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ORIGINAL PAPER

Low phylogeographic structure in a wide spread endangered Australian frog *Litoria aurea* (Anura: Hylidae)

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Abstract The green and golden bell frog (*Litoria aurea*) has a widespread distribution along the south-east coast of Australia. The species range, however, is highly fragmented and remaining populations are predominately isolated and restricted to the coastline. Previously, the range extended further inland and the species was considered common. Here we report a study designed to identify the phylogeographic and conservation genetic parameters of *L. aurea*. Mitochondrial DNA sequences were examined from 263 individuals sampled from 26 locations using both phylogenetic and population analyses. Despite a general consensus that amphibians are highly structured we found no phylogeographic divisions within the species, however, there was significant structure amongst extant populations (F_{ST}=0.385). Patterns of haplotype relatedness, high haplotypic diversity (mean h=0.547) relative to low nucleotide diversity (mean π=0.003) and mismatch distribution analysis supported a Pleistocene expansion hypothesis with continued restricted dispersal and gene flow. We conclude that the genetic structure of the species may permit 'well managed' intervention to mediate gene flow amongst isolated populations and provide some guidelines for the implementation of such conservation strategies.

Keywords mtDNA · Phylogeography · Population structure · Amphibian · Hylid · *Litoria aurea*

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Introduction

An understanding of the evolutionary history and genetic structure of species is of critical importance when designing conservation strategies. This knowledge allows for the definition of management units (e.g. Moritz 1994a) and the design of strategies aimed at minimising genetic erosion whilst preserving genetic distinctiveness (Hedrick 2001).

Historical and contemporary processes shape the genetic structure of species and therefore a combination of analyses targeted at different temporal scales is needed to help delineate not only genetic structure but also the historic and contemporary processes that have shaped it (Althoff and Pellmyr 2002; Godoy et al. 2004).

Cyclical climatic fluctuations, particularly during the Pleistocene, have been highly influential in shaping the current distribution and population genetics of many plants and animals (Hewitt 2000). During the colder, more arid cycles of glacial maxima mesic habitats retracted into refugia, which is believed to have fostered allopatric divergence between isolated populations (Avise 2000). In

the northern hemisphere a major feature of the Quaternary was the advance and retreat of large ice sheets and numerous phylogeographic studies have now illustrated the effect of this period on the genetic structure of current biota (reviewed in Hewitt 2000). In contrast, Australia experienced limited glaciation (Markgraf et al. 1995; McKinnon et al. 2004) but nevertheless glacial-interglacial fluctuations in sea level, rainfall, humidity and temperature caused significant changes in vegetation communities resulting in complex patterns of habitat fragmentation, expansion and contraction across the continent (Kershaw 1981; Nix 1982; Schauble and Moritz 2001). Typically, mesic habitats retracted to coastal regions and previous studies in coastal Queensland (QLD) and far northern New South Wales (NSW) have shown that rainforest restricted species show intraspecific genetic structuring strongly influenced by historical contractions of rainforests in response to climate change (Joseph et al. 1995; McGuigan et al. 1998; Schneider et al. 1998).

Relative to rainforest species there is little known about the phylogeography of south-eastern Australian fauna, however, recent studies have begun to address this deficiency. For example, Schauble and Moritz (2001) investigated the phylogeographic structure of two frog species *Limnodynastes peronii* and *Lim. tasmaniensis* and compared resulting intraspecific phylogenies to an initial study in *Litoria fallax* (James and Moritz 2000). Despite limited sampling across NSW Schauble and Moritz (2001) detected a phylogeographic break positioned somewhere south of the McPherson Range (at the NSW/QLD border) and north of Sydney. James and Moritz (2000), however, did not find a break in this area for *L. fallax*. More recently, Nicholls and Austin (2005) investigated the phylogeography of the satin bowerbird (*Ptilonorhynchus violaceus*) where the southern subspecies was sampled comprehensively across NSW and Victoria. Although there was significant population structure the study found limited phylogeographic structure across NSW and Victoria.

Historically the distribution of *L. aurea* extended from northern NSW, as far as Ballina, to East Gippsland in Victoria, with inland populations as far west as Bathurst and Tumut (Goldingay 1996; White and Pyke 1996, Fig. 1). Through to the early 1980s *L. aurea* was considered common but since has undergone dramatic declines with disappearances reported from 80% of its former range (White and Pyke 1996 as cited in Pyke et al. 2002). Remaining populations are mostly fragmented and typically restricted to the coastline, extending from Yuraygir National Park (northern NSW) to East Gippsland (Victoria); the two most inland populations are from the Southern Tablelands near Queanbeyan and the Upper Hunter near Mt. Owen (A. White and R. Wellington pers. com., see also Pyke and White 2001, Fig. 1 and Table 1). Currently

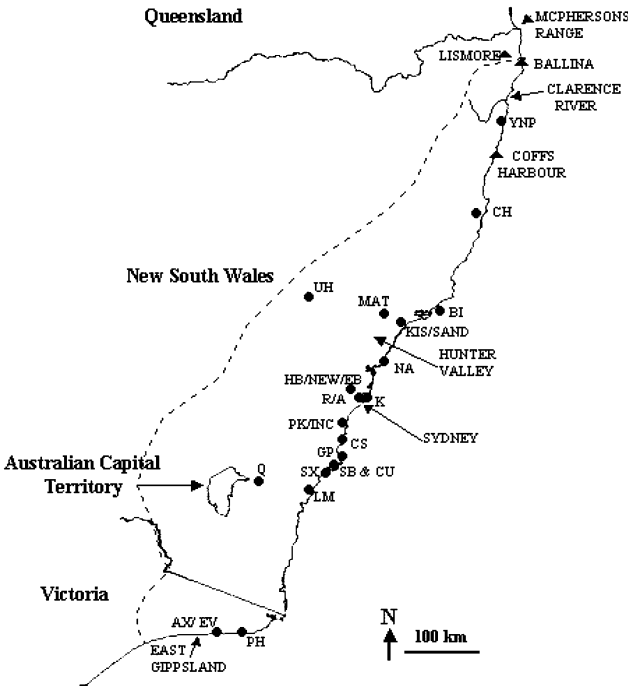

Fig. 1 Map of the south eastern coastline of Australia. The map details the location of 26 green and golden bell frog populations (●) sampled for this study. The location of the sampled sites ranged from the far north coast of New South Wales (NSW) to east Gippsland in Victoria (Vic). Due to the large scale of the study area a single point represents some sites sampled within close geographic proximity. The corresponding geographical regions for these locations are given in Table 1. Note BI is an island situated approximately 3 km off the coast. Important geographical references made in the text are also shown (← or ▲). The dashed line indicates the approximate historic range of the species

L. aurea is listed as 'Vulnerable' nationally (*Environmental Protection and Biodiversity Conservation Act 1999*) and 'Endangered' in New South Wales (*Threatened Species Conservation Act 1995*).

Litoria aurea inhabits wetlands, open forest and grassland habitat; breeding site characteristics vary but generally consist of still, relatively unshaded water bodies low in salinity (Pyke et al. 2002). The species is thought to have a high dispersal capability with recorded movement in excess of 10 km from known breeding ponds (Pyke and White 2001) but most frequent capture-recapture data is within a 500 m radius (Christy 2001, Pyke and White 2001). A previous microsatellite study detected significant population structuring throughout the species range, however, the authors also found evidence of weak genetic structuring within areas of continuous habitat (Burns et al. 2004).

Typically amphibians are expected to show pronounced phylogeographic structure because they are small bodied and presumed to be relatively immobile (Avise 2000; Palo et al. 2004). However, limited phylogeographic structure has been detected in frog species (or other terrestrial vertebrates) south of the McPherson Range (McGuigan et al.

