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Biogeography and speciation of a direct developing frog from the coastal arid zone of Western Australia

Danielle L. Edwards

School of Animal Biology, The University of Western Australia, Crawley, WA 6009, Australia
School of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia

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Abstract

Within the southwestern Australian biodiversity hotspot, the Shark Bay region displays high levels of plant and animal endemism, particularly in the herpetofauna. The region has been subjected to dramatic climatic fluctuations and has been geologically active from the Late Miocene to the present. The myobatrachid frog *Arenophryne rotunda*, a Shark Bay endemic, provides an ideal opportunity to examine the relative effects of fluctuating climates and geological activity on the biota of Shark Bay. A comprehensive phylogeographic analysis of *A. rotunda*, based on data comprising 1154 bp of the mitochondrial gene *ND2*, is presented. My results demonstrate a major genetic break that divides this species at the northern edge of the Victorian Plateau into northern and southern species lineages, dating to the Late Miocene, with a further division of the southern species lineage across the Murchison Gorge dating to the Plio-Pleistocene border. Both of these periods are related to prominent geological activity and climatic shifts in the Shark Bay region. Interpretation of phylogeographic results point to the prominent role of fluctuating Pleistocene climates and associated coastal landscape evolution in the generation of phylogeographic structure within the distinct *A. rotunda* species lineages. Similar processes have been invoked to explain the diversity of other Shark Bay biota.

Keywords: Phylogeography; Southwestern Australia; Anura; Nested clade phylogeographic analysis; Mitochondrial DNA; Shark Bay; *Arenophryne rotunda*

1. Introduction

Southwestern Australia, which includes the Shark Bay region at its northern end, has been identified as one of 25 of the world's "biodiversity hotspots" based on both high levels of endemism and conservation concern (Cincotta et al., 2000). The region is well known for extreme plant diversity and endemism (Hopper, 1979; Hopper and Gioia, 2004) but less well known for its fauna, which also show high levels of endemism. For example, the southwest has a large number of endemic invertebrate, (Main, 1996), mammal, reptile and amphibian faunas (Hopper et al., 1996). Climatic fluctuations of the late-Tertiary and Quaternary have been implicated in explaining the extreme diversity of southwestern Australian endemic flora (Hopper, 1979; Hopper and Gioia, 2004). Studies conducted on myobatrachid frogs endemic to southwestern Australia also have suggested that climatic fluctuations have played a role in speciation within several genera, some of which are particularly diverse in the region (Morgan et al., 2007; Wardell-Johnson and Roberts, 1993; Roberts, 1997). Also from the limited phylogeographic studies conducted on southwestern Australian frogs, climate (Driscoll, 1997, 1998; Davis and Roberts, 2005; Edwards et al., 2007) and to a certain extent geological features (Berry, 2001) have influenced the current genetic architecture of endemic species.

The Shark Bay and surrounding region has undergone some of the most dramatic climate fluctuations of the entire southwest, as it is the border area between Hopper's Transitional Rainfall Province and the arid zone (Hopper, 1979;

E-mail address: dan@cyllene.uwa.edu.au

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Hopper and Gioia, 2004). Climate has shifted from a tropical/subtropical climate to one of aridity and winter seasonal rainfall (Hocking et al., 1987). The onset of aridity in Australia became entrenched in the southwest during the Late Miocene and was associated with an overall drop in sea-levels as well as a general drying of the continent (Macphail, 1997; Dodson and Macphail, 2004); consequently, vast new areas of coastal sand habitats formed and dune-building processes began (Hocking et al., 1987). The Pliocene saw a brief retreat of arid conditions (Dodson and Macphail, 2004) then a general trend of increasing fluctuation between wet to arid climates, with arid pulses gradually increasing in intensity across the Australian continent (Bowler, 1976; Kershaw et al., 1991; Macphail, 1997). Pleistocene climate fluctuations were associated with eustatically controlled sea-level transgression/regression cycles leading to massive changes in the occurrence and area of coastal sandplain and sand-dune habitats (Hocking et al., 1987; Mory et al., 2003). Dune-building episodes occurred during arid (glacial) cycles intersected with transgressive episodes during interglacial wet periods (240 000 yrs ago and 120 000–130 000 yrs ago) in the Shark Bay region (Van de Graaff et al., 1980; Hocking et al., 1987). The most recent transgressive cycle occurred at the height of the last interglacial and produced the final flooding of Shark Bay, beginning ~10 000 yrs ago and reaching its peak ~6000 yrs ago (Butcher et al., 1984; Hocking et al., 1987).

Pleistocene coastal landscape evolution driven by climatic fluctuations have been used to explain diversity and recent speciation in the Shark Bay region biota (Storr and Harold, 1978, 1980; Hopper and Gioia, 2004; Rabosky et al., 2004). However, older and more fundamental geological evolution also may play a part in shaping current genetic architecture, particularly in fossorial anurans and reptiles common in the area. While southwestern Australia in general is considered to have been geologically stable since the Tertiary (Hopper, 1979; Hopper and Gioia, 2004) coastal areas of the Shark Bay region have undergone a complex series of geological processes leading to the evolution of the current landscape (Van de Graaff et al., 1980; Hocking et al., 1982, 1987; Butcher et al., 1984; Mory et al., 2003). After a period of long stability reactivation of pre-existing faults in the area began in the Miocene and a period of tectonic instability continued through to the Pleistocene. This tectonic instability has been linked to the formation and dissection of the Victoria Plateau, the incision of the Murchison Gorge (Hocking et al., 1982, 1987), general uplift (Haig and Mory, 2003; Mory et al., 2003) and the gentle folding of anticlines, which are now a controlling factor in shaping the coastline of the Shark Bay area (Hocking et al., 1987).

