimate than the more restricted, southern Australian *A. castanea*. Caution is certainly warranted in interpreting the absolute values estimated by our analysis. We do suggest, however, that an estimate of approximately one million for a continent-wide Australian waterbird subject to massive population fluctuations is not beyond reason.

The stages that mtDNA polymorphism can be expected to go through while sorting from ancestral polymorphism to reciprocal monophyly in daughter species have been described and elaborated by Neigel and Avise (1986), Avise (2000) and Omland et al. (2006). Early stages in this process have been identified at which some haplotypes are still shared but at which mutation is starting to generate novel haplotypes in each species. *A. gracilis* and *A. castanea* seem to fit well with that stage of intermediate divergence (Fig. 2).

Our data provide another example from Australian birds of two closely related species, here the grey teal *A. gracilis* and chestnut teal *A. castanea*, with paraphyletic mtDNA gene trees. The pattern in our data of almost zero net divergence between two species recalls similar findings in two Australian species of woodswallows (Passeriformes: *Artamus* spp) (Joseph et al. 2006, see also Joseph and Wilke 2006). A review of Australian avian phylogeography to date (Joseph and Omland 2009) suggested that paraphyletic relationships among mtDNAs of well-marked, widespread taxa such as teal, woodswallows and parrots is ultimately to be expected as a consequence of the Australian continent not having been glaciated in the Pleistocene. That is, *A. gracilis* and *A. castanea* likely did not undergo as severe population bottlenecks as north temperate species during Pleistocene glacial maxima. Furthermore, this would have been at times when the mtDNA diversity of the teal was only in initial stages of sorting to eventual reciprocal monophyly, which clearly still has not been attained. Note here that the IMa analysis also suggested population expansion after a bottleneck. This suggests that relatively high nucleotide diversity may have been present in their ancestor. Population expansion from refugia following climatic amelioration after the Last Glacial Maximum at 18–20,000 ybp, especially in recently diverged birds capable of spreading across the continent's interior such as *A. gracilis*, would result in the geographically unstructured low diversity we have recorded here in that species.

A. castanea, on the other hand, being ecologically more restricted to temperate southern habitats appears to have experienced geographical isolation between its eastern and western populations very recently. Multilocus data would be helpful in pursuing this result further (Edwards and Beerli 2000, Jennings and Edwards 2005).

The question arises of how to reconcile our findings from molecular data about population structure (or its absence) and historical demography with taxonomy and fossil evidence. Taking our earlier conclusion that incomplete sorting is feasibly involved in explaining our data with the biological differences between the two teal (reviewed in Marchant and Higgins 1990), we do not advocate changing the taxonomic recognition of *A. gracilis* and *A. castanea* as separate species under any species concept.

Next we note that phenotypically *A. gracilis* most closely resembles the Indonesian Teal *A. gibberifrons* from which it is diagnosed as distinct principally on characters of skull osteology (Parker et al. 1985) and plumage of downy young (Young and Brayshaw 2004, Young and Kear 2006). Like earlier authors who have studied molecular genetics of the various southern hemisphere teal (see Young et al. 1997, Young and Brayshaw 2004), we have been unable to include *A. gibberifrons* in our analyses. Confirmation of near-zero divergence between the two Australian teal *A. gracilis* and *A. castanea* nonetheless signals the need for further work to examine the following questions: 1) What are the relationships among the various species and populations of ''austral teal'' especially those in the Australian and Indo-Malay region that have been considered to be forms of *A. gracilis* and/or *A. gibberifrons* (see Young et al. 1997, Young and Kear 2006)? 2)Are *A. gracilis* and *A. gibberifrons* similar to each other in external phenotype because of retained ancestral character states or close relationship?

Concerning fossils, more work is needed to meet the challenge of understanding the connections between fossils of Australia anatids and extant species, especially Pliocene fossils identified as species closely resembling *A. gracilis* and *A. castanea* (Olson 1977, Worthy 2008). Extinction of closely related similar species, rapid homoplastic morphological change and complex demographic histories, or a combination of these factors could be involved in reconciling molecular and fossil data sets.

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Appendix 1. Specimens examined of *A. gracilis* and *A. castanea* with their localities shown for brevity (full details available from Australian National Wildlife Collection) and Genbank accession numbers of sequences. Latitudes (south) and longitudes (west) are decimalized and rounded to two decimal places. Abbreviations (other than conventional abbreviations of directions such as N for north, and NW for northwest): ANU-Australian National University Botany and Zoology Northern Connections project; ANWC-Australian National Wildlife Collection; DPIW – Department of Primary Industries and Water, Tasmania; NSW-New South Wales; WA-Western Australia; SA-South Australia; Tas – Tasmania; QLD – Queensland; ca-approximately.