Table 1 Summary of samples used in this study

Sample location	Region	AMG Easting (m)	AMG Northing (m)	*n*
Yuraygir National Park (YNP), NSW	Upper North Coast	523,600	6,687,400	2
Crescent Head (CH), NSW	Upper North Coast	501,009	6,558,000	13
Broughton Island (BI), NSW	Lower North Coast	436,400	6,391,100	15
Kooragang Island (KIS), NSW	Hunter	379,700	6,363,500	10
Sandgate (SAND), NSW	Hunter	378,586	6,362,935	10
Maitland (MAT), NSW	Hunter	343,947	6,409,566	7
Upper Hunter (UH), NSW	Hunter	321,857	6,412,214	2
North Avoca (NA), NSW	Central Coast	354,488	629,6251	11
Homebush (HB), NSW	Sydney	321,700	6,253,700	25
Newington (NEW), NSW	Sydney	321,500	6,255,500	18
Enfield Brickpit (EB), NSW	Sydney	321,242	6,247,237	2
Rosebery (R), NSW*	Sydney	333,569	6,247,461	5
Arncliffe (A), NSW**	Sydney	329,250	6,242,800	9
Kurnell (K), NSW	Sydney	331,300	6,233,700	10
Port Kembla (PK), NSW	Illarwarra	306,800	6,181,350	19
Incitec-Port Kembla (INC), NSW	Illarwarra	307,840	6,182,781	5
Coomonderry Swamp (CS), NSW	Shoalhaven	291,833	6,145,613	10
Brundee Swamp (SB), NSW	Shoalhaven	286,168	6,132,202	16
Greenwell Point (GP), NSW	Shoalhaven	292,100	6,134,400	10
Culburra (CU), NSW	Shoalhaven	296,416	6,131,281	9
Sussex Inlet (SX), NSW	Shoalhaven	279,300	6,105,600	10
Lake Meroo (LM), NSW	Shoalhaven	262,947	6,070,124	10
Captain-Flat Queanbeyan (Q), NSW	Southern Tablelands	171,196	6,074,574	10
Point Hicks-Cann River (PH), VIC	North East Victoria	162,147	5,846,114	10
Alcox-Bemm River (AX), VIC	North East Victoria	145,846	5,814,335	10
Evans-Bemm River (EV), VIC	North East Victoria	146,101	5,815,053	5

Geographical coordinates of sampled locations in Australian Map Grid units (zone 56 Easting and Northing) and the number of individuals sampled (n) per location are detailed. Sample location abbreviations are given in parentheses

*All captive individuals held at Taronga Zoo, Sydney

**Five samples from captive individuals held at Taronga Zoo, Sydney

1998; James and Moritz 2000; Schauble and Moritz 2001) and from what we know of the ecology and population structure (see Burns et al. 2004) of *L. aurea* we may expect little phylogeographic structure to be present.

Here we comprehensively describe the phylogeographic structure of *L. aurea*. Extant populations are examined from throughout the species' current range and the resulting pattern of haplotype relatedness, in conjunction with demographic analyses, is used to infer the recent evolutionary history of the species. We also analyse population structure to compliment the previous microsatellite study (see Burns et al. 2004) and to further investigate current genetic structuring and the history of dispersal. Finally, the findings are used to make conservation recommendations.

Material and methods

Tissue sampling and outgroup choice

Litoria aurea tissue samples (toe clips) were collected from 263 individuals from 26 locations. Sampling was extensive, covering nine regions from throughout the species' range from the far north coast of New South Wales to East Gippsland in Victoria (Fig. 1 and Table 1). These geographical regions were proposed by the NSW National Parks and Wildlife Service (NPWS) for conservation management and were based on the local government boundary areas in which remaining known populations occur but also includes adjoining local government areas within the historic distribution and hence the full extent of known habitat for *L. aurea* (NSW NPWS 2002).

In the phylogenetic analyses, *Litoria raniformis* was used as the outgroup taxon based on the findings of Burns and Crayn (in press) which demonstrated *L. aurea* and *L. raniformis* are sister taxa.

Mitochondrial DNA extraction, amplification and sequencing

Total genomic DNA was extracted using a standard phenol–chloroform extraction protocol (Sambrook et al. 1989), or a High Pure PCR Template Preparation Kit (Roche Applied Science, Australia) following the manufacturers instructions.

Two protein-coding genes *cytochrome oxidase 1 (COI)* and *NADH dehydrogenase subunit 4* (*ND4*) were chosen for sequencing because both had previously proven informative

in studies of frog phylogeography (James and Moritz 2000; McGuigan et al. 1998; Schauble et al. 2000; Mahony et al. 2001). These genes were therefore expected to be evolving at rates appropriate to provide sufficient information to investigate phylogenetic relationships among *L. aurea* samples. Two genes were considered necessary because initial analyses of *ND4* indicated comparatively low levels of variation in *L. aurea* (Burns and Crayn, in press).

COI was amplified for ten *L. aurea* and two *L. raniformis* samples using primers COX and COY described in (Schneider et al. 1998). These sequences were then used to design internal *L. aurea* specific primers COI-smallF (Forward primer 5′-TTGGCCTGCTAGGTTTTATTG-3′) and COI-smallR (Reverse primer 5′-CAAATACGGCCCC-CATAGAT-3′), which amplified an approximate 330 bp product. *ND4* was amplified using the same approach. Initially primers 'Limno2' (Schauble et al. 2000) and 'ND4' (Arèvalo et al. 1994) were employed and then internal *L. aurea* specific primers (designed by the first author) 'ND4-3' (forward, 5′-TTAGCAGGAACACTTCTAAAACTAG-3′) and 'ND4-1' (reverse, 5′-GAAAGTGTTTAGCTTTCATC TCTAG-3′) were used which amplified an approximate 750 bp product.

The target regions for both *COI* and *ND4* were sequenced for all 263 *L. aurea* sampled. Target DNA was usually amplified in 25 μl PCR reactions, which comprised: template DNA (~50–200 ng), 2.5 μl 10×reaction buffer, 3 mM $MgCl_2$, 1 mM dNTPs, 0.5 μM each primer and 0.5 U Tth^+ (Biotech Australia) DNA polymerase. Most often the following MJ Research PTC-100 'step down' thermal cycling profile was employed with the hot lid enabled: (1) initial 2 min denaturation at 94°C, (2) 37 cycles of denaturation (30 s at 94°C), annealing (1 min at 60°C—2 cycles; 58°C—2 cycles; 56°C—10 cycles; 54°C—23 cycles), and extension (45 s at 72°C), (3) 5 mins at 72°C. Amplified products were visualised by ethidium bromide staining of 1% agarose gels.

Following purification by PEG precipitation, sequencing reactions were performed in both directions using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems v3.1, Foster City, USA) according to the manufacturers specifications, on a MJ Research PTC-100 (GeneWorks, Hindmarsh, Australia). Sequencing fragments were purified by ethanol precipitation and visualised using an ABI 3730 capillary sequencer (Applied Biosystems v3.1). For each individual forward and reverse sequences were evaluated and a consensus derived using AUTOASSEMBLER 1.3.0 (Applied Biosystems).

