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ORIGINAL ARTICLE

Climatic fluctuations shape the phylogeography of a mesic direct-developing frog from the south-western Australian biodiversity hotspot

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ABSTRACT

Aim To assess phylogeographic pattern throughout the range of *Metacrinia nichollsi* in order to develop specific biogeographical hypotheses for the wet forests of south-western Australia. This was carried out by contrasting a direct-developing frog species, *M. nichollsi*, that breeds independently of free surface water with conventional, aquatic breeders and highly specialized direct developers.

Location Wet forests of the south-western Australian biodiversity hotspot – an area of high species richness and endemism for myobatrachid frogs and many other faunal groups.

Methods We compiled an extensive phylogeographic data set from field-collected samples based on mitochondrial *ND2* sequences. Phylogenetic analyses combined with estimates of divergence times were used to build a model of major biogeographical events affecting the species. Phylogeographic analyses were used to provide insights into smaller-scale processes acting within each major lineage.

Results Phylogenetic analysis recovered three major lineages, with divergence dates coincident with late Miocene–early Pliocene arid cycles. One lineage was confined to geographically isolated populations in the Stirling Ranges (Stirling Ranges Lineage, SRL). The continuous range of *M. nichollsi* was split into two: the Main Range Lineage (MRL) and the Southern Coastal Lineage (SCL). The SCL displays a strong drainage-based population structure, whereas the MRL displays a strong signature of recent expansion, suggesting that these two lineages have had very different biogeographical histories.

Main conclusions Late Miocene–Pliocene aridity appears to have isolated populations in the Stirling Ranges and resulted in the formation of two additional lineages on a north–south gradient that are independent of southward-flowing drainage systems. Our results demonstrate that climatic fluctuations are likely to have generated fine-scale phylogeographic structure within *M. nichollsi* and that catchment regions are important refugia during arid cycles.

Keywords

Biogeography, *Metacrinia nichollsi*, mitochondrial genealogy, Myobatrachidae, phylogeography, south-western Australia.

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INTRODUCTION

The south-western corner of Australia provides an interesting biogeographical conundrum. The region is a centre of endemism and a biodiversity hotspot of global significance owing to its high species diversity and highly threatened environments (Cincotta *et al.*, 2000; Myers *et al.*, 2000). However, the region lacks the obvious vicariant forces commonly evoked to explain speciation events, such as glaciation or major tectonic or volcanic activity, and the region is topographically subdued (Hopper & Gioia, 2004). South-western Australia is world-famous for its extreme

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diversity of plant species (Hopper, 1979; Hopper & Gioia, 2004), but it is also home to a great diversity of endemic invertebrates (Main, 1996), mammals, reptiles and amphibians (Hopper *et al.*, 1996). Although phylogenetic and phylogeographic investigations into speciation mechanisms in the plants of the south-west have accumulated rapidly over the last 30 years (Hopper, 1979; Hopper & Gioia, 2004; Byrne, 2007), our understanding of the processes resulting in speciation and genetic diversity within south-western faunal assemblages remains comparatively poor. Given the levels of human habitat modification in the region, an understanding of speciation processes and the distribution of genetic diversity is paramount for competent conservation efforts; it is also of inherent evolutionary interest (Moritz & Faith, 1998; Moritz *et al.*, 2001; Moritz, 2002).

The patterns and mechanisms of speciation in south-western flora provide a significant foundation for investigations into faunal speciation. Recent plant studies, particularly molecular studies, have generally focused on Pleistocene processes to explain the generation of diversity in the transitional climatic zones of the south-west (Lamont & Markey, 1995; Byrne & MacDonald, 2000; Byrne *et al.*, 2003a,b; Coates *et al.*, 2003; Broadhurst *et al.*, 2004; Byrne & Hines, 2004). The few studies conducted within the wet forested areas of the south-west have suggested that climatic fluctuations, particularly those in the Plio-Pleistocene period (Dodson & Ramrath, 2001), may be shaping biodiversity (Coates & Hamley, 1999; Wheeler & Byrne, 2006). However, our understanding of speciation processes for endemic south-western Australian fauna relative to that for plants is limited. This represents a major gap, and our understanding is particularly poor across the wet forested regions of the south-west. The wet forests of south-western Australia are rich in Gondwanan relicts, such as onychophorans (Reid, 2002), myglamorph spiders (Main, 1996) and myobatrachid frogs (Roberts, 1993; Roberts *et al.*, 1997).

Myobatrachid frogs represent an ancient anuran lineage endemic to Australia and Papua, and have long been recognized as being particularly diverse in the south-west (Roberts & Maxson, 1985a,b). There are six described endemic relictual species with geographic ranges from 6 km^2 to > 1000 km^2 in the mesic southern forest (Wardell-Johnson & Roberts, 1993; Roberts *et al.*, 1997), suggesting allopatric speciation in isolated populations; however, the details of the mechanisms of speciation in south-western myobatrachids are still unclear. There is increasing evidence that climatic fluctuations may have been particularly important in shaping the distributions and diversity of south-western Australian myobatrachids (Driscoll, 1998a,b; Edwards, 2007; Edwards *et al.*, 2007).

Historical climate fluctuation may also have contributed to patterns of range contraction and expansion in species of the *Geocrinia rosea* group (Driscoll, 1998a,b). The *G. rosea* species complex is a series of allopatric, highly restricted and specialized species distributed across the wet forests, along the southern coast of Western Australia. The development of geographically isolated populations, followed by local differentiation, could account for the known patterns of species diversity and geographic range in this complex, but most of that divergence is strongly catchment-based (Driscoll, 1998a,b). However, the *G. rosea* complex may not be representative of broader processes acting on south-western Australian frogs as these direct-developing frogs are highly reliant on surface water/moisture for successful recruitment; additionally, timing of divergence estimates are uncertain (Wardell-Johnson & Roberts, 1993; Driscoll, 1997, 1998a). Pleistocene climate fluctuations appear to have been important in shaping the historical and current distribution of subspecific lineages in the widespread frog *Crinia georgiana*, but little of this variation can be specifically attributed to catchment-based, genetic structure along the southern coast (Edwards *et al.*, 2007). The generation of a comprehensive view of the historical biogeography of the region requires data from multiple species with varying life histories and habitat relationships (Cracraft, 1988; Avise *et al.*, 1998; Riddle *et al.*, 2000; Moritz *et al.*, 2001; Zink, 2002), but data so far are from conventional aquatic breeders (Edwards *et al.*, 2007) or direct developers with very specialized wet forest requirements (Driscoll, 1998a,b).