Arenophryne rotunda, a highly arid adapted and fossorial direct-developing frog endemic to Shark Bay, provides an ideal model species to investigate the influences of both geology and climate change/sea-level fluctuations on Shark Bay fauna. While nothing is known about the history of this species, given the considerable molecular genetic divergence between *Arenophryne* and it's closest relatives, *Myobatrachus gouldii* and *Metacrinia nichollsi* (Read et al., 2001), older climatic and geological events may have impacted the current genetic architecture of *A. rotunda*. The distribution of *A. rotunda* crosses many significant geological entities within the Shark Bay region, namely the northern border of the Victoria Plateau and the Murchison Gorge (Fig. 1). The species also occupies much of the coastal Shark Bay region and Dirk Hartog Island, which permits an assessment of the impacts of coastal landscape evolution and the flooding of Shark Bay. Additionally, given the fossorial habit of the species and its preference for sandplain and dune habitats, Pleistocene dune-building episodes may well have influenced population structure within *A. rotunda*. I compiled sequence data from an 1154 bp fragment of the mitochondrial gene encoding *ND2* from 47 individuals, across 19 localities and covering the whole known range of the species. This study provides the first comprehensive dataset specific to the Shark Bay region and a comparison for biogeographic hypotheses developed for plants and herpetofauna of the Shark Bay region.

2. Materials and methods

2.1. Animal and tissue samples

Arenophryne rotunda is a small, fossorial, direct-developing frog endemic to the southwest (Roberts, 1990), from Shark Bay south to Kalbarri—Fig. 1. It occupies sand dune and sandplain habitats, encompassing several different substrate types and crossing several climatic zones. Its distribution is thought to be continuous across its range, with some of the highest levels of anuran abundance ever recorded (Roberts, 1985). A total of forty-seven individuals were sampled (toe-clips) from 13 sites across the entire species distribution, with 2–4 animals per site (Fig. 1 and Table 1). Samples from EL1, ZU2 and ZU5 were taken from the WA Museum Tissue Collection, WAM collection numbers 122520–122522, 123493–123495 and 123523–123526, respectively. Outgroup sequences used in this current study were: *Metacrinia nichollsi* (34°59′38″ 116°39′22) and *Myobatrachus gouldii* (30°01′57″ 115°49′06″).

2.2. Molecular genetic methods

Template DNA was extracted from toe samples using a modified CTAB method, suspended in TE and stored at 0 °C. Targeted DNA was amplified using a touch-down PCR profile: 94 °C for 5 min—1×; 94 °C for 30 s, 70–45 °C (decreasing in 5 °C increments) for 20 s, 72 °C for 90 s—each of these cycles were repeated 2× for each extension temperature; 94 °C for 30 s, 40 °C for 30 s, 72 °C for 45 s—40×; 72 °C for 4 min—1×; 4 °C held. Primers used to amplify *ND2* were L4221 5′-AAGGRCCTCCTTGA TAGGGA-3′; modified from Macey et al. (1998), and

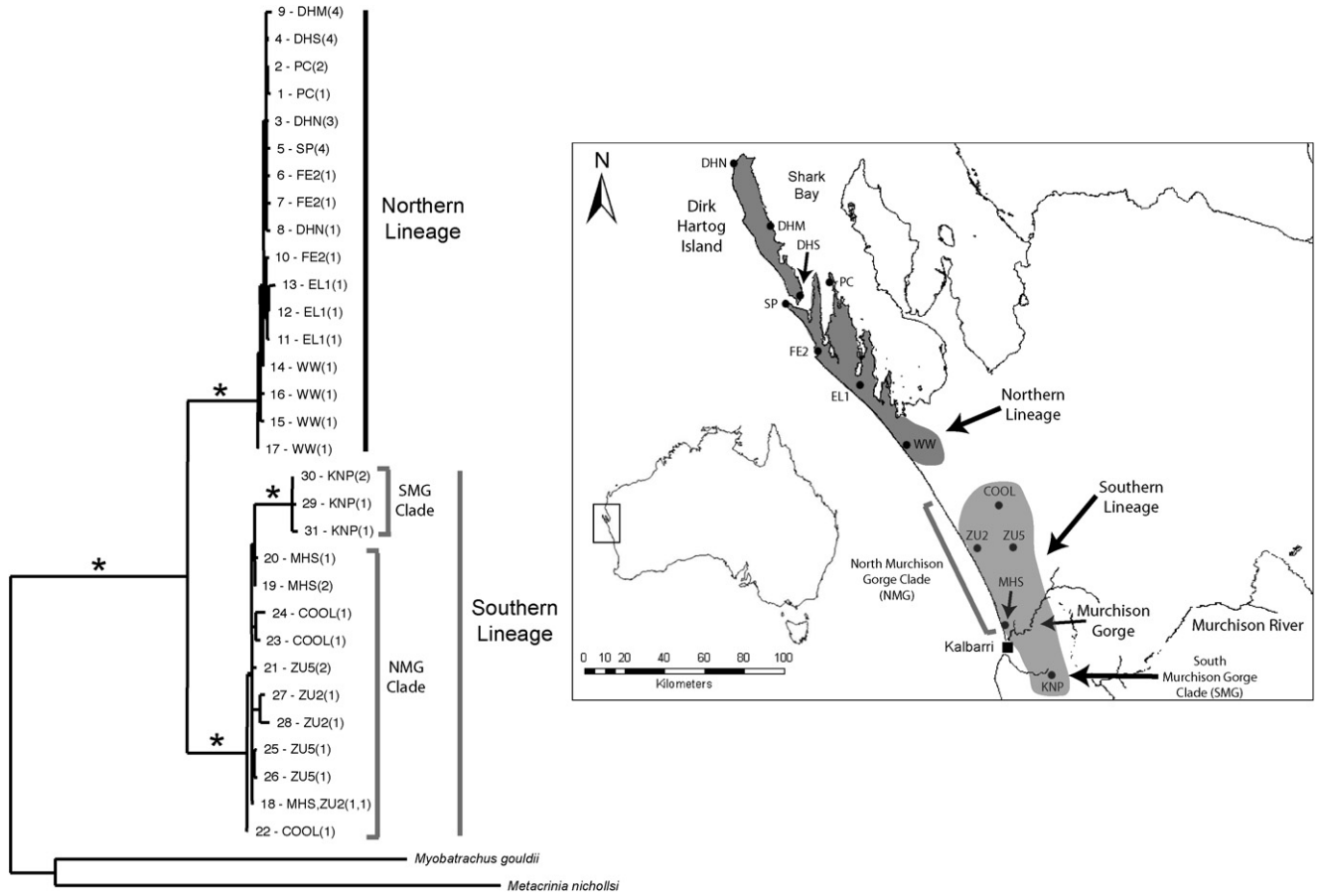

Fig. 1. Maximum-likelihood phylogram of 31 *Arenophryne rotunda ND2* haplotypes showing two major lineages with *Metacrinia nichollsi* and *Myobatrachus gouldii* as outgroups. Haplotype numbers are displayed with the sample site from which they came and their frequency in brackets. Bootstrap values for clades above 70 are represented by * and were calculated from 100 replicates. The TrN + I model of DNA evolution was enforced in maximum-likelihood analyses as suggested by AIC tests in ModelTest 3.7. Map of the mid-western Australian coast is shown with map of the Australian continent inset and with shaded areas representing the distribution of both the northern and southern species lineages. Tissue collection locations [●] for the *Arenophryne rotunda* phylogeographic study cover the entire known distribution of the species.