Sequence alignment and haplotype designation

Consensus sequences were aligned by eye and trimmed to 324 bps (*COI*) and 524 bps (*ND4*); there were no internal gaps so the alignment for both genes was unproblematic. Variable sites were verified from the chromatograms and sequences translated (using MacClade version 4.0, Maddison and Maddison 2000) and aligned in accordance with previously deposited *Litoria* sequences in GenBank (e.g. *COI* – AF198261-347, published in James & Moritz 2000; *ND4* – AF282598-608, published in Mahony et al. 2001). Haplotypes for each gene and both genes combined were identified using MacClade version 4.0. Sequence data was assumed to be mitochondrial and not nuclear homologs because of the absence of stop codons, the bias against guanine, a notable third codon substitutional bias and an overall conservation of codons among the 263 ingroup samples and two outgroup samples. To test for substitutional saturation, plots of pairwise uncorrected ('p') distances against maximum likelihood distances (model TrN+I see below; Tamura and Nei 1993) were generated for the third codon position of both genes.

Phylogenetic analyses

Phylogenetic relationships among mtDNA haplotypes of *COI*, *ND4* and combined data were estimated under maximum parsimony (MP), maximum-likelihood (ML) and neighbour-joining (NJ) criteria; analyses were performed using PAUP* 4.0 (version b10) (Swofford 1998). However, only combined data results are shown. MP analyses were conducted using heuristic search under the Fitch model (all character-state changes equally weighted) (Fitch 1971) with tree-bi-section-reconstruction (TBR) branch swapping; 100 random taxa addition replicates were also run to search for multiple islands of optimal trees (Maddison 1991). For ML and NJ analyses we employed MODELTEST (Posada and Crandall 1998) to select the simplest model of character evolution (and associated model parameter values) that adequately explained the data, as determined by the hierarchical likelihood test. For ML analysis, a heuristic search was employed with 10 random taxa addition replicates, employing TBR branch swapping.

For all resulting phylogenies, non-parametric bootstrap analyses (1000 pseudoreplicates for MP, NJ and single gene ML analyses; 500 pseudoreplicates for combined data ML analyses) were performed to assess relative node support (Felsenstein 1985). In addition, MP and NJ analyses were repeated (for the combined dataset only) excluding outgroup samples to further assess ingroup topology.

Population analyses

All population-based analyses were performed using the combined sequence data for *L. aurea*. Haplotype and nucleotide diversity along with all other analyses were calculated using Arlequin 2.0 (Schneider et al. 2000); and

where appropriate the K2P model (Kimura 1980) with gamma correction ($\alpha = 0.016$) was chosen. Evidence of genetic structure was assessed with consideration of molecular distance (Φ_{ST}) and for haplotype frequencies only (F_{ST}). Under both conditions a number of different methods were employed: (1) analysis of molecular variance (AMOVA) (Excoffier et al. 1992); (2) F_{ST}/Φ_{ST} values between pairs of populations and regions; (3) an exact test for population differentiation (Raymond and Rousset 1995a); and (4) isolation by distance using linearised pairwise differentiation (F_{ST}/Φ_{ST}) and geographical distance (ln km). For AMOVA, only sites with five or more samples were used and significance was tested using 20,000 permutations. For pairwise F_{ST}/Φ_{ST} and exact tests, only sites with five or more samples were used for population-based analyses but all samples were used for region-based analyses, and significance for these was tested using 10,000 permutations. The significance of the log-linear association for isolation by distance analysis was tested using Mantel's procedure (Mantel 1967) with 10,000 permutations of the data, in GENEPOP (Raymond and Rousset 1995b).

A minimum-spanning network (MSN) (Excoffier and Smouse 1994) was also constructed using all samples to depict phylogenetic, geographical and potential ancestor–descendant relationships among haplotypes (Eizirik et al. 2001). Networks were constructed with a K2P ($\alpha = 0.016$) matrix and an absolute difference matrix using the program MINSPNET included in the Arlequin 2.0 package.

Inference of past population expansion events was tested using mismatch distribution (Rogers and Harpending 1992; Rogers 1995; Schneider and Excoffier 1999), Tajima's *D* test for selective neutrality (Tajima 1989a, b) and Fu's F*s* (Fu 1997) which detects excesses of low-frequency alleles in growing populations as compared to the expected number in stationary populations. Each of these analyses was conducted using the total sample combined in Arlequin 2.0. We also estimated the time of demographic expansion by the mode of mismatch distribution, $\tau = 2\ ut$ where t is the expansion time in number of generations and u is the mutation rate per generation for the whole sequence (Rogers 1995). We used a generation time of 18 months (Pyke and White 2001) and the rate of sequence change estimated for *Bufo bufo* mtDNA of 0.69% per lineage per million years (Macey et al. 1998).

Results

COI Sequence variation

The *COI* dataset ceonsisted of 324 bp of sequence from 263 *L. aurea* samples (ingroup) and two *L. raniformis* samples (outgroup). From these, 19 ingroup and one outgroup haplotypes were observed (GenBank AY835886–AY835905). Levels of sequence divergence among ingroup samples ranged from 0.3% to 1.5% (average 0.8%±SE 0.002). There were 13 variable sites and of these six were parsimony informative. Intraspecific sequence divergence was low compared to previous studies of *Litoria* species employing this gene where up to 12.1% (James and Moritz 2000) and 6.8% (McGuigan et al. 1998) were detected.

Across the complete dataset there were 25 variable sites (4% first position and 96% third position) of which 9 were parsimony informative; levels of sequence divergence ranged from 0.3% to 8.6%, with a mean sequence divergence between ingroup and outgroup haplotypes of 7.9%±SE 0.013. Saturation plots of the third position (data not shown) showed no evidence of saturation.

ND4 Sequence variation

The *ND4* dataset consisted of 524 bp of sequence from 263 *L. aurea* samples (ingroup) and two *L. raniformis* samples (outgroup). From these sequences 26 ingroup and two outgroup haplotypes were observed (GenBank AY755415–27; AY835906–21). Levels of sequence divergence among ingroup samples ranged from 0.19% to 2.1% (average 0.96%±SE 0.002). There were 28 variable sites and of these 15 were parsimony informative. Similarly to *COI*, intraspecific sequence divergence in *ND4* was low compared to a previous study of two *Limnodynastes* species employing this gene where up to 17% sequence divergence was detected. However our maximum divergence was greater than that reported for an elapid snake *Hoplocephalus stephensii* (1.7%; Keogh et al. 2003).

Across the complete dataset there were 90 variable sites (12.2% first position, 4.4% second position and 83.3% third position) of which 81 were parsimony informative; levels of sequence divergence ranged from 0.19% to 15.1%, with a mean sequence divergence between ingroup and outgroup haplotypes of 14.35% ± SE 0.015. Mean pairwise ti/tv ratios=7.51 (excluding comparisons where no transversions were detected) and as with *COI* base composition (A = 28.47%, C = 27.09%, G = 11.14% and T = 33.3%) indicated base G was slightly depauperate and a base T bias. Saturation plots of the third position (data not shown) indicated there was no evidence of saturation.