The current study describes the phylogeography of the south-western Australian endemic *Metacrinia nichollsi* (Harrison, 1927). *Metacrinia* is a monotypic genus closely aligned with two other south-western Australian endemics, namely *Arenophryne rotunda* and *Myobatrachus gouldii*, which are also monotypic (Read *et al.*, 2001). *Metacrinia nichollsi* is a direct developer with non-specific breeding-site requirements (Anstis, 2007). It is distributed widely across a range of landscapes from relatively dry coastal heaths to the wettest karri and tingle forest throughout the high-rainfall zone of south-western Australia (Tyler *et al.*, 2000). There are also populations in the eastern Stirling Range, which are geographically isolated from the wet forests to the south-west by *c.* 100 km (Tyler *et al.*, 2000). The species is not obviously tied to drainage systems and therefore may not show the same extreme fragmenting effects of climate change as seen in the *G. rosea* species complex (Driscoll, 1998a,b). *Metacrinia nichollsi* has a summer breeding pattern and is therefore reliant on access to moist environments for egg development (Anstis, 2007). Given the continuous distribution and the life-history characteristics of *M. nichollsi*, the phylogeography of this species is likely to display the general effects of long-term climate change across the 'relictual' wet forests along the south-western Australian coast, providing a contrast to studies so far conducted on restricted specialist species and more widespread generalist species.

MATERIALS AND METHODS

Tissue samples

Sixty-eight individuals were sampled (toe-clips) from 16 sites across the entire species distribution, with two to five animals sampled per site (Fig. 1). The gap that exists between the Stirling Ranges population and the main range of the species is real: both current and historical surveys have failed to find the

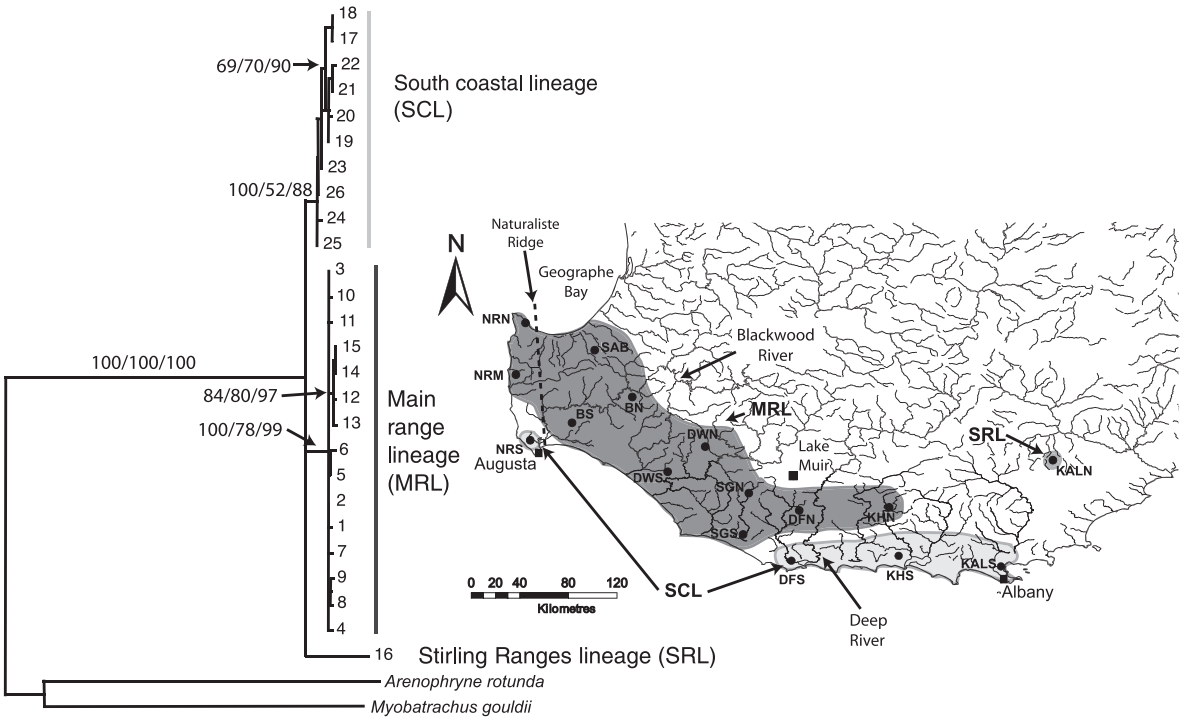

Figure 1 The maximum likelihood (ML) phylogram of 26 unique *M. nichollsi ND2* haplotypes shows three major lineages with *Arenophryne rotunda* and *Myobatrachus gouldii* as outgroups. Clade support is provided by maximum parsimony bootstrap/ML bootstrap/Bayesian posterior probabilities. The TIM+I+G model of DNA evolution was enforced in ML analyses selected by Akaike information criterion tests in MODELTEST ver. 3.7. A map of the south-western Australian coastline is shown inset with shaded areas representing the distribution of the main range, south coastal and Stirling Ranges lineages. Tissue collection locations [●] for the *Metacrinia nichollsi* phylogeographic study cover the entire known distribution of the species. Site names and abbreviations are as follows: Naturaliste Ridge Nth (NRN, $n = 3$); Naturaliste Ridge Mid (NRM, $n = 4$); Naturaliste Ridge Sth (NRS, $n = 4$); Sabina (SAB, $n = 3$); Blackwood Nth (BN, $n = 4$); Blackwood Sth (BS, $n = 4$); Donnelly-Warren Nth (DWN, $n = 4$); Donnelly-Warren Sth (DWS, $n = 4$); Shannon-Gardner Nth (SGN, $n = 2$); Shannon-Gardner Sth (SGS, $n = 3$); Deep-Frankland Nth (DFN, $n = 5$); Deep-Frankland Sth (DFS, $n = 5$), Kent-Hay Nth (KHN, $n = 3$); Kent-Hay Sth (KHS, $n = 5$); Kalgan Nth (KALN, $n = 10$); Kalgan Sth (KALS, $n = 5$).

species in intervening areas. Past and present surveys in the Porongurup Mountains (34°40′46″, 117°52′23″) have recovered no records of the species (B.Y. Main has pit-trapped for spiders for > 50 years with no captures of *M. nichollsi*; the same methods catch *M. nichollsi* in all other parts of the species range; Main, personal communication). Surveys by D. Edwards in 2003 and 2004 did not detect *M. nichollsi* either, despite what is apparently ideal habitat for the species. Two populations, *c.* 15 km apart, were sampled within the Stirling Ranges, with five animals sampled from each. Because of the lack of genetic diversity between these two populations, they are considered together below. *Arenophryne rotunda* (27°49′59″, 114°21′53″) and *Myobatrachus gouldii* (30°01′57″, 115°49′06″) sequences were used as outgroups for this study (Read *et al.*, 2001).