Table 1
Arenophryne rotunda sampling location names, abbreviations, sample sizes and exact GPS co-ordinates in degrees, minutes, seconds

Site	Abbreviation	Sample size	Latitude	Longitude
Dirk Hartog Island Nth	DHN	4	25°31′21″	112°55′49″
Dirk Hartog Island Mid	DHM	4	25°48′29″	113°05′58″
Dirk Hartog Island Sth	DHS	4	26°07′12″	113°13′55″
Steep point	SP	4	26°09′21″	113°10′00″
False entrance #2	FE2	3	26°22′16″	113°18′36″
Pearler's camp	PC	3	26°03′45″	113°21′46″
Edel land #1	EL1	3	26°31′32″	113°30′07″
Whale well	WW	4	26°47′41″	113°42′45″
Cooloomia nature reserve	COOL	3	27°03′59″	114°07′39″
Zyutdorp cliffs #2	ZU2	3	27°15′36″	114°01′53″
Zyutdorp cliffs #5	ZU5	4	27°15′20″	114°11′26″
Murchison house station	MHS	4	27°36′22″	114°09′27″
Kalbarri national park	KNP	4	27°49′59″	114°21′53″

All points are in geodetic WGS84.

tRNA-Asn 5′-CTAAAATRTTRCGGGATCGAGGCC-3′ (Read et al., 2001), or Myo tRNA-trp 5′-GGGGTA GYATHCCACAAGTC-3′; this paper. Targeted fragments were amplified in 40 μl reactions comprising of ~100 ng template DNA, 4 μl of 10× reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTPs, 10 pmol each primer and 2 units of Platinum *Taq* polymerase (Life Technologies, Gaithersburg, MD).

Samples were run on a 2% Agarose gel, targeted fragment were then excised and cleaned using a Mo Bio UltraClean DNA Purification Kit (Mo Bio Laboratories, Inc). Approximately 100 ng of PCR product was added to sequencing reactions using either DYEnamic ET Terminator (Amersham Pharmica Biotech) or Big Dye Terminator 3.1 (Applied Biosystems) sequence mix and run according to manufacturer's specifications. Internal primers, L4437 5′-AAGCTTTCGGGGCCCATACC-3′ (Macey et al., 1998), and Myo-L4882 5′-MACVTGRCAAAAAY THGCCCC-3′; modified from Melville et al., 2004, were used in addition to PCR primers to obtain reliable sequence across the entire gene. Cleaned reactions were then resuspended in a loading dye/formamide mix. Sequences were visualised on an ABI 3010 Capillary sequencer (Applied Biosystems). DNA sequence data were then edited using Sequencher 3.0 (Gene Codes Corporation).

Sequences were aligned using ClustalX (Thompson et al., 1997). Alignments were then checked by eye. Sequences were translated using the mammalian mitochondrial genetic code option in Sequencher 3.0, and an open reading frame was observed in all sequences. Thus sequences were assumed to be genuine mitochondrial copies and not nuclear paralogues (Sunnucks and Hales, 1996). Haplotype sequences have been deposited in GenBank (Accession Nos. EF606703–EF606760).

2.3. Phylogenetic analysis

Phylogenetic techniques were employed to describe major phylogenetic structure within the *A. rotunda* phylogeographic dataset. Maximum likelihood (ML) analyses of haplotype sequences were used to assess overall phylogenetic structure and support for major clades in PAUP*4.0b10 (Swofford, 2002). Akaike Information Criterion (AIC) tests, performed in Modeltest 3.7 (Posada and Crandall, 1998), were employed to select the best-fit model of evolution from the data. The model selected was then applied to calculate the nucleotide frequencies, substitution rates, gamma distribution and proportion of invariant sites for the ML analysis. To assess branch support for relationships in the trees, bootstrap values were calculated from 100 replicates. Starting trees were obtained by step-wise addition and the TBR method of branch swapping was employed in a heuristic search.

A molecular clock estimate was used to calculate approximate timing of major divergence events. Divergence between major clades was calculated using the formula of Nei and Li for d_A (the average number of nucleotide substitutions per site between clades/lineages) (Nei, 1987). The d_A parameter estimates and their standard errors were calculated using DnaSP v4.10.8 (Rozas and Rozas, 1999). There are no appropriate external calibration points or fossils that can be used to calibrate a molecular clock for any southwestern frog genera, despite the existence of some fossils found in recent to Pleistocene cave deposits (Roberts and Watson, 1993; Price et al., 2005). Therefore, I adopted the molecular clock rate of 0.957%/lineage/million years, model-corrected by Crawford (2003) from the uncorrected genetic distances of Macey et al. (Macey et al., 1998). To test the hypothesis of clock-like evolution in the *A. rotunda ND2* sequences, a maximum-likelihood search was conducted in PAUP* 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 3.7 (Posada and Crandall, 1998).

2.4. Population genetic and phylogeographic analysis

The aims of phylogeographic analyses were to provide a measure of geographical genetic structuring of populations and to gain inferences about the evolutionary history of *A. rotunda*. These analyses sought to identify important events leading to the development of genetic structure within this species. Nested Clade Phylogeographic Analysis (NCPA) provides a test of the non-random geographic distribution of haplotype variation and a method of inference to distinguish current population structure versus historical processes leading to an association between the gene tree and geography (Templeton, 1998). The use of NCPA has been criticized (Knowles and Maddison, 2002), but it remains a powerful phylogeographic technique particularly when all events and processes affecting a species evolutionary history are not known *apriori* (Templeton, 2004). This was the case for *A. rotunda*, as there was no prior knowledge of the species history.