Combined data phylogeny

Sequence variation

We combined all unique *COI* and *ND4* sequences (848 bp) for a total of 51 ingroup (Table 2) and two outgroup haplotypes. The geographical distribution of

Table 2 Variable sites in 848 bases of Green and Golden Bell Frog mitochondrial DNA from ND4 and COI genes

HAP	ND4 (524 bp)	COI (324 bp)	*n*
H1	CTCCAACCATACAACCTAACAGAAAAAA	CCATCATCTTACT	2
H2	T.....T.....G.	....T.....	2
H3	.....G.	.....	9
H4	.....A.....G.	.....	2
H5	...T.....T.....G.	.....	15
H6	.....G...G.	.....	36
H7	T.....T.....T....G..G..G.	.....G.T.....	41
H8	T.....T.....G..G.	.....G.....T.	7
H9	TC.....T.....G..G.	.....G.....T.	7
H10	T.....T.G.....G..G.	.T...G.....	4
H11	T.....G.T.....G..G.	.....GC..C...	9
H12	T.....T.....T....G..G..G.	.....	2
H13	T.....T.....G..G.	.....	2
H14	T.....T.....G..G.	.....G.....	15
H15	T.....T.....G..G.	.T...G.....T.	5
H16	T.....T.....GG.G.	.....G...C...	3
H17	T.....T.....G..G.	.....G...C...	4
H18	T.....G.T.....G..G.	.....G...C...	15
H19	T.....T..C.....T.G...A.G..GG	.....G.....	3
H20	T.....T.G.....G..G.	..G..G.....	4
H21	T.....G....T.G.....G.GG.	.....G.....	4
H22	T.....T.G.....G..G.	.....G.....	15
H23	T.....T..C.T...T.G...A.G..GG	.....G.....	3
H24	T...G.....G.T.....G..G.	.....G...C...	8
H25	T...G.....G.T...T...G..G.	.....G...C...	3
H26	T.....T..C.....T.G.....G..GG	.....G.....	9
H27	T.....T.G.....G..G.	.....G....G..	4
H28	T.....T.G.....G..G.	.....G.....C	2
H29	.....G...G.	.....G.T.....	1
H30	.....G...G...G.	.....	1
H31	.....C.....G...G.	.....	5
H32	.....G...G.	T..C.....	1
H33	T.....T.....T.....G..G.	.....G.T.....	1
H34	T.....T.....T....G..G..G.	....T.....	1
H35	TC.....T.....G..G.	....T.....	1
H36	TC.....T.....G..G.	.....	1
H37	T.....T.....T....G..G..G.	.....G.T...T.	1
H38	T.....G.T.....G..G.	.....G.T.....	1
H39	T..T.....T.....G.	.....G.T.....	1
H40	T.....T.....T....G..G..G.	.....G.....	1
H41	T.....G.T.....G..G.	T....G...C...	1
H42	T.....T.....G..G.	T....G...C...	1
H43	T.T....T.....T....G..G..G.	T....G.T.....	1
H44	T.....T.....T....G..G..G.	..G..G.T.....	1
H45	T.....T.....GG.G.	T....G...C...	1
H46	T.....T.....G..G.	T....G.....	1
H47	T.....T.....T....G..G..G.	.....G.TC....	1
H48	T....T.T.....T....G..G..G.	.....G.T.....	1
H49	T.....T.G.....G..G.	.....G...C...	1
H50	T.....T.....G..G.	.....G.T.....	1
H51	T.....TT.G.....G..G.	.....G.....	1

Numbers (*N*) at the end of each haplotype (HAP) indicates the frequency of that haplotype. The geographical distribution of these haplotypes across regions is given in Table 3

these haplotypes across regions for *L. aurea* is shown in Table 3. Across all data there were 125 variable sites of which 113 were parsimony informative. Levels of sequence divergence ranged from 0.12% to 12.3%, with a mean sequence divergence between ingroup and outgroup haplotypes of 11.99% ± SE 0.011. Across ingroup sequences there were 41 variable sites and 25 sites were parsimony informative; levels of sequence divergence ranged from 0.12% to 1.3% (average 0.65% ± SE 0.001).

Table 3 Geographical distribution of 52 *L. aurea* haplotypes (combined data) summarised across regions

Region	Haplotype																									
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26
Upper North Coast	2	2	9	2																						
Lower North Coast					15																					
Hunter						22																				
Central Coast						8	2																			
Sydney						6	17	5	7	4	8	2	2	5	5											
Illawarra							3							8		2	2	7								
Shoalhaven							19	2			1			2		1	3	8	3	4	4	6	3	1		
Southern Tablelands																								7	3	
Northeast Victoria																						9				9

Region	Haplotype																									
	H27	H28	H29	H30	H31	H32	H33	H34	H35	H36	H37	H38	H39	H40	H41	H42	H43	H44	H45	H46	H47	H48	H49	H50	H51	
Upper North Coast																										
Lower North Coast																										
Hunter			1	1	5																					
Central Coast						1																				
Sydney							1	1	1	1	1	1	1	1												
Illawarra															1	1										
Shoalhaven																	1	1	1	1	1	1	1	1		
Southern Tablelands																										
Northeast Victoria	4	2																							1	

Geographical distribution of 52 *L. aurea* haplotypes (combined data) summarised across regions

Phylogenetic analyses

Phylogenetic analyses (MP, ML and NJ) of the combined dataset did not support the existence of any major intraspecific phylogeographic breaks or well supported clusters (Fig. 2). However, when MP and NJ analyses were repeated excluding outgroup samples, greater nodal support was provided to ingroup topology. In both analyses a clade excluding haplotypes 19, 23 and 26 was strongly supported (94%). These haplotypes are from the southern regions Shoalhaven and Victoria. However, other haplotypes from these two regions were also nested within the major clade. There was also weak support (MP 55%; NJ 60%) for a clade consisting of haplotypes from Sydney and regions north of Sydney, however, samples from these regions also cluster outside this clade (Fig. 2).

MSN and demographic history

The MSN (Fig. 3) of haplotypes provided further support to the phylogenetic analyses. The network depicts a high number of common haplotypes found across multiple regions with little obvious geographical clustering. There was some clustering of northern regions, however, haplotype 6 was also detected in Sydney and haplotype 7, the most common haplotype, was found across a large portion of the range from the Central Coast to the Shoalhaven. So although there was some tendency for geographic