Molecular genetic methods

DNA extraction, polymerase chain reaction (PCR) amplification and DNA sequencing procedures were conducted as outlined in Edwards (2007). Primers used to amplify the mitochondrial gene *ND2* were L4221 (5′-AAGGRCCTCCTT GATAGGGA-3′, modified from Macey *et al.*, 1998) and tRNA-trp (5′-CTCCTGCTTAGGGSTTTGAAGGC-3′, modified from Read *et al.*, 2001). Internal primers, L4437 (5′-AAGCTTTCG GGGCCCATACC-3′, Macey *et al.* 1998) and H4980 (5′-ATT TTTCGTAGTTGGGTTTGRTT-3′, Macey *et al.* 1998), were used for sequencing in addition to PCR primers in order to obtain reliable sequences across the entire gene. DNA sequence data were edited using SEQUENCHER ver. 3.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were aligned individually using CLUSTALX (Thompson *et al.*, 1997). Alignments were then checked by eye. Sequences were translated using the mammalian genetic code option in SEQUENCHER ver. 3.0, and a clear reading frame was observed in all sequences. Thus, sequences were assumed to be genuine mitochondrial copies and not nuclear paralogues. Distinct haplotype sequences have been lodged in GenBank (EU432130–EU432155).

Phylogenetic analysis

Phylogenetic analyses and sequence divergence estimates calculated using a molecular clock were used to assess the

overall phylogenetic structure and to approximate the timing of major splits within the *M. nichollsi* phylogeographic data set. Maximum likelihood (ML) and maximum parsimony (MP) analyses (both using PAUP* ver. 4.0b10; Swofford, 2002) and Bayesian Markov chain Monte Carlo (MCMC) analyses (using MrBayes ver. 3.1.2; Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) of haplotypes were carried out to resolve and assess support for relationships between the major clades and overall phylogenetic structure. The Akaike information criterion was used to select the best-fit model of evolution from the data for ML analyses using MODELTEST ver. 3.7 (Posada & Crandall, 1998), and to calculate the nucleotide frequencies, substitution rates, gamma distribution and proportion of invariant sites for the data under the selected model. Branch support for the ML and MP trees is provided in the form of likelihood bootstrap values calculated from 100 bootstrap replicates. For ML and MP analyses, starting trees were obtained by step-wise addition, and the tree bisection–reconnection (TBR) method of branch swapping was employed in each heuristic search. Bayesian analyses were conducted using the GTR + I + G model using default priors for MCMC analyses in MrBayes ver. 3.1.2. Four independent runs of four chains each were run for 4×10^6 generations sampling every 100 generations, with burn-in set at 400,000 generations. Convergence of posterior probabilities and stationarity of likelihood scores between the two runs were assessed in TRACER ver. 1.3 (Rambaut & Drummond, 2005). Descriptive statistics such as haplotype diversity (Hd) and nucleotide diversity (π) were calculated in DnaSP ver. 4.10.8 (Rozas & Rozas, 1999).

Divergence between major *M. nichollsi* lineages was calculated using the formula of Nei and Li for d_A (the average number of nucleotide substitutions per site between lineages; Nei, 1987). The d_A parameter estimates and their standard errors were calculated using DnaSP ver. 4.10.8 (Rozas & Rozas, 1999). There are no appropriate external calibration points/fossils to calibrate a specific molecular clock for any south-western frog genera, despite the existence of some fossils found in recent to Pleistocene cave deposits (Roberts & Watson, 1993; Price *et al.*, 2005). Therefore, we adopted the molecular clock rate of 0.957% Myr^{-1}, calibrated for *ND2* in eleutherodactylid frogs (Crawford, 2003); this estimate is closely aligned with an estimate for a similar mtDNA fragment, from *ND1-ND2*, in the *Bufo viridis* subgroup (Stöck *et al.*, 2006). To ensure that the *M. nichollsi ND2* sequences were evolving in a clock-like manner, a ML search was conducted in PAUP* ver. 4.0b10 (Swofford, 2002) enforcing a molecular clock. A likelihood ratio test was then performed to assess if there were any significant differences between the likelihood scores of trees with and without a molecular clock enforced (Felsenstein, 1981) in MODELTEST ver. 3.7 (Posada & Crandall, 1998).

Phylogeographic analysis

Phylogeographic analyses provided estimates of the geographical significance of genetic pattern and an inference about the evolutionary history of *M. nichollsi*. Phylogeographic results were compared with the known climatic history to determine the likely impact, if any, of climate fluctuations on *M. nichollsi*. Unrooted statistical parsimony haplotype networks or gene trees were created using TCS ver. 1.21 (Clement *et al.*, 2000), and the network was then nested according to the nesting rules outlined in Templeton & Sing (1993), Templeton *et al.* (1995) and Crandall *et al.* (1994). Where interior/tip status was ambiguous, particularly at the final nesting level of the separate networks, nested clade outgroup probability (Castelloe & Templeton, 1994) and position in relation to outgroups in the phylogenetic tree (Fig. 2) were used to determine the interior nested clade. Tests for geographical association were carried out on the nested haplotype networks in GeoDis ver. 2.4 (Posada *et al.*, 2000) using the latitude and longitude coordinates for each sampling location. Nested clades with significant phylogeographic structure were specified by a significant χ^2 value from contingency tests calculated over 1000 random permutations. The distance values (D_C and D_N) from the nested clades with significant phylogeographic structure were then used in conjunction with the nested clade phylogeographic analysis (NCPA) inference key (http://darwin.uvigo.es/software/geodis.html) to reconstruct population histories.