Unrooted statistical parsimony haplotype networks were created using TCS 1.21 (Clement et al., 2000). The separate networks, where connections between divergent haplotypes could not be made under the 95% probability criterion, were nested according to the nesting rules outlined in Templeton and Sing (1993), Templeton et al. (1995) and Crandall et al. (1994). Where interior/tip status was ambiguous, particularly at the final nesting level, clade outgroup probability (Castelloe and Templeton, 1994) and position in relation to outgroups in the phylogenetic tree (Fig. 1) were used to determine the interior clade. Tests for geographical association were performed on the nested haplotype networks in GeoDis v2.4 (Posada et al., 2000) using the latitude and longitude coordinates for each sampling location. Clades with significant phylogeographic structure, determined by χ^2 contingency tests after 1000 random permutations, were identified and the significant D_C and D_N values within these clades were then used in conjunction with the November 2005 NCPA inference key (http://darwin.uvigo.es/software/geodis.html) to reconstruct population histories.

Various techniques have been used to complement the results of the NCPA inference key. Tajima's D (D_T) was calculated to test the hypothesis of neutrality (Tajima,

1989). Where NCPA requires confirmation of recent population expansion in certain nested clades (e.g., step 21 of the key), R_2 tests (Ramos-Onsins and Rozas, 2002) were used to test the hypothesis of population growth under the neutral model using coalescent simulations permuted 1000 times. The R_2 test for population growth is based on the difference between the number of singleton mutations and the average number of nucleotide differences among sequences. It is a powerful test compared to other measures such as mismatch distributions and Fu's F_S (Ramos-Onsins and Rozas, 2002), and more effective than these methods with small sample sizes. Both D_T and R_2 were calculated in DnaSP v4.10.8 (Rozas and Rozas, 1999). Where secondary contact between distinct haplotype lineages was suspected, the supplementary test described by Templeton (2001) was used. This test requires the calculation of average pairwise distances (km) between geographical centres of clades (provided by the GeoDis 2.4 output) found at each sampling location and is calculated for every nesting level of the network. Sites where geographically divergent haplotype clades (i.e., high distance values) co-occur mark sites of secondary contact between divergent population lineages. For principles and methodology behind this test for NCPA refer to Templeton (2001, 2004).

Several techniques were used to test the relative contribution of known potential geographic barriers to population-genetic structure within each of the major *A. rotunda* species lineages diagnosed by methods described in the preceding paragraph. DnaSP v4.10.3 was used to calculate Hudson's 'nearest neighbour' statistic (Snn) with 1000 permutations via the coalescent, to provide a measure of population differentiation within each major species lineage, as well as for populations north of the Murchison Gorge within the southern species lineage. Hudson's Snn 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). An Analysis of Molecular Variance (AMOVA) was calculated in GenAlEx v6 (Peakall and Smouse, 2004) with 100 permutations. AMOVA analyses within the northern *A. rotunda* lineage were used to assess the proportion of genetic variability explained by island versus mainland populations. AMOVA analyses were carried out on the southern *A. rotunda* species lineage to quantify the amount of genetic variation that could be explained by the separation of populations on either side of the Murchison Gorge.

3. Results

3.1. Phylogenetic Analyses

Complete sequences of the mitochondrial *ND2* gene are reported for all 47 individuals sampled (1154 base pairs), yielding 31 haplotypes with a total of 113 variable sites, 87 of which were parsimony informative. Strong anti-G bias (11.8% G) suggested the fragment was a genuine mitochondrial sequence and not a nuclear paralogue (Zhang and Hewitt, 2003). AIC scores, in Modeltest, selected the TrN + I model as the model of substitution which best fit the data. Parameters defined under this model were as follows: Base frequencies (0.2862, 0.3526, 0.1179), Rmat = (1.0000, 24.0631, 1.0000, 1.0000, 10.4843), and Pinvar = 0.7723. The parameters from this model were enforced in likelihood analyses with 100 bootstrap replicates to assess branch support for major clades. The maximum-likelihood tree (Fig. 1) shows a strongly supported divergence between haplotypes from the northern and southern populations of *A. rotunda* separated by the northern edge of the Victoria Plateau. Nested within the southern haplotype clade is a strongly supported grouping of haplotypes sampled south of the Murchison Gorge. Within the northern haplotype clade 17 haplotypes were recovered from 29 individuals. Twenty-seven sites from these northern lineage haplotype sequences were variable, with a total haplotype diversity (Hd) of 0.946 ± 0.0005 and nucleotide diversity (π) of 0.00374 ± 0.0000001. For the southern haplotype clade 14 haplotypes were recovered from 18 individuals with 53 variable sites and $\mathrm{Hd} = 0.974 \pm 0.00064$ and $\pi = 0.01131 \pm 0.0000042$.

Pairwise genetic distances between the northern and southern haplotype clades range from 5.5% to 6.4% sequence divergence (uncorrected *p*). Sequence divergences within the northern haplotype clade were 0.09–0.78%, and 0.09–2.9% within the southern haplotype clade. Within the southern haplotype clades, haplotypes corresponding to populations either side of the Murchison River differed by 1.9–2.9%; divergences among haplotypes sampled north of Murchison Gorge (NMG) were 0.09–1.5% and those among haplotypes sampled south of Murchison Gorge (SMG) were 0.09–0.26%. The score of the likelihood tree, without enforcing a molecular clock, was $-\mathrm{In}\, L = 3346.4254$. The score for the likelihood tree enforcing a molecular clock was $-\mathrm{In}\, L = 3368.8046$. Likelihood ratio tests suggested that sequences did not depart from a clocklike model of evolution (n.s.; $P = 0.0524$). The proportion of nucleotide substitutions (d_A) between the northern and southern haplotype clades was 0.05390 ± 0.00392. The d_A between the NMG and SMG haplotypes of the southern haplotype clade was 0.01965 ± 0.00406. This provides divergence estimates of ~5.63 million years ago (MYA) ±410 000 yrs and ~2.05 MYA (±424 000 yrs) for the split between the northern and southern haplotype clades and between clades of the southern haplotypes sampled on either side of the Murchison Gorge, respectively.