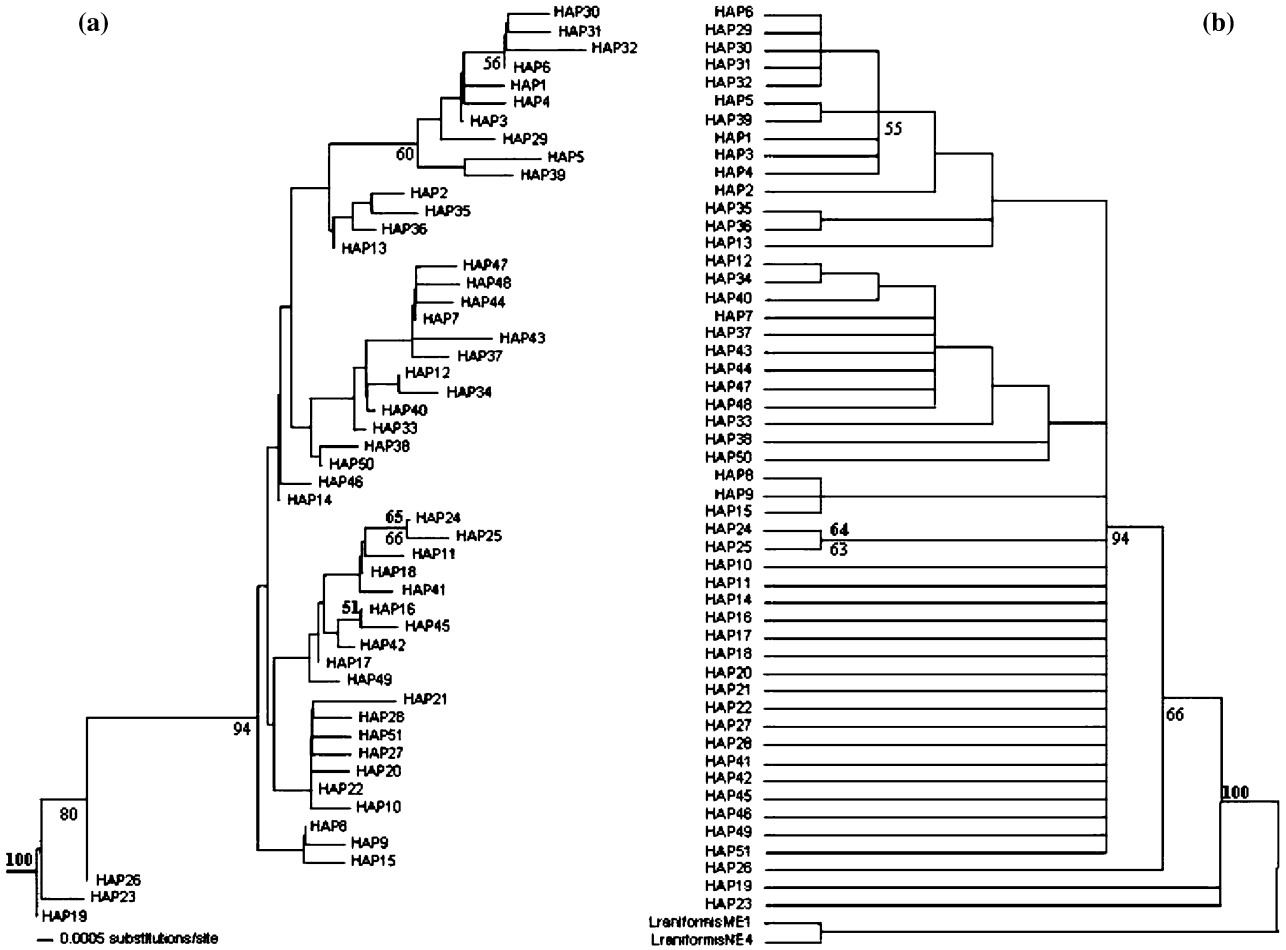

Fig. 2 Phylogenetic relationships among *L. aurea* mtDNA haplotypes based on combined (*COI* and *ND4*) sequence data. (**a**) Neighbour-joining (NJ) tree based on the TrN+I+G (Tamura and Nei 1993) model of evolution with bootstrap (1,000 pseudoreplicates) values >50% shown above the branches. This tree was re-drawn without the outgroup samples to better represent ingroup topology, the distance from outgroup samples to the first node was 0.535. NJ analysis excluding outgroup samples was also performed (under the HKY+I+G model; Hasegawa et al. 1985) and bootstrap values >50% are shown under the branches. (**b**) MP bootstrap (1000 pseudoreplicates) consensus tree with support values >50% shown above the branch in bold. MP analysis revealed 1078 most parsimonious trees, 61 steps, CI=0.556, RI=0.833. As with the NJ tree values below a branch indicate bootstrap support (>50%) for analysis excluding the outgroup samples (which found 1076 trees, 160 steps, CI=0.784, RI=0.864). The ML tree for the data including outgroup samples (using the TrN+I+G model) was similar in topology to the above trees with bootstrap support >50% (500 pseudoreplicates) found for three clades only: Hap19, Hap 23, Hap 26 (58%); Hap 24, Hap 25 (59%); and Hap 16, Hap 45 (57%) (data not shown)

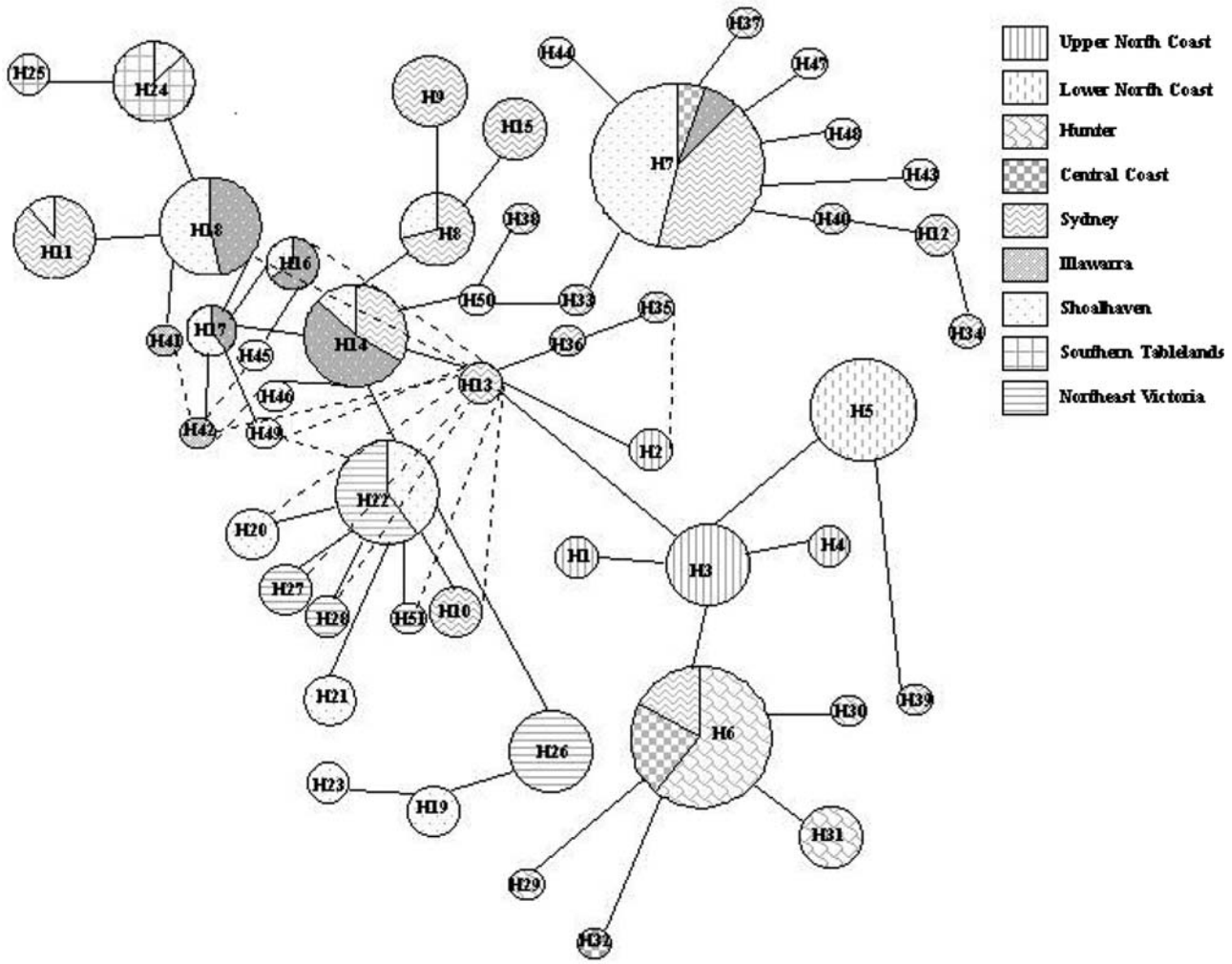

Fig. 3 Minimum-spanning network depicting relationships among *L. aurea* haplotypes based on combined data sequences. The area of the circle is proportional to the haplotype frequency, and the length of the connecting line is proportional to the genetic distance (K2P, $\alpha = 0.016$) between haplotypes. Dash lines indicate alternative topology. Shadings indicate what region each haplotype was detected in and if a haplotype was detected in more than one region the area of shading is proportional to its prevalence in the region. Analysis using the raw number of nucleotide differences among haplotypes produced a concordant result

structuring between the regions north of Sydney versus the regions south of Sydney the signal was weak and this level of structuring was poorly supported by phylogenetic analyses (Figs. 2, 3).