Reg. No.	Species	State	Locality	Latitude	Longitude	GenBank accession
ANWC 29939	*A. castanea*	NSW	ca 6 km SW Dapto	34.54	150.75	EU846131
ANWC 42512	*A. castanea*	SA	Kangaroo Isl.	35.90	137.42	EU846132
ANWC 45602	*A. castanea*	TAS	Flinders Isl.	40.13	148.20	EU84612
ANWC 45603	*A. castanea*	TAS	Flinders Isl.	40.13	148.20	EU846128
ANWC 45604	*A. castanea*	TAS	Flinders Isl.	40.13	148.20	EU846127
ANWC 45700	*A. castanea*	TAS	Flinders Isl.	40.13	148.20	EU846129
ANWC 50193	*A. castanea*	WA	ca 17 km E Esperance	33.82	122.07	EU846133
ANWC 50259	*A. castanea*	WA	Mt Barker area	34.24	117.65	EU846134
ANWC 50285	*A. castanea*	WA	Mt Barker area	34.47	117.30	EU846135
ANWC 50437	*A. castanea*	WA	ca 52 km W Bremer Bay	34.44	118.81	EU846136
ANWC 50438	*A. castanea*	WA	ca 52 km W Bremer Bay	34.44	118.81	EU846137
ANWC 50439	*A. castanea*	WA	ca 52 km W Bremer Bay	34.44	118.81	EU846138
DPIW CT127	*A. castanea*	TAS	Waterhouse Lake	40.95	147.60	EU846119
DPIW CT132	*A. castanea*	TAS	Waterhouse Lake	40.95	147.60	EU846125
DPIW CT135	*A. castanea*	TAS	Ross	42.02	147.48	EU846124
DPIW CT136	*A. castanea*	TAS	Ross	42.02	147.48	EU846123
DPIW CT146	*A. castanea*	TAS	Rushy Lagoon	42.33	147.58	EU846122
DPIW CT147	*A. castanea*	TAS	Rushy Lagoon	42.33	147.58	EU846121
DPIW CT150	*A. castanea*	TAS	Rushy Lagoon	42.33	147.58	EU846120
DPIW CT151	*A. castanea*	TAS	Rushy Lagoon	42.33	147.58	EU846117
DPIW CT154A	*A. castanea*	TAS	Ross	42.02	147.48	EU846118
ANWC 34160	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846152
ANWC 34161	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846156
ANWC 34162	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846154
ANWC 34163	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846149
ANWC 34164	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846155
ANWC 34165	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846153
ANWC 34166	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846150
ANWC 34167	*A. gracilis*	NSW	ca 55 km E Albury	35.99	147.58	EU846151
ANWC 50087	*A. gracilis*	NSW	ca 6 km SE Mathoura	35.83	144.93	EU846164
ANWC 50097	*A. gracilis*	NSW	Mathoura	35.87	144.94	EU846163
ANWC 50098	*A. gracilis*	NSW	Mathoura	35.87	144.94	EU846159
ANWC 50102	*A. gracilis*	NSW	Mathoura	35.87	144.94	EU846157
ANWC 50116	*A. gracilis*	NSW	Mullawoolka Basin	30.49	143.79	EU846165
ANWC 50139	*A. gracilis*	NSW	Mullawoolka Basin	30.49	143.79	EU846160
ANWC 50140	*A. gracilis*	NSW	Mullawoolka Basin	30.49	143.79	EU846161
ANWC 50172	*A. gracilis*	NSW	Mullawoolka Basin	30.49	143.79	EU846158
ANWC 50175	*A. gracilis*	NSW	Mullawoolka Basin	30.49	143.79	EU846162
ANWC 51156	*A. gracilis*	NSW	ca 84 km W Moree	29.23	149.08	EU846169
ANWC 51158	*A. gracilis*	NSW	ca 84 km W Moree	29.23	149.08	EU846167
ANWC 51286	*A. gracilis*	NSW	ca 55 km E Albury	35.97	147.52	EU846166
ANWC 51288	*A. gracilis*	NSW	ca 55 km E Albury	35.97	147.52	EU846168
ANWC 51290	*A. gracilis*	NSW	ca 55 km E Albury	35.97	147.52	EU846170
ANU 07121101	*A. gracilis*	NSW	Wagga Wagga	35.13	147.40	EU846144
ANU 07121102	*A. gracilis*	NSW	Wagga Wagga	35.13	147.40	EU846145
ANU 07121103	*A. gracilis*	NSW	Wagga Wagga	35.13	147.40	EU846148
ANU 07121107	*A. gracilis*	NSW	Wagga Wagga	35.13	147.40	EU846146
ANU 07121109	*A. gracilis*	NSW	Wagga Wagga	35.13	147.40	EU846142
ANU 09154211	*A. gracilis*	NSW	Wagga Wagga	35.13	147.40	EU846143
ANU 09154213	*A. gracilis*	NSW	Wagga Wagga	35.13	147.40	EU846147
ANWC 29560	*A. gracilis*	QLD	ca 4 km S Karumba	17.50	140.85	EU846139
ANWC 51597	*A. gracilis*	QLD	Kowanyama	15.45	141.64	EU846141
ANWC 45701	*A. gracilis*	TAS	Flinders Isl.	40.13	148.20	EU846140
ANWC 32999	*A. gracilis*	WA	ca 300 km N Meekatharra	24.44	119.68	EU846181
ANWC 33000	*A. gracilis*	WA	ca 300 km N Meekatharra	24.44	119.68	EU846182
ANWC 50204	*A. gracilis*	WA	ca 20 km N Esperance	33.68	121.91	EU846186
ANWC 50258	*A. gracilis*	WA	Mt Barker area	34.21	117.67	EU846188
ANWC 50283	*A. gracilis*	WA	Mt Barker area	34.42	117.24	EU846176
ANWC 50284	*A. gracilis*	WA	Mt Barker area	34.47	117.30	EU846184
ANWC 50318	*A. gracilis*	WA	ca 11 km N of Manjimup	34.14	116.16	EU846172
ANWC 50370	*A. gracilis*	WA	ca 5 km NW of Dandaragan	30.64	115.67	EU846173
ANWC 50724	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846187
ANWC 50725	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846180
ANWC 50757	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846178
ANWC 50758	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846174
ANWC 50759	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846175

Appendix 1 (*Continued*)

Reg. No.	Species	State	Locality	Latitude	Longitude	GenBank accession
ANWC 50760	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846183
ANWC 50825	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846171
ANWC 50826	*A. gracilis*	WA	73–83 km E Broome	17.77	122.86	EU846177
ANWC 50827	*A. gracilis*	WA	73–83 km E Broome	17.78	122.89	EU846179
ANWC 50828	*A. gracilis*	WA	73–83 km E Broome	17.78	122.89	EU846185