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Impact of Plio-Pleistocene arid cycling on the population history of a southwestern Australian frog

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Abstract

Southwestern Australia is regarded as a global biodiversity hotspot. The region contains a high number of endemic species, ranging from Gondwanan relicts to much more recently evolved plant and animal species. Myobatrachid frogs are diverse in southwestern Australia, and while we know they have speciated *in situ* in the southwest, we know little about the temporal and geographical patterning of speciation events. *Crinia georgiana* is an ideal subject to test hypotheses concerning the effect of climatic history on southwestern Australian anurans, as it is an old lineage with a broad distribution covering the entire region. We compiled an extensive phylogeographical data set based on 1085 bp of the mitochondrial gene ND2 for 68 individuals from 18 sites across the species' range. Two major genetic clades were identified which were largely confined to the high rainfall and southeast coastal biogeographical zones, respectively. The clades appear to have diverged around the Plio-Pleistocene border (1.26–1.72 million years ago), concordant with increasing intensity and frequency of arid climate cycles. Subsequent phylogeographical structure appears to have developed primarily during the Pleistocene climatic fluctuations that also have been integral in generating species diversity in the endemic southwestern Australian flora. Phylogeographical analyses identified several dispersal routes, possible refugial areas within the range of the species and also regions of secondary contact. Dispersal routes identified may now be closed to the species because of habitat destruction and salinity problems in inland regions, posing concerns about the evolutionary potential of the species in light of predicted climate change.

Keywords: biogeography, *Crinia georgiana*, mitochondrial genealogy, Myobatrachidae, nested clade analysis, phylogeography

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Introduction

Southwestern Australia is an iconic region known for its extreme endemicity, high species diversity and its threatened environments (Cincotta *et al*. 2000; Myers *et al*. 2000). It is widely recognized for its extreme diversity and high level of endemism of plant species (Hopper 1979; Hopper & Gioia 2004). Less known but equally spectacular is the high level of faunal diversity, particularly invertebrates (York Main 1996), mammals, reptiles and amphibians (Hopper *et al*. 1996). The region has long been a biogeographical enigma. It lacks obvious historical geographical barriers arising from events such as glaciation and mountain building, events that are common in many vicariant speciation models. It has been geologically stable since the Tertiary (Hopper & Gioia 2004). For animals particularly, our understanding of the processes leading to speciation and endemism in southwestern Australian fauna is poor. Understanding the processes generating diversity, both between and within species, is important to the long-term conservation of conditions that might promote future diversification and preserve the evolutionary potential of existing species (Moritz 2002).

Processes generating botanical diversity in the southwest are reasonably well understood. The late Tertiary and Quaternary have been identified as periods of intense speciation in southwestern Australian flora (Hopper 1979; Hopper & Gioia 2004). During northern hemisphere glacial cycles of the Quaternary southern Australia experienced expanding semi-arid conditions with corresponding humid periods during interglacial cycles (Dodson & Ramrath 2001). Studies along the southern margin of Australia also

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have shown sea level fluctuations that correspond to interglacial wet and glacial arid cycling, respectively (Galloway & Kemp 1981). Climatic fluctuations led to landscape evolution, through differential soil erosional/depositional histories and coastal dune and sandplain development, which contributed to the high levels of diversity and endemicity observed in southwestern flora (Hopper & Gioia 2004). Extreme levels of plant diversity are found particularly in the northwestern and southeastern coastal areas of the region, areas that are more complex topographically than the wider southwestern forest system [high rainfall zone (HRZ)] (Hopper & Gioia 2004). Comparatively little work has been carried out to investigate the processes involved in generating diversity both within and between species of endemic southwestern Australian fauna. The processes acting on terrestrial vertebrates might be quite different from those involved in the speciation of southwestern Australian plants, for example range sizes are often higher, habitat specializations less marked.