The overall picture of haplotype relatedness implied from resulting trees, and the MSN is one of no robust structure, suggesting a recent origin for most haplotypes and a relatively recent population expansion (Eizirik et al. 2001; Althoff and Pellmyr 2002). This pattern is suggestive of a widespread species that recovered from a small number of individuals (Avise 2000).

Mismatch distribution analysis further supports a sudden expansion hypothesis as the parameters of the mismatch distribution did not differ significantly from the sudden expansion model (P=0.648). This was consistent with a significant Fu's F*s* (−23.92, $P < 0.001$), however, Tajima's D (−0.657), although negative was not significantly different from the simulated data (P=0.298).

The approximate timing of the demographic expansion, estimated by the mode of mismatch distribution ($\tau = 6.4$/ 95% CI = 2.2–10.8), was 213,000 years ago (95% CI = 73,000–360,000 years ago). When considering the lower and upper 95% confidence limits this places the time of the estimated population expansion during the late Pleistocene.

Genetic diversity and population structure

Genetic diversity

Overall the mitochondrial sequences of *L. aurea* exhibited high haplotype diversity relative to low nucleotide diversity (Table 4). At four sampled sites (YUR, BI, UH and R)

only single haplotypes were detected and in general centrally located regions (Sydney, Illawarra and Shoalhaven) showed the highest levels of diversity (Table 4).

Genetic structure

Population-based analyses indicated that haplotypes were not distributed randomly with respect to geography as was alluded to in the phylogenetic analyses. AMOVA analysis considering the 9 geographical regions and 23 sampling locations (sites with ≥5 samples) revealed significant genetic structuring across all hierarchical levels ($P < 0.0001$) whether or not nucleotide differences were taken into account. Over all, 52.15% of genetic variance was observed amongst regions ($\Phi_{CT} = 0.52$), with 13.3% observed amongst sampled sites within regions ($\Phi_{SC} = 0.28$) and 34.6% observed amongst individuals within sampled sites ($\Phi_{ST} = 0.65$). However, when we used frequency data without consideration of molecular distance the result varied considerably with 17.94% of genetic variance observed amongst regions ($F_{CT} = 0.179$), 20.57% amongst sampled sites within regions ($F_{SC} = 0.251$) and 61.49% amongst individuals within sampled sites ($F_{ST} = 0.385$).

Pairwise F_{ST} and Φ_{ST} analyses and exact tests ($P < 0.0001$) also indicated significant structuring between regions although there were also non-significant comparisons (Table 5). For pairwise population based analyses (i.e. not pooled) 159 out of 276 comparisons were significant after corrections (151/276 for Φ_{ST} comparisons). For F_{ST} comparisons BI (all significant), PH (2 not significant), CH (3 not significant) and Q (5 not significant) were the most differentiated (data not shown), whilst for Φ_{ST} comparisons BI (all significant), Q (2 not significant), CH (3 not significant) and PH (4 not significant) were the most differentiated (data not shown).

An isolation by distance pattern was detected over all sampled sites and regions when using linearised pairwise Φ_{ST} differentiation estimates (Fig. 4), and over all sampled sites using linearised pairwise F_{ST} estimates (data not shown, $P < 0.001$). However, this pattern was not observed across regions when using linearised pairwise F_{ST} estimates (data not shown, P=0.71).

Table 4 Diversity measures for sample locations and regions of *L. aurea* using combined sequence data and K2P (α=0.016) substitution model

Region	Sample Location	No. of samples	No. of haplotypes	No. of polymorphic sites	Haplotype diversity	Nucleotide diversity×10^{-3}
Upper North Coast		**15**	**4**	**5**	**0.623 (0.124)**	**1.779 (0.001)**
	YUR	2	1	0	0	0
	CH	13	3	2	0.513 (0.144)	0.717 (0.001)
Lower North Coast	BI	**15**	**1**	**0**	**0**	**0**
Hunter		**29**	**4**	**4**	**0.406 (0.101)**	**0.652 (0.001)**
	KIS	10	2	2	0.200 (0.154)	0.552 (0.001)
	SAND	10	2	1	0.200 (0.154)	0.255 (0.0004)
	MAT	7	2	1	0.476 (0.171)	0.595 (0.001)
	UH	2	1	0	0	0
Central Coast	NA	**11**	**3**	**10**	**0.473 (0.162)**	**6.827 (0.004)**
Sydney		**69**	**18**	**18**	**0.899 (0.020)**	**7.424 (0.004)**
	HB	25	6	10	0.793 (0.046)	6.681 (0.004)
	NEW	18	11	10	0.941 (0.033)	6.276 (0.004)
	EB	2	2	4	1.000 (0.500)	6.510 (0.005)
	R	5	1	0	0	0
	A	9	6	11	0.833 (0.123)	6.040 (0.003)
	K	10	2	5	0.533 (0.095)	4.729 (0.002)
Illarwarra		**24**	**7**	**7**	**0.804 (0.015)**	**3.028 (0.001)**
	PK	19	6	7	0.795 (0.060)	3.420 (0.002)
	INC	5	3	2	0.700 (0.218)	1.337 (0.001)
Shoalhaven		**65**	**21**	**22**	**0.886 (0.023)**	**7.508 (0.003)**
	CS	10	6	13	0.778 (0.137)	7.450 (0.004)
	SB	16	9	12	0.850 (0.077)	6.859 (0.004)
	GP	10	3	5	0.733 (0.076)	4.355 (0.003)
	CU	9	4	7	0.778 (0.110)	3.761 (0.002)
	SX	10	6	13	0.889 (0.075)	11.01 (0.006)
	LM	10	7	8	0.933 (0.062)	4.850 (0.003)
Southern Tablelands	**Q**	**10**	**2**	**1**	**0.467 (0.132)**	**0.595 (0.001)**
Victoria		**25**	**5**	**6**	**0.737 (0.051)**	**2.976 (0.001)**
	PH	10	2	4	0.200 (0.154)	1.302 (0.001)
	AX	10	2	1	0.356 (0.159)	0.453 (0.001)
	EV	5	3	2	0.800 (0.164)	1.613 (0.001)

Numbers in parentheses give standard deviations

Table 5 Genetic differentiation between *L. aurea* regions (sampled sites pooled)

	UNC	LNC	HUNT	CC	SYD	ILLAW	SHOAL	ST	VICT
UNC	–	0.765	0.605	**0.168**	0.504	0.760	0.593	0.920	0.813
LNC	0.686	–	0.910	0.607	0.666	0.880	0.723	0.990	0.903
HUNT	0.503	0.744	–	**0.161**	0.650	0.873	0.718	0.969	0.899
CC	0.443	0.796	**0.024**	–	0.450	0.648	0.550	0.802	0.735
SYD	0.212	0.414	0.268	0.184	–	**0.090**	0.076	0.474	0.369
ILLAW	0.276	0.540	0.403	0.319	0.095	–	**0.075**	0.554	0.538
SHOAL	0.220	0.423	0.321	0.232	0.032	0.071	–	0.460	0.250
ST	0.443	0.809	0.573	0.530	0.260	0.333	0.261	–	0.797
VICT	0.312	0.572	0.434	0.371	0.174	0.230	0.154	0.370	–

Pairwise F_{ST} and Φ_{ST} values are shown (lower left hand matrix based on haplotype frequencies, upper right hand matrix accounts for molecular distance using K2P, $\alpha = 0.016$). Non-significance comparisons are shown in bold. Significance levels were evaluated by 10,000 permutations and then adjusted for multiple tests using Bonferroni Correction