The utility of NCPA has been questioned owing to the apparent lack of statistical power of the biological inferences obtained using this technique (Knowles & Maddison, 2002), but it remains a powerful phylogeographic analysis technique, particularly where the events and processes affecting species evolutionary histories are not known *a priori* (Templeton, 2004). However, this analysis should be used in conjunction with supplementary testing, particularly when either restricted gene flow with isolation by distance or contiguous range expansion inferences are obtained for the data (Panchal & Beaumont, 2007). Where these inferences were obtained they were treated with caution unless supported by secondary lines of evidence. We employed several analytical techniques to complement the NCPA analyses. Initially, Tajima's D (D_T) was calculated to ensure that sequence data fitted the assumption of neutral evolution (Tajima, 1989), using DnaSP ver. 4.10.8 (Rozas & Rozas, 1999). Where NCPA inferred recent population expansion (e.g. step 21 of the current key), R_2 tests (Ramos-Onsins & Rozas, 2002) were conducted to test the hypothesis of constant population size vs. population growth using coalescent simulations with values permuted 1000 times in DnaSP ver. 4.10.8 (Rozas & Rozas, 1999). R_2 tests for population growth based on the difference between the number of singleton mutations and the average number of nucleotide differences between sequences, and is a powerful test, especially with limited sample sizes (Ramos-Onsins & Rozas, 2002). Where secondary contact between divergent nested clades/lineages was suspected, the supporting tests described in Templeton (2001) were carried out. These involve the calculation of pairwise distances between the geographical centres of each nested clade (provided by the GeoDis ver. 2.4 output) found at each sampling site for every nesting level of

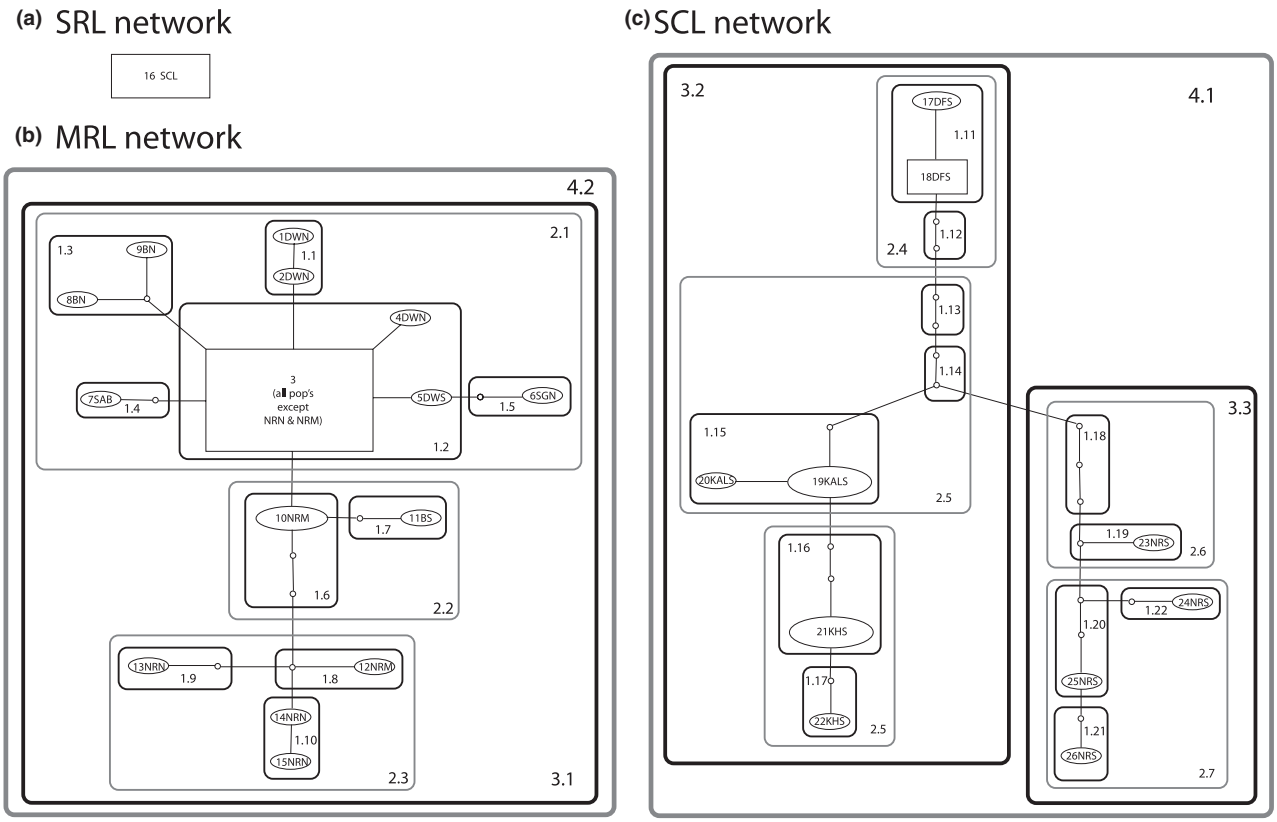

Figure 2 Haplotype networks (including site references) for 26 unique *Metacrinia nichollsi ND2* haplotypes, created in TCS ver. 1.21. Three distinct networks were created, corresponding to the (a) Stirling Ranges Lineage (SRL), (b) Main Range Lineage (MRL), and (c) South Coastal Lineage (SCL). Each line represents a single mutational change. Ellipse size is proportional to haplotype frequency, with small open circles representing missing haplotypes and the square representing the ancestral haplotype as inferred by TCS using outgroup weights. Connections up to 10 and 16 steps are within the 95% confidence limits of a parsimonious connection for the SCL and MRL networks, respectively. The SRL differs from the MCL and SCL by 49 and 52 mutational steps, respectively, and the MRL and SCL differ by 31 mutational steps. Clades are nested according to the rules outlined in Templeton *et al.* (1987), Crandall (1994) and Templeton *et al.* (1995). Site names and abbreviations are as follows: Naturaliste Ridge Nth (NRN, $n = 3$); Naturaliste Ridge Mid (NRM, $n = 4$); Naturaliste Ridge Sth (NRS, $n = 4$); Sabina (SAB, $n = 3$); Blackwood Nth (BN, $n = 4$); Blackwood Sth (BS, $n = 4$); Donnelly-Warren Nth (DWN, $n = 4$); Donnelly-Warren Sth (DWS, $n = 4$); Shannon-Gardner Nth (SGN, $n = 2$); Shannon-Gardner Sth (SGS, $n = 3$); Deep-Frankland Nth (DFN, $n = 5$); Deep-Frankland Sth (DFS, $n = 5$), Kent-Hay Nth (KHN, $n = 3$); Kent-Hay Sth (KHS, $n = 5$); Kalgan Nth (KALN, $n = 10$); Kalgan Sth (KALS, $n = 5$).

the cladogram. Secondary contact can be inferred if haplotypes/clades with divergent geographical centres are found at the one location (Templeton, 2001, 2004).