The Myobatrachidae, an ancient anuran family endemic to Australia, show high levels of diversity and endemism in southwestern Australia (Roberts & Maxson 1985a, b). There are a number of endemic and relictual anuran species found in the southwest, particularly in the southern forests, reflecting the ancient history of the region. The genera *Heleioporus*, *Crinia*, *Geocrinia* and *Neobatrachus* are highly speciose within southwestern Australia and this diversity is known to have evolved *in situ* (Barendse 1984; Roberts & Maxson 1985b; Read *et al*. 2001; Morgan *et al*. 2007), but little is known about the specific speciation mechanisms in most of these genera. Speciation via polyploidy is known to have occurred within *Neobatrachus* (Mahony & Robinson 1980; Mable & Roberts 1997; Roberts 1997); however, polyploidy does not occur in other myobatrachid genera (Mahony & Robinson 1986). Fragmentation of populations into drainage systems, associated with periods of drying, may have led to allopatric speciation in the highly specialized and geographically restricted *Geocrinia rosea* species complex (Driscoll 1997, 1998). However the same processes seem less likely to have generated the observed diversity in *Crinia* or *Heleioporus* as many species within these genera have broad distributions that cover semi-arid areas and many congeneric species are broadly sympatric (Read *et al*. 2001; Morgan *et al*. 2007). Thus, it is important that biogeographical history is assessed in a diversity of species: in particular those that are widespread across a bioregion (Cracraft 1988; Avise *et al*. 1998; Riddle *et al*. 2000; Zink 2002).

We extend the limited data on processes generating intraspecific diversity in frogs from southwestern Australia with a comprehensive phylogeographical data set for *Crinia georgiana* (the quacking frog). This species has been the subject of numerous sexual selection and sperm competition studies (e.g. see Byrne & Roberts 2004; Byrne 2004; Hettyey & Roberts 2006) and its breeding success is highly dependent on a predictable hydrological regime (Dziminski & Roberts 2006). The distribution of *C. georgiana* covers the entire southwest forest system (or the HRZ) and extends into the topographically complex transitional rainfall zone on the southeastern coast [southeastern coastal zone (SECZ)]. This distribution thus covers two botanical provinces and an important biogeographical track described in Hopper & Gioia (2004) as a path 'along which congruent patterns of speciation have occurred within the southwest'. *Crinia georgiana* is the sister taxon to four other endemic *Crinia* species from southwestern Australia and one from eastern Australia (Read *et al*. 2001), suggesting it is an old lineage.

Given the antiquity of this lineage, *C. georgiana* is likely to have experienced multiple climate fluctuations during the Miocene and Plio-Pleistocene eras, and given its geographical range and sensitivity to changes in rainfall, the impacts of past climate change should be reflected in the phylogeography of this species. Also considering the sensitivity of this species to predictable hydrological regimes (Dziminski & Roberts 2006), this species also serves as an excellent model for investigating the potential effects of future climate change (Hughes 2003) on a widespread generalist species. These data will be the first comprehensive data set for fauna to contrast with patterns in southwestern Australian plants which show higher genetic structure and diversity in the SECZ compared to the HRZ (Hopper & Gioia 2004).

Materials and methods

Tissue samples

Sixty-eight frogs (toe-clips) were sampled from 18 sites across the species distribution, two to four animals per site (Fig. 1, Table 1). There is a large gap in our sampling between Bremer Bay and Cape Le Grand on the southeastern coast. Despite extensive fieldwork in the area, we found neither animals nor suitable habitat so we conclude that this reflects a real gap in the species' distribution. Furthermore, there are no historical records (over the last 150 years) of the species in this region (WA Museum records) with far eastern populations apparently disjunct from the main range (Tyler *et al*. 2000). Mondrain Island samples were from the WA Museum Tissue Collection (151200-151201-WAM). The *Crinia pseudinsignifera* outgroup used in phylogenetic analyses (32°43′58″, 116°6′17″).