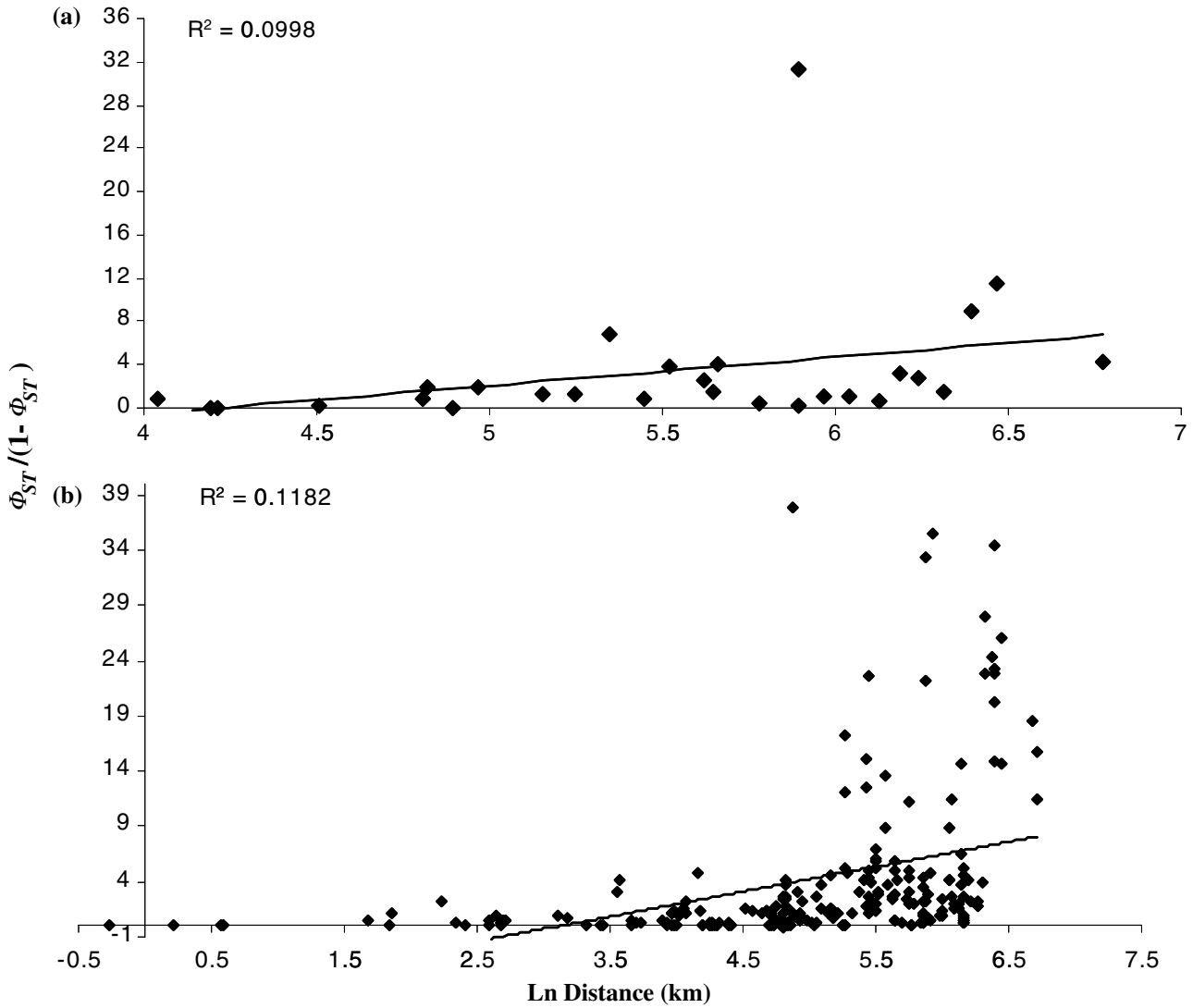

Fig. 4 Isolation by distance plots of $\Phi_{ST}/(1-\Phi_{ST})$ plotted against the natural log of geographical distance (km) for all samples pooled into regions (**A**) and sampled sites with greater than five samples (**B**). In both analyses BI was excluded because of its isolation from gene flow and the Φ_{ST} matrix was formulated based on the K2P ($\alpha = 0.016$) substitution model. The solid line represents the best-fit linear regression based on all points. For both analyses Mantels test was significant (regions $P < 0.05$; all sampled sites $P < 0.001$)

Discussion

L. aurea phylogeography

Analyses of the phylogeographic structure of *L. aurea* showed no evidence of major geographic partitions or substantial barriers to historical gene flow through out the species' range (Fig. 2). This lack of phylogeographic structure supports the findings of a recent microsatellite study of *L. aurea* which found that populations within continuous habitat showed evidence of only weak genetic structuring (Burns et al. 2004). Interestingly several other studies of frog species have detected strong phylogenetic structure (e.g. Bos and Sites 2001; Austin et al. 2002; Palo et al. 2004) and as a result it has previously been suggested that frog species generally have limited vagility and are likely to be insufficiently motile to cross moderate barriers of unfavourable habitat (see for e.g. Austin et al. 2002). However, the results of this study and the previous microsatellite study indicate that such generalisations can be inaccurate.

At present, it is highly likely that extensive habitat fragmentation would limit successful dispersal in *L. aurea*. However in the past, if habitat was relatively continuous and climatic conditions favourable, then there would have been few barriers to gene flow. Our current understanding of the ecology of this species (see introduction and Pyke and White 2001) and the findings of Burns et al. (2004) supports this idea.

Schauble and Moritz (2001) conducted a phylogeographic study of two frog species *Limnodynastes peronii* and *Lim. tasmaniensis*. This study covered the southeast coastline but sampling effort south of the McPherson Range to East Gippsland was extremely limited (Schauble and Moritz 2001, Fig. 1). The phylogeographic structure of *Lim. peronii* is particularly relevant to *L. aurea* because both species inhabit similar habitats (Pyke et al. 2002) and are more often than not recorded at the same water bodies (E. Burns pers. obs.). In their study, Schauble and Moritz (2001) detected a distinct phylogenetic break positioned somewhere south of the McPherson Range (at the border of NSW and QLD) and north of Sydney (Fig. 1). No other breaks were detected along the NSW and Victorian south-eastern coastline and the detected break was more pronounced in *Lim. peronii* than *Lim. tasmaniensis* (Schauble and Moritz 2001).

Schauble and Moritz (2001) speculate that the break may constitute geographically shifted breaks that once centred on the McPherson Range or alternatively may be positioned in one of two areas. (1) The Clarence River, which has a tidal reach extending 100 km inland (Schauble and Moritz 2001, Fig. 1) and where Donnellan et al. (1999) previously recorded a mtDNA lineage split in the *Litoria citropa* species-group; and (2) the Hunter Valley, which previously has been a suggested boundary between southern and northern coastal faunal elements (Cracraft 1991, Fig. 1).

Our data failed to support the Hunter Valley as a potential biogeographic barrier, which is also consistent with a recent study of the satin bowerbird (Nicholls and Austin 2005). However, we were unable to test the Clarence river hypothesis because our sampling was limited to extant populations south of the Clarence River (most northern population at YNP; Fig. 1). Historically *L. aurea* was recorded as far north as Ballina (White and Pyke 1996) and museum samples may permit further examination of the Clarence River hypothesis. Two other studies that sampled either side of the Clarence River did not have highly concordant results. *Litoria fallax* showed little phylogenetic structure south of the McPherson Range (James and Moritz 2000); however, *Hoplocephalus stephensii* (elapid snake) showed support (87%) for a clade including Lismore samples but excluding Coffs Harbour and Newcastle samples (Keogh et al. 2003, Fig. 1). If the Clarence River does constitute a long-term barrier to gene flow, then based on our data and that of the aforementioned studies, it may constitute the only distinctive break to terrestrial vertebrates along the south-eastern coastline of NSW and Victoria.