Population genetic analysis

Population genetic statistics were used to investigate and describe genetic structure within the identified lineages. DnaSP ver. 4.10.8 (Rozas & Rozas, 1999) was used to calculate Hudson's Snn 'nearest neighbour' statistic with 1000 permutations via the coalescent, to provide a quantitative measure of population genetic structure for the entire species data, the major lineages specified above and the population genetic data. Hudson's Snn 'nearest neighbour' statistic is specifically designed for haplotype sequence data and has been shown to outperform a range of other statistics used to estimate genetic differentiation (Hudson, 2000). Values of Snn are expected to be close to 0.5 if populations are panmictic, and closer to 1 if populations are highly differentiated (Hudson, 2000). Analyses of molecular variance (AMOVAS) were calculated in GenAlEx ver. 6 (Peakall & Smouse, 2004) with 1000 permutations. AMOVAS for the phylogeographic data set were calculated between and among populations across the major lineages specified to assess genetic variation amongst populations. For the population genetic data set the distribution of genetic variation amongst catchment regions was calculated using an AMOVA.

RESULTS

Phylogenetic analysis

The 1125-bp sequence fragment of *ND2* from 68 individuals recovered 26 haplotypes with 93 variable sites, 49 of which

were parsimony-informative. Total haplotype diversity (Hd) was 0.861 ± 0.034, and total nucleotide diversity (π) was 0.02316 ± 0.0021. For phylogenetic analysis the TIM+I+G model of DNA evolution was selected using AIC tests in MODELTEST. The parameters Base = (0.2825; 0.3564; 0.1272), Nst = 6, Rmat = (1.0000; 23.0322; 0.3433; 0.3433; 5.8514), Rates = gamma, Shape = 0.7316 and Pinvar = 0.6805 were enforced in a likelihood analysis with 100 bootstrap replicates to assess branch support. Parallel Bayesian runs were identical, and major lineages were all well supported. The phylogram presented in Fig. 1 shows three main lineages. The first lineage has a disjunct distribution across some south coastal catchments (SCL), a second is endemic to the Stirling Ranges (SRL), and a third covers the remainder of the species main range (MRL). The SRL had no genetic diversity, despite the fact that samples came from two sites across the range of the species in that region, and therefore was considered as one site/population in further analysis. There was no sharing of haplotypes at any of the sampling sites; neither were there any observable gross morphological differences between any of these lineages (D. Edwards, personal observation). Branches lacking support in the various phylogenetic analyses were collapsed, and thus relationships amongst the distinct lineages could not be resolved.

Pairwise differences in haplotypes between the SRL and MRL were 4.36–4.71% sequence divergence (uncorrected p), and between the SRL and SCL were 4.62–5.42% sequence divergence. Sequence divergence between the MRL and SCL haplotypes, the two lineages occupying the bulk of the *M. nichollsi* range, ranged from 2.76% to 3.56% in pairwise comparisons. For the SCL, Hd = 0.906 ± 0.04 and π = 0.00654 ± 0.00067. All haplotypes from the SRL were the same (Hd and π = 0). The MRL had Hd = 0.655 ± 0.088 and π = 0.00179 ± 0.0004. Divergences within the MRL ranged from 0.09% to 0.8%, and divergences within the SCL ranged from 0.09 % to 1.33%. The score of the likelihood tree without enforcing a molecular clock was −InL = 2200.1561, and the score for the tree enforcing a molecular clock was −InL = 2180.8659. The likelihood ratio tests showed that sequences did not depart from a clock-like model of evolution (P = 0.03021; not significant (n.s.) using the default and conservative value of α = 0.01). When this same test was run on all samples excluding the SRL haplotype, the molecular clock assumption was accepted much more strongly (P = 0.07771; −InL[clock] = 2010.1618; −InL[no clock] = 1993.5639). The average number of nucleotide substitutions per site (d_A) between SRL and MRL was 0.0454 ± 0.00322, providing a divergence estimate of 4.74 Ma ± 330,000 years between these two lineages. Between SRL and SCL, d_A = 0.04963 ± 0.00527, and therefore divergence between these two lineages was estimated at 5.19 Ma ± 551,000 years. Finally, a more recent divergence was obtained between SCL and MRL, namely 2.89 Ma ± 0.177 years (d_A = 0.02764 ± 0.00169).

Phylogeographic analysis

Intraspecific analysis techniques were used to provide information on the biogeographical and historical inferences contained within the data. Tajima's D for the *M. nichollsi* data set showed that sequences were evolving neutrally (D_T = 1.02547; n.s. $P > 0.1$). Three separate networks were joined at the 95% probability of a parsimonious connection. The first contained the haplotypes from the SRL (Fig. 2a). The second contained haplotypes from the majority of the species range, excluding some of the southern catchment areas (MRL); haplotypes in this network were connected by a maximum of 10 mutational steps (Fig. 2b). Finally, haplotypes from the NRS, DFS, KHS and KALS sites (or the SCL) were all joined in another separate network (Fig. 2c) connected by up to 16 mutational steps. The SRL network differs from the MRL and SCL networks by 49 and 52 mutational steps, respectively. The MRL and SCL networks differ by 31 mutational steps. Owing to the large divergence between each of the separate networks they were not joined for the NCPA.

The GeoDis 2.4 output showed several nested clades with significant distance values. A summary of the nested clades with significant phylogeographic signal and of the subsequent biological inferences obtained is given in Table 1. Nested clade 2.1 showed evidence of restricted gene flow with isolation by distance amongst all sites represented by the MRL network, except for the NRM and NRN sites. Also in the MRL network, an inference of either long-distance colonization with fragmentation or fragmentation followed by range expansion in

Table 1 Biogeographical inferences for nested *Metacrinia nichollsi* clades from the Main Range and South Coastal lineages with significant phylogeographic structure, specified by a χ^2 nested contingency test.