3.2. Population genetic and phylogeographic analyses

Tajima's *D* showed that neutrality could not be rejected for either the northern or southern haplotypes in the *A. rotunda* dataset ($D_T = -1.428$ and -0.633 for the northern

and southern species lineages, respectively). Three separate networks were formed at the 95% probability of parsimonious connection, the first coinciding with the northern haplotype clade (Fig. 2). The remaining two networks corresponded to the two major clades (NMG and SMG) within the southern haplotype clade (Fig. 3). Networks from the distinct northern and southern haplotype clades differed by an estimated 63 mutational steps.

Significant phylogeographic structure was detected in several clades within the northern haplotype clade (significant χ^2 P-value: Table 2). Inferences for the northern haplotype clade network suggest allopatric fragmentation within nested clade 2.1, or between several of the mainland (PC and SP/FE2) and all island populations. In nested clade 3.1 (all populations excluding EL1 and 1 haplotype from FE2) there is an inference of either long distance colonisation with subsequent fragmentation or past fragmentation with range expansion. Demographic expansion, which can indicate range expansion, is detected from R_2 values calculated for nested clade 2.4 (WW only), ($R_2 = 0.0866$; $P \leqslant 0.01$), but not for nested clade 2.1, ($R_2 = 0.1065$; P-n.s.). Using the supplementary tests outlined by Templeton (2001) there is strong evidence for secondary contact between haplotype clades at the FE2 site (a distance of 54 km relative to the ~140 km distribution of the northern lineage). With estimated dispersal distances of up to 14.8 m per night (Tyler et al., 1980), movement is generally not in a straight line and populations are generally patchy across the landscape (pers. obs.). Therefore, it is unlikely that an individual would move over 40 km within its lifetime. The most likely inference is past fragmentation of WW and EL1 from northern populations followed by gradual range expansion north with secondary contact at the FE2 site. An overall inference of restricted gene flow is attained at the total network level for the northern haplotype clade of *A. rotunda*.

Haplotypes within each of the two southern haplotype networks were connected by a maximum of 14 mutational steps at the 95% probability level (Fig. 3). These two separate networks were joined at the final nesting level and differed by an estimated 22 mutational steps. There is evidence for significant phylogeographic structure within the southern haplotype clade networks (Table 2). Restricted gene flow is inferred among NMG populations (Nested clades 2.1 and 4.1), suggesting limited dispersal among these populations. There is also an inference of allopatric fragmentation between populations on either side of the Murchison Gorge, between the nested clades 4.1 and 4.2, when both networks are joined at the final nesting level.

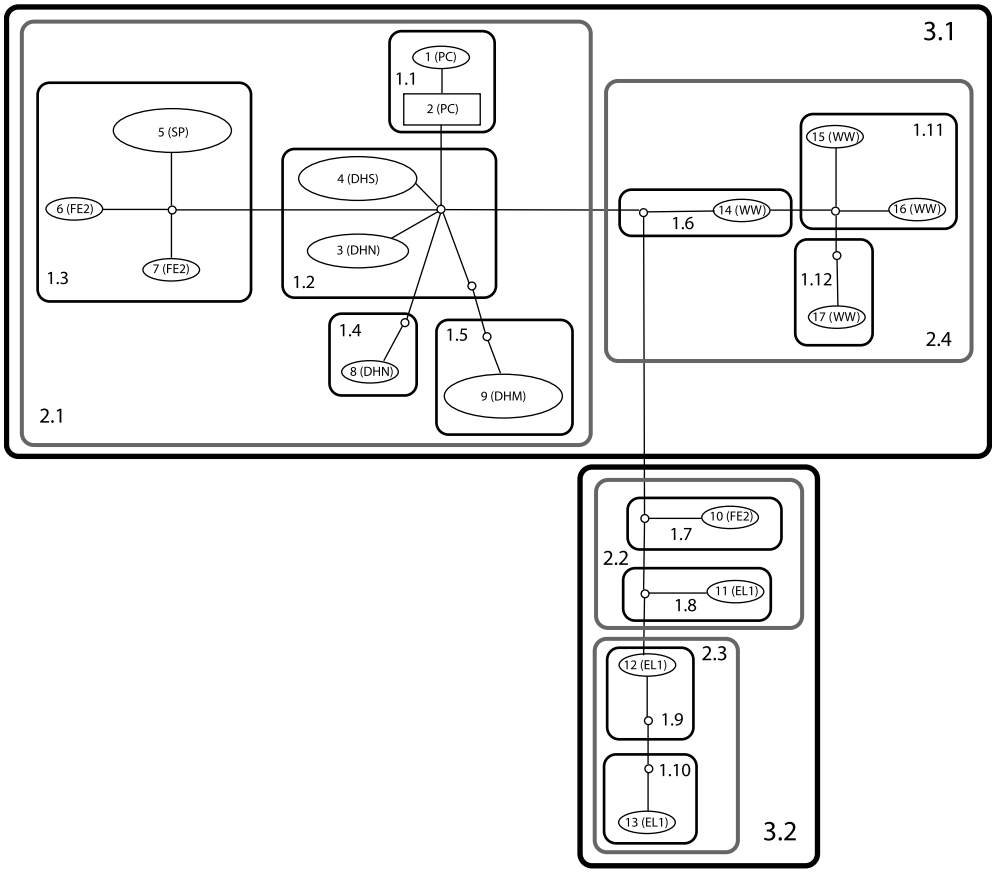

Fig. 2. Haplotype network for 17 *Arenophryne rotunda ND2* haplotypes (including site references) from the northern species lineage created in TCS 1.21. Each line represents a single mutational change. Ellipse size is proportional to haplotype frequency with small open circles representing missing haplotypes and the square representing the ancestral haplotype as inferred by TCS using outgroup weights. All connections, up to 11 steps, are within the 95% confidence limits of a parsimonious connection. Clades are nested according to the rules outlined by Templeton et al. (1987, 1995) and Crandall (1994).