Recent evolutionary history of *L. aurea*

The phylogeographic pattern of *L. aurea* mtDNA was characterised by a diverse and shallow intraspecific phylogeny where common haplotypes were widespread and to a limited extent closely related haplotypes were geographically clustered, albeit on a large geographic scale (Figs. 2, 3). This phylogeographic pattern, according to Avise (2000), intimates contemporary gene flow between populations that are tightly connected in history, where common haplotypes are likely ancestral (plesiomorphic) and rare haplotypes apomorphic.

Relative to other intraspecific Australian frog studies, employing *COI* or *ND4* genes, the level of sequence divergence in *L. aurea* was low (see results for specific comparisons). A shallow intraspecific phylogeny in conjunction with high haplotype diversity, relative to low nucleotide diversity, as found in *L. aurea*, is indicative of a recent population expansion (e.g. Eizirik et al. 2001; Althoff and Pellmyr 2002; Joseph et al. 2002; Stamatis et al. 2004). Mismatch distribution analysis and Fu's F*s* test support an expansion hypothesis and the estimated date of expansion was approximately 213,000 years ago with upper and lower bounds of 73,000 and 360,000 years respectively. If this time estimate is realistic, the expansion occurred during the late Pleistocene but prior to the last glaciation maximum 18,000 years ago (Markgraf et al. 1995).

Based on our data, we suggest that this population expansion occurred from an area extending from the Hunter to the Shoalhaven, where the two most common haplotypes were detected (Hap 6 and 7, Table 3 and Fig. 3). This would explain the current high haplotype diversity in this area (Tables 3, 4), the central section of the species range, and the tendency for haplotypes to be distributed Sydney-south and Sydney-north.

During the Pleistocene the Australian environment, unlike the northern Hemisphere (reviewed in Markgraf et al. 1995; Hewitt 2000), experienced limited glaciation with ice sheets confined to the Snowy Mountains (Barrows et al. 2001) and sections of Tasmania (reviewed in McKinnon et al. 2004). However, during this period the continent experienced increased aridity and climatic oscillations with wetter climates during the interglacial periods and more arid environments during the glacial maxima (Bowler 1982). This resulted in the expansion and contraction of arid habitats, reducing Australia's more mesic habitats that dominated the Tertiary (Kemp 1981), to minimal areas along coastal strips (Kershaw 1981; Nix 1982).

Litoria aurea is not a habitat specialist, however, like most amphibians it is dependent on freshwater bodies for breeding and wet conditions for dispersal (ecology reviewed in Pyke and White 2001). It is therefore possible that arid cycles during the Pleistocene would have limited dispersal, colonisation, breeding activity and recruitment and could have resulted in some localised extinctions.

However, a lack of phylogeographic structure in *L. aurea* suggests that Pleistocene glaciation cycles had a limited effect on the genetic structure of *L. aurea* implying that there was little habitat contraction throughout the species range, which is in contrast to rainforest taxa from north east and south east QLD (McGuigan et al. 1998; Schneider et al. 1998; James and Moritz 2000; Schauble and Moritz 2001). Therefore, it is likely that there was mostly continuous habitat throughout *L. aurea*'s range until the European invasion of Australia a little over 200 years ago (consistent with Keogh et al. 2003; Nicholls and Austin 2005).

Population structure and variability

Analyses of population structure on the large geographical scale of regions was performed to further investigate genetic structuring and the history of dispersal throughout the species range.

Overall population-based analyses indicate that there was significant structuring throughout the species range both within and between regions, and in general restricted gene flow was the result of geographic distance (Table 5 and Fig. 4). The correlation between genetic divergence and geographic separation was weak, however (Fig. 4). An isolation by distance pattern, across all populations, was also detected in a previous microsatellite study (Burns et al. 2004).

As expected the more isolated NSW populations (BI, CH and Q) were highly differentiated and the Victoria populations were not significantly structured (data not shown), consistent with microsatellite data (Burns et al. 2004). However, Victorian populations AX and EV were not significantly different from all NSW populations (pairwise F_{ST} and Φ_{ST} analyses; raw data not shown) as was the case for microsatellite data (Burns et al. 2004) but this may be due to small sample sizes.

Levels of genetic diversity were generally highest in populations within the centre of the species range (Table 4). Interestingly, Victorian populations were not found to be comparatively depauperate as was found in the microsatellite study (Burns et al. 2004), again this may be an artefact of small sample sizes. However, Broughton Island in both studies showed low levels of diversity as would be expected of an island population (Frankham 1997, Table 4).

Taken together, phylogenetic and population-based analyses indicate that since a Pleistocene expansion there has been continued (albeit restricted) dispersal and gene flow which has resulted in a weak isolation by distance pattern and the significant population structure evident today. However, current dispersal is likely to be increasingly restricted or non-existent between fragmented populations (see also Burns et al. 2004).

Conservation Implications

Phylogeographic analyses of intraspecific sequence variation, coupled with population-based analyses, provide valuable information on how genetic variation is partitioned within species and can therefore aid in the implementation of effective conservation strategies.

Our data provide no support for the existence of distinct phylogeographic breaks within the species' range, implying that there are no historically isolated groups that should be viewed as separate evolutionary significant units (Ryder 1986; Moritz 1994b). However, below this level there is significant genetic structuring both within and between the regions nominated for conservation management in the species Draft Recovery Plan (NSW NPWS 2002).

AMOVA analysis indicated (when considering molecular distance) these regions account for 52.15% of genetic variance, however, when considering frequency data alone variation amongst regions was reduced to 17.94%. So it is difficult to determine the suitability of these regions as conservation management units based on these data. However, we prefer not to suggest 'management units' for this species but rather give a generalised approach based on the natural dynamic of the species as was suggested by Burns et al. 2004. That is, priority should be given to conserving areas

of connecting habitat to promote population connectivity and maintain adaptive diversity and evolutionary potential.

Our results potentially support 'well managed' intervention to mediate gene flow amongst isolated populations, however, based on both mtDNA and microsatellite data, there is no evidence to suggest that supplementation through artificial immigration is necessary at this time, given current levels of genetic variation within populations (Burns et al. 2004). If however, for demographic and ecological reasons, it is thought necessary to supplement or reintroduce populations then there is no genetic evidence to suggest this is an inappropriate management strategy *per se*. This conclusion, however, is based on the following principles. First, given the overall isolation by distance pattern for microsatellite and mitochondrial data, source populations should be within geographical proximity (i.e. the closest neighbouring population). This will act to reduce impacts of localised adaptations and maintain or restore 'natural' levels of gene flow. Second, source populations should have equivalent or greater levels of genetic diversity than the recipient population to prevent a loss of genetic diversity and no single source should be used extensively. Last, source populations should not be so genetically distinct as to significantly alter the genetic integrity of recipient populations by disrupting localised adaptations or resulting in loss of evolutionary potential through a loss of genetic diversity (see for review Moritz 1999; Storfer 1999; Johnson 2000).

Under the above criterion I would suggest that Broughton Island, the Southern Tablelands, and Crescent Head populations should not currently be used as source populations because they are highly differentiated (mtDNA and microsatellite data). Furthermore, based on microsatellite data, Broughton Island, Kurnell and Victorian populations would not be appropriate source populations because they have levels of genetic variation that are comparatively depauperate (Burns et al. 2004).

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