Lineage	Nested clade	χ^2 permuted *P*-value	Chain of inference	Inferred process
MRL	2.1	0.0044	1-2-3-4	RGF w/IBD
	Total cladogram	> 0.001	1-2-11-12-13-21	PF w/RE
SCL	3.2	> 0.001	1-2-11-12	CRE
	Total cladogram	> 0.001	1-19-20-2-11-12-13-21	PGRE w/F

P-values are calculated from 1000 random permutations and are considered significant if permuted expected χ^2 values are greater than or equal to the observed values. RGF, restricted gene flow; IBD, isolation by distance; PF, past fragmentation; RE, range expansion; CRE, contiguous range expansion; PGRE, past gradual range expansion; F, fragmentation; w/, with.

nested clade 3.1 was obtained. Nested clades 2.1 and 2.3 showed evidence of range expansion using independent tests ($R_2 = 0.13531$; $P \leq 0.01$, and $R_2 = 0.30728$; $P \leq 0.05$, respectively), whereas clade 2.2 did not ($R_2 = 0.34418$; n.s.). Using the tests for secondary contact outlined in Templeton (2001), the BS site showed strong evidence of contact between divergent nested clades, with some slight signal for the NRM site (results not shown). Long-distance colonization seems unlikely for an animal of this size (up to 25 mm snout–vent length), with no potential for larval tadpole dispersal (Tyler *et al.*, 2000), so biologically contiguous range expansion is a more realistic conclusion. Therefore the most likely inference is past fragmentation across the Naturaliste Ridge and southern Blackwood area followed by range expansion with secondary contact at BS and NRM.

Inferences for the SCL network include evidence for contiguous range expansion from DF across the southern coast to KALS in nested clade 3.2. At the final nesting level for the SCL network there was an inference of either long-distance colonization with fragmentation or past fragmentation with range expansion. Using independent tests for demographic expansion, nested clade 3.3 showed evidence for range expansion ($R_2 = 0.2662$; $P \leq 0.05$), whereas nested clade 3.2 did not ($R_2 = 0.14472$; n.s.). There was also no evidence for secondary contact (results not shown). Long-distance colonization is not likely (reasons outlined above), and therefore past gradual expansion across the southern coast followed by fragmentation was adopted as the appropriate biological inference.

Population genetic analysis

Table 2 provides a summary of the population genetic analyses carried out on the MRL and SCL within *M. nichollsi* separately. SRL data were not analysed in this manner owing to a lack of polymorphism. AMOVA results from the SCL of *M. nichollsi* show extremely high levels of population structure, with 86% of genetic variation accounted for between populations/catchments (each population of this lineage being in a different catchment). Hudson's Snn corroborate these results, suggesting that populations/catchments are completely differentiated (Snn = 1.000). The AMOVA results for the MRL of *M. nichollsi* show lower levels of population genetic structure (accounting for 56% of the variation), with Snn suggesting that populations are panmictic (Snn = 0.238). AMOVAs considering catchment groups within the MRL show that there is much more variation among populations within catchments (34%) than between catchment groups (10%).

DISCUSSION

There are two major phylogenetic divergence events within the *M. nichollsi* lineage that account for the majority of the genetic diversity observed. The first is the separation of the relictual Stirling Ranges populations (SRL) from the populations in the remainder of the species range during the late Miocene–early Pliocene (Figs 1 and 3). The second splits the remainder of the species distribution into a lineage with a disjunct distribution across the south coast (SCL) and another covering the majority of the species range (MRL), with divergence estimates dating this split during the mid- to late Pliocene (Figs 1 and 3). The biogeographical patterns observed in each of these clades appear to be vastly different. Within the SCL, a pattern of restricted dispersal between catchment groups exists, and fragmentation between the Blackwood and Deep Rivers was inferred. Within the MRL, there was evidence of recent expansion with some restriction of gene flow across the Naturaliste Ridge. The biogeographical history of the major divergence events and the phylogeographic history within the various lineages are examined below with reference to how the climatic history of the south-west region may have impacted on the genetic structure of *M. nichollsi*. As noted in the Introduction, there have been no geological events of any significance in relevant historical time frames.

Table 2 Summary table of population genetic statistics for the South Coastal and Main Range *Metacrinia nichollsi* lineages.

Source	d.f.	SS	MS	Est. var.	Percentage	Stat.	Value
South Coastal Lineage population genetic analysis							
Among populations	3	56.861	18.954	3.870	86		
Within populations	15	9.350	0.623	0.623	14	ΦPT	0.861***
Total South Coast Lineage						Snn	1.000***
Main Range Lineage population genetic analysis							
Among regions	6	14.472	2.412	0.103	10	ΦRT	$0.099^{n.s}$
Among populations/regions	4	7.233	1.808	0.353	34	ΦPR	0.374***
Indiv./within populations	28	16.500	0.589	0.589	56	ΦPT	0.436***
Total Main Range Lineage						Snn	0.238***

Analyses of molecular variance (AMOVA) results for each lineage are presented separately. Within the Main Range Lineage (MRL) the distribution is divided up into catchment regions; within the South Coastal Lineage discrete populations are already in separate catchment regions. Hudson's 'nearest neighbour' statistic (Snn) is also shown for each lineage as a whole. *P*-values were calculated from 1000 permutations. $n = 19$ and $n = 39$ for the SCL and MRL, respectively; n.s. = $P > 0.05$; ***$P \leq 0.001$. SS, sum of squares; MS, mean square; ϕPT, among population variance; ϕRT, variance among regions; ϕPR, variance among populations within a designated region.