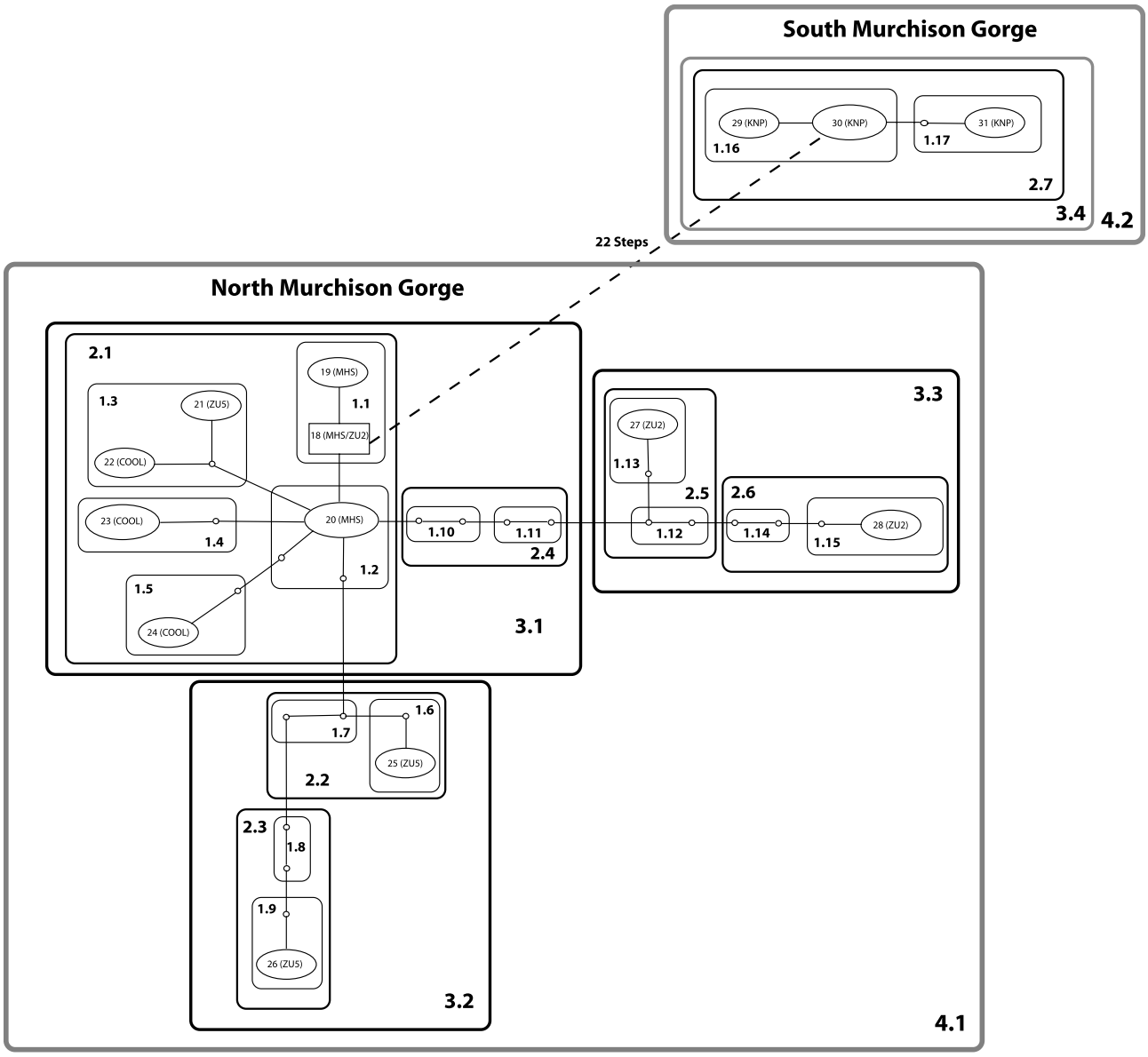

Fig. 3. Haplotype network for 14 *Arenophryne rotunda ND2* haplotypes (including site references) from the southern species lineage created in TCS 1.21. Each line represents a single mutational change. Ellipse size is proportional to haplotype frequency with small open circles representing missing haplotypes and the square representing the ancestral haplotype as inferred by TCS using outgroup weights. Connections, up to 17 steps, are within the 95% confidence limits of a parsimonious connection. A connection between nested clades 4.1 and 4.2 is not within the 95% confidence limits, but are joined by 22 mutational steps. Clades are nested according to the rules outlined in Templeton et al. (1987, 1995) and Crandall (1994).

Table 2
Biogeographical inferences for nested *Arenophryne rotunda* clades from both the northern and southern species 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
Northern	1.2	0.024	Int/tip status not determined	N/A
	2.1	<0.001	1-2-3-4-9	AF
	3.1	<0.001	1-19-20-2-11-12-13-LDC	PF w/ GRE
	Total network	<0.001	1-2-3-4	RGF w/ IBD
Southern	2.1	0.238	1-2-3-4	RGF w/ IBD
	4.1	<0.001	1-2-3-4	RGF w/ IBD
	Total network	<0.001	1-19	AF

P-values are calculated from 10 000 random permutations and are considered significant if permuted expected χ^2 values are greater than or equal to the observed.
AF, allopatric fragmentation; PF, past fragmentation; GRE, gradual range expansion; RGF, restricted gene flow; IBD, isolation by distance; w/, with.

Table 3
AMOVA evidence for high levels of population genetic structure and differentiation within both the northern and southern *Arenophryne* species lineages

Source	df	SS	MS	Est. var.	%	Stat	Value
Northern lineage population genetics analysis							
Island versus mainland	1	7.899	7.899	0.083	4	ϕRT	$0.035^{n.s.}$
Among pops./regions	6	38.515	6.419	1.603	68	ϕPR	0.706^{***}
Indiv./within pops.	21	14.000	0.667	0.667	28	ϕPT	0.717^{***}
Total northern lineage						Snn	0.887^{***}
Southern lineage population genetics analysis							
North versus south Murchison	1	70.567	70.567	10.411	78	ϕRT	0.784^{**}
Among pops./regions	3	16.071	5.357	1.004	8	ϕPR	0.35^{*}
Indiv./within pops.	13	24.250	1.865	1.865	14	ϕPT	0.86^{***}
Total southern lineage						Snn	0.648^{***}
North of Murchison pops.						Snn	0.548^{**}

Analyses assessed the partitioning of variation attributed to the flooding of Shark Bay isolating populations on Dirk Hartog Island from mainland populations (Northern Lineage), and the incision of Murchison Gorge isolating populations to the north and south of this barrier (Southern Lineage). Hudson's 'nearest neighbour' statistic (Snn) measures the population divergence within geographic regions as indicated. *P*-values were calculated via 1000 permutations.
n.s. = $P > 0.05$.
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.
*** $P \leqslant 0.001$.