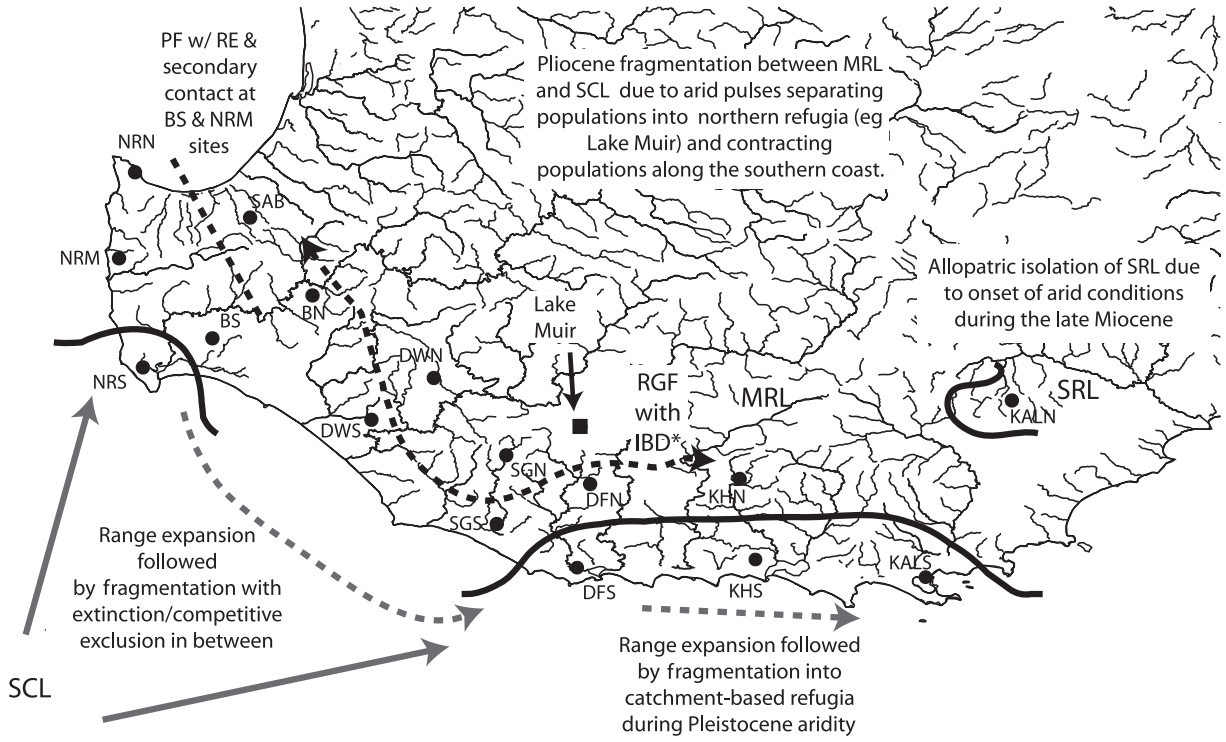

Figure 3 Biogeographical hypotheses relating to the *Metacrinia nichollsi* phylogeographic data set. Hypotheses are generated from both the nested clade phylogeographic analysis results and the known geological and climatic history of the region. MRL, Main Range Lineage; SRL, Stirling Ranges Lineage; SCL, South Coastal Lineage; PF, past fragmentation; RE, range expansion; ●, sampled populations; *, a cautious inference owing to limitations of the method; RGF, restricted gene flow; IBD, isolation by distance. See Fig. 1 for site names.

Isolation of the Stirling Ranges populations

The Stirling Ranges is a bioregion of significance within south-western Australia: it contains a very large number of recently evolved plant species, and a large number of Gondwanan relicts (Burbidge, 1960; Dirnböck *et al.*, 2002; Hopper & Gioia, 2004). The high topographical relief and 'wet, moist' upland regions and gullies provide refuge microhabitats for formerly widespread Gondwanan relicts, such as myglamorph spiders (Main, 1999, 2001). The isolation of Stirling Range *M. nichollsi* lineages from those occurring throughout the wetter forests is a pattern observed also in myglamorph spiders (Main, 1999). Distributional patterns and persistence in wet microhabitats in the Stirling Ranges may be a result of the contraction of higher rainfall to the south-western coast from the Late Miocene onwards (Archer, 1996; Main, 1999, 2001). Our estimates of divergence date the isolation of the SRL during the late Miocene–early Pliocene period; however, this may be an overestimate if this population has suffered one or more bottlenecks, leading to genetic drift and an overestimate of mutation rates (Bromham & Penny, 2003; Welch & Bromham, 2005). A severe bottleneck event is indicated by the complete lack of genetic diversity in the two Stirling Ranges populations sampled (Nei *et al.*, 1975). While the separation of the SRL dates to the late Miocene, isolation could just as easily have occurred during Pliocene arid pulses as arid climatic fluctuations increased in south-western Australia from the late Miocene through to the present (Macphail, 1997; Dodson & Macphail, 2004).

In the Stirling Ranges, *M. nichollsi* survives in a few gully systems and mountaintops on the eastern side of the range (personal observation). The SRL fits the criteria of an Ecologically Significant Unit for conservation purposes because these populations are genetically distinct, geographically isolated and genetically depauperate (Moritz, 2002). Forecast climate change models predict higher temperatures in the future, similar to those seen at the Plio-Pleistocene border (Cronin & Dowsett, 1993), but current trends suggest that warmer temperatures will be associated with reduced rainfall (Bureau of Meteorology – http://www.bom.gov.au/), which is likely to lead to increased fire frequency and intensity in the Stirling Ranges. Such increases in fire frequency and intensity in the area, primarily human-induced, have already been linked to population bottlenecks and local extinctions in myglamorph spiders (Main, 1999), which often occupy the same microhabitats as *M. nichollsi* (Main, personal communication). Therefore, impending climate change is likely seriously to threaten the viability not only of this relictual and distinct population of *M. nichollsi*, but also of many other relictual species currently found in the Stirling Ranges.

Biogeography within the south-western clades of *M. nichollsi*

Divergence estimates suggest that the two lineages within the continuous range of *M. nichollsi* separated 2.6–3.4 Ma, consistent with divergences between sister-species in other myobatrachid genera (Read *et al.*, 2001). Palynological evidence suggests that significant arid pulses occurred during the mid- to late Pliocene period in south-western Australia, with severe arid pulses occurring 2.6 and 2.9 Ma (Dodson & Ramrath, 2001; Dodson & Macphail, 2004), closely matching our estimated divergence dates. Pliocene arid events may have led to isolated populations in the north and south of the species range, forming the MRL and SCL. In the south (SCL), the species is likely to have contracted towards the coast, where rainfall remained high, albeit reduced, during arid cycles, as indicated by the large number of relictual animals and plants (Hopper *et al.*, 1996). In the north (MRL), individuals may have persisted in a number of sites, for example Lake Muir or parts of the upper Blackwood catchment (see Fig. 1), which may have remained wet enough during severe arid pulses in the Pliocene. The individual phylogeographic histories of the two lineages, SCL and MRL, give some indication of the possible historic scenarios that might explain the current distribution of these divergent lineages.