Table 3 is a summary of population-genetic analyses performed on the two main haplotype clades within the *A. rotunda* dataset. AMOVA results from the northern *A. rotunda* haplotype clade show very little genetic variation (4%) explained by the separation of Dirk Hartog Island populations from the mainland and the majority of genetic variability distributed among populations (68%) with high levels of population structure ($\phi_{PT} = 0.717; P \leqslant 0.001$). High levels of population divergence among populations of the northern haplotype clade are also indicated by the highly significant Snn value ($0.887; P \leqslant 0.001$). Within the southern *A. rotunda* haplotype clade most genetic variation is explained by the separation of populations either side of the Murchison Gorge (78%). AMOVA (8% among populations) and Snn (0.648—all populations; 0.548—NMG populations only) results suggest little population divergence, verging on panmixia, within the NMG and SMG populations on either side of the Murchison Gorge (Fig. 1).

4. Discussion

I have inferred a molecular phylogeny for a fossorial frog that sheds light on the factors that have produced geographic structuring of populations in this taxon. A major divergence event has occurred between northern and southern populations of *A. rotunda* across the northern edge of the Victoria Plateau (Figs. 1 and 4) approximately ~5.63 MYA (±410 000 yrs), or in the Late Miocene period. Within the southern *A. rotunda* populations (Figs. 1, 3 and 4) haplotypes sampled north versus south of Murchison Gorge form distinct clades with an estimated divergence of ~2.05 MYA (±424 000 yrs), or the Late Pliocene period. Here, I first consider the biogeography and speciation of *A. rotunda* at a broad level, and then turn to each of the two lineages, with particular reference to examining how geological and climatic history have influenced current genetic structure.

4.1. Biogeography and speciation in Arenophryne

The major genetic break between the northern and southern *Arenophryne* haplotypes is consistent with the genetic differences observed between sister species in other groups within the Myobatrachidae (Morgan et al., 2007; Read et al., 2001). As a result of this work clear morphological differences have been measured and a new species description corresponding to the southern haplotype clade is forthcoming (Doughty and Edwards, submitted ms). We refer to the populations located above and below the northern edge of the Victoria Plateau as the northern species lineage and the southern species lineage, respectively. There has been much discussion of the role sea-level and climatic fluctuations, occurring predominately during the Plio-Pleistocene, have played in imposing geographic isolation among populations of herpetofauna in the Shark Bay and wider Carnarvon Basin region. Fluctuating climates and sea-levels seem plausible explanations for vicariance in many of the species with separate populations that have split in the northern Carnarvon Basin, such as *Rankinia adelaidensis* (Melville and Doughty, in press) and several other skink and gecko species (Storr and Harold, 1978, 1980). However, divergence estimates suggest that the major split within *Arenophryne* predates many of the Plio-Pleistocene sea-level fluctuations resulting in coastal dune evolution in the region (Hocking et al., 1987). While molecular clock estimates are fraught with difficulties (Rambaut and Bromham, 1998), the date obtained in this

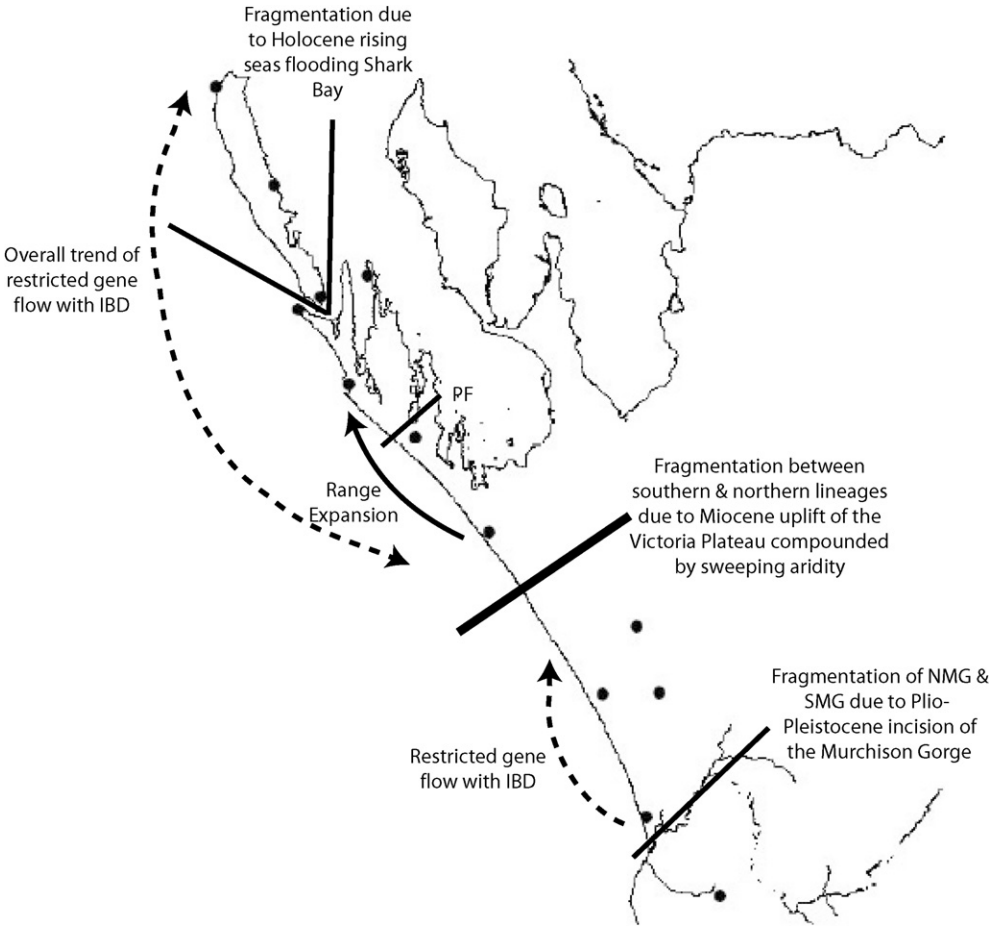

Fig. 4. Biogeographic hypotheses regarding the history of the northern and southern species lineages within *Arenophryne rotunda*. Hypotheses are synthesized by a combination of phylogenetic, phylogeographic and population-genetic analyses, which were interpreted with the aid of the known geological and climatic history of the region. PF, past fragmentation; IBD, isolation by distance; [●] sampled populations.

instance provides an estimate that matches predictions of known climatic and geological changes.

The formation of the Victoria Plateau, combined with sweeping aridity, is likely to have produced the Late Miocene divergence between the northern and southern *A. rotunda* species lineages. Tectonic instability reactivated pre-existing faults producing the uplift and formation of the Victoria Plateau; with the Kalbarri region of the Victoria Plateau uplifted by as much as 60 m (Haig and Mory, 2003). The northern border of the Victoria Plateau roughly corresponds to the geographic position of the genetic break between the two *Arenophryne* species lineages and without the presence of the thick coastal sand deposits of the Edel group not formed until the Plio-Pleistocene (Hocking et al., 1987); an alternative avenue for dispersal was not available. The role of tectonic instability in inducing vicariance, particularly in fossorial psammophillic species, has largely been ignored in treatments of the region's diversity to date in favour of hypotheses relating to fluctuating sea-levels resulting in coastal dune and sandplain development (Hopper and Gioia, 2004; Rabosky et al., 2004; Melville and Doughty, in press).

I hypothesize a scenario that incorporates both geological activity and climate shifts (Fig. 4): I suggest that *Arenophryne*, a formerly widespread taxon, was split by geological activity disrupting effective dispersal through sand habitat, a break that was compounded and reinforced by range contraction westwards with increasingly arid conditions about 5.63 MYA (Fig. 4). The onset of aridity also intensified in the Late Miocene, causing a change from a subtropical climate to one that oscillated between arid and temperate conditions (Dodson and Macphail, 2004). Hopper and Gioia (2004) argue that throughout these climatic fluctuations, the Shark Bay region suffered the most severe climate change due to the massive differences in rainfall experienced in these regions during glacial maxima and minima. *A. rotunda* is heavily reliant on soil moisture for dermal rehydration (Cartledge et al., 2006); limited rainfall may have diminished soil moisture which thereby restricted populations to coastal areas in the west, reinforcing fragmentation via uplift of the Victoria Plateau.

4.2. Phylogeography and population structure—Southern Species Lineage

Divergence estimates suggest a split within the southern *A. rotunda* species lineage (NMG and SMG—Fig. 1) across

the Murchison Gorge at ~2.05 MYA (Fig. 4). The Murchison Gorge is the overriding biogeographic feature within the range of the southern *A. rotunda* lineage. The final incision of the deep sandstone gorge in the lower Murchison River is estimated to have occurred between the late Pliocene and early Pleistocene (Hocking et al., 1987). North of the Murchison River/Gorge remaining populations in the southern lineage show consistent evidence for restricted gene flow with isolation by distance, suggests that dispersal is limited across relatively short distances (~50 km).

4.3. Phylogeography and population structure—Northern Species Lineage

Allopatric fragmentation was shown (NCPA results) between different prongs (the north–south oriented finger-like projections seen throughout the Shark Bay coastline) and between these populations and those on Dirk Hartog Island (Fig. 4). This suggests that fragmentation is associated with the flooding of the region and formation of Shark Bay, as the prong regions and the island would have been interconnected prior to Holocene sea-level rises, rather than just simply separation of Dirk Hartog Island from the mainland. Results also suggest that due to the relatively recent flooding of Shark Bay, comparatively little of the genetic variation within the northern haplotype clade is explained by the geographical separation of Dirk Hartog Island from the mainland (Table 3). The sea-level in Shark Bay is known to have reached its current levels ~5–6000 yrs ago (Playford, 1990). This rise led to the separation of Dirk Hartog Island and the formation of gulfs between the anticlinal dune ridges, which are now the various prongs in the western Shark Bay region (Butcher et al., 1984).

An overall inference of restricted gene flow among haplotypes in the northern species lineage (Fig. 4) is explained by a combination of sea-level rises causing both the flooding of Shark Bay and former isolation of the prong areas along the Shark Bay coast. Sea-level rises, associated with interglacial periods, are implicated in fragmentation events separating the population around the bay area from those further south along the coast in the northern species lineage (Fig. 4). Repeated episodes of higher sea-levels than present during the Pleistocene have been shown to have practically isolated the anticlinal ridges (underlying the several prominent prongs along the coast) during interglacial maxima. Two specific events during the Pleistocene have been noted to have dissected the prongs around 240 000 yrs ago and 130–120 000 yrs, evidenced by the deposition of distinct limestone formations (Van de Graaff et al., 1980). An inference of population expansion from coastal sites northwards is likely to have occurred in response to newly available habitat during the Pleistocene arid maxima (Fig. 4). Extensive dune systems formed on the coast during periods of severe aridity (Hocking et al., 1987). Climatic fluctuations associated with glacial maxima, which were frequent in the Pleistocene (Dodson and Macphail, 2004), are also known to have lowered sea-levels, permitting the expansion of coastal dune complexes in the Shark Bay region (Hocking et al., 1987; Playford, 1990).

5. Conclusions

Interactions between tectonic activity and climatic fluctuations provide a historical explanation for geographic genetic variation in *Arenophryne*. Tectonic activity during the late Miocene uplifted the Victoria Plateau and reactivated faults in this area. This geological activity coupled with the onset of aridity (intensifying ~6 MYA) in Australia is likely to have produced the most prominent phylogenetic break of *A. rotunda* into northern and southern species lineages. Morphological evidence supports species-level divergence of the northern and southern species lineages, and a new species description for the latter is forthcoming. Pleistocene dune-building episodes, incision of the Murchison Gorge, and climatic fluctuations explain geographic genetic structure observed in the species lineages. Coastal landscape evolution and climatic change have been well documented in the evolutionary diversification of plants and herpetofauna of Shark Bay; however, tectonic activity is an important and formerly overlooked influence on speciation and evolution of this region's biota.

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