The SCL has a disjunct distribution across the Pingerup Plains on the south coast. Populations within this lineage occur from the Deep River through to the Kalgan River catchment, with an isolated population situated to the north of Augusta (NRS). Fragmentation of the NRS population from the other SCL populations occurs across the area between the Scott River Coastal Plain (east of the Blackwood River) and the Pingerup Plains (west of the Deep River). The Pingerup Plains and Scott Coastal Plains define the geographic breaks between the ranges of *G. rosea* and *G. lutea*, and between those of *G. alba/G. vitellina* and *G. rosea*, respectively (Wardell-Johnson & Roberts, 1993). The Pingerup Plains are extremely waterlogged, with swampy ground during winter that dries rapidly in spring into summer, an environment thought to be incompatible with survival and reproductive success in the wetter-adapted *Geocrinia* species (Wardell-Johnson & Roberts, 1993). The Scott Coastal Plain has a similar pattern of seasonal surface-water fluxes (Strategen, 2005). Although *M. nichollsi* is less reliant on moisture in drainage systems than species in the *G. rosea* complex, it is still dependent on soil moisture in summer, the driest season, for breeding. Discrete genetic groups, restricted to specific catchments, within the SCL of *M. nichollsi* and in the *G. rosea* species complex (Driscoll, 1998a,b), highlight a probable role for catchments as important refugial areas during periods of reduced rainfall throughout the Pleistocene.

The MRL occupied the remaining region covered by the continuous range of *M. nichollsi*, including the intervening area between the disjunct SCL populations. Phylogeographic inferences within the MRL suggest that rapid dispersal occurred across this region after initial isolation of the lineage. A broad pattern of dispersal across the southern coast is also reflected in the phylogeography of *C. georgiana* (Edwards *et al.*, 2007). However, expansion in the MRL within *M. nichollsi* is likely to have preceded that seen in *C. georgiana*, a more broadly distributed species with a phylogeographic history that is much more recent than that of *M. nichollsi* (Edwards *et al.*, 2007). This expansion may well have been associated with 'warm, wet' periods during the Pliocene (Dodson & Macphail, 2004), which allowed the species to move out of wetter refuges to occupy its current distribution.

Subsequent inferences within the MRL suggest that dispersal has been restricted between Naturaliste Ridge populations and those within the remaining range of the MRL, with some secondary contact mainly at the BS site, and a smaller signal indicating secondary contact at the NRM site. The lower Blackwood River may have disrupted gene flow within the MRL in the region north of Augusta, as well as contributing to the maintenance of divergence between the MRL and SCL directly east of Augusta. During interglacials, higher rainfall (Dodson & Macphail, 2004) and higher sea levels (Hodgkin & Hesp, 1998; Sircombe & Freeman, 1999) would have led to higher river flows that cut off dispersal, with the potential for dispersal across the riverine barrier and secondary contact only available during drier times. Higher sea levels and subsequent changes in the coastline in the Geographe Bay region (Sircombe & Freeman, 1999; Hageman *et al.*, 2003) may also have contributed to the restriction of gene flow between populations in the northern Naturaliste Ridge and the remaining MRL. An inference of restricted dispersal, following an inference of range expansion in the MRL (excluding Naturaliste Ridge populations), suggests a pattern of dispersal across the landscape that is restricted in current times. However, this inference may be confounded by the NCPA method itself and should be treated with caution (Panchal & Beaumont, 2007).

Given the disparate history of the two lineages within the continuous distribution of *M. nichollsi*, there are several equally plausible explanations that may explain the biogeography of the species. SCL populations may have became extinct between the Naturaliste Ridge and the Deep River during a climatic extreme, either wet (Sircombe & Freeman, 1999) or dry (Galloway & Kemp, 1981), during the Pleistocene, with expanding northern populations then quickly moving to occupy available habitat in more favourable mesic periods. Another plausible explanation is that SCL populations moved out onto the coastal plain exposed by lower sea levels during the Pleistocene, and may have been held in place by an expanding MRL, only to be forced to extinction by rises in sea level. Alternatively, MRL individuals may have a selective advantage with hybridization only occurring between northern females and southern males. Changes in colour/pattern and the potential for sexual selection should also be considered as viable explanations. *Metacrinia nichollsi* is highly variable across its range in body size, and in dorsal and ventral colour and pattern; however, the distribution of this variability in light of the current genetic results has not been documented. An assessment of the extent of lineage distributions, and of patterns of change in body size, pattern, colour and call should

be carried out in combination with genetic testing using both mitochondrial and nuclear markers before any conclusions can be drawn about the historical reasons for the current distribution of these two divergent lineages.

CONCLUSIONS

Metacrinia nichollsi presents an important case study for the biogeography of the south-western Australian wet forest system. The biogeography of *M. nichollsi* has elements of: (1) patterns of geographic isolation in the Stirling Ranges, comparable to the patterns of isolation seen in myglamorph spiders; (2) patterns seen in the *G. rosea* species complex, with some local catchment-based differences in the SCL; and (3) broad geographic patterns of expansion across parts of the southern coast, seen also in *Crinia georgiana* but differing in biogeographical detail, location and particularly age. There are no simple, common patterns in the biogeography of south-western Australian frogs, as might have been expected given the history of adaptation to gross climate change (e.g. from summer to winter rainfall patterns). The deep lineage splits within *M. nichollsi* reflect patterns seen in other south-western Australian frogs and indicate the potential for speciation at very small geographic scales in areas that are not concordant across species. Emerging biogeographical models for south-western Australian frogs are increasingly showing that the conservation of biodiversity in south-western Australia is an ongoing and complex problem that must consider the evolutionary history and evolutionary potential of all biotic groups.

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BIOSKETCHES

The study presented formed part of the PhD research of Danielle Edwards, supervised by Dale Roberts, with molecular work completed in Scott Keogh's molecular laboratory.

Danielle Edwards is interested in the application of molecular techniques to problems in Australian biogeography, population genetics and the conservation of herpetofauna. She is currently a postdoctoral associate with Scott Keogh at ANU.

Dale Roberts studies sexual selection, call evolution, and the biogeography of Australian frogs.

Scott Keogh's research interests cover the taxonomy and biogeography of a range of herpetofauna.

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