Molecular genetic methods

Template DNA was extracted from samples using a modified cetyltrimethyl ammonium bromide (CTAB) method, suspended in TE and stored at 0 °C. Targeted DNA was amplified using a touchdown polymerase chain reaction (PCR) profile [94 °C-5 min-1×; followed by a series of touchdown

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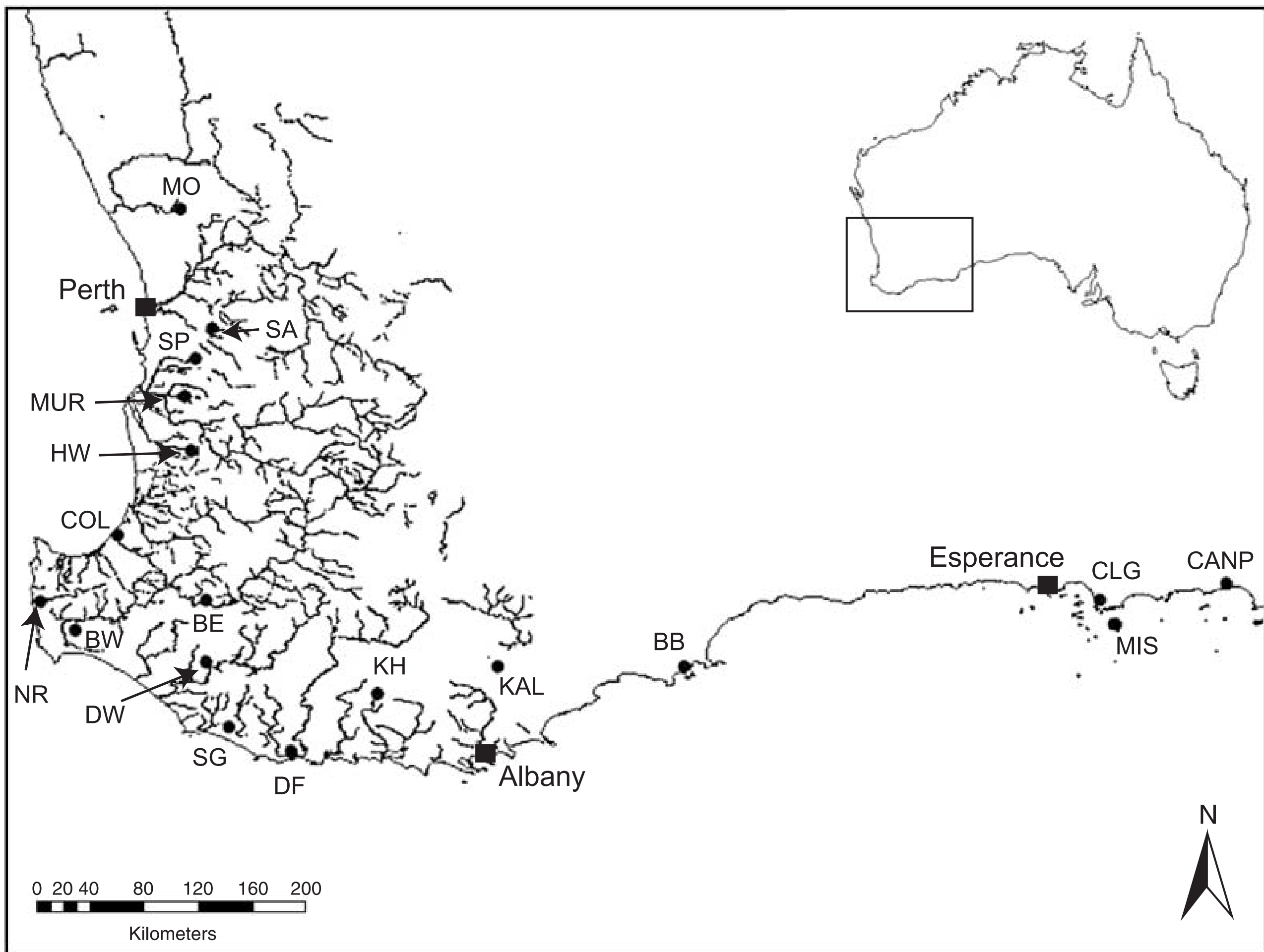

Fig. 1 Map of southwestern Australia showing all major southwestern drainage systems with map of the Australian continent inset. Tissue collection locations (•) for the *Crinia georgiana* phylogeographical study cover the entire known range of the species. The gap between the Bremer Bay (BB) and Cape Le Grand (CLG) sites is a known gap in the species distribution from both current and historical records. See Table 1 for details on sample sizes, abbreviations and exact locations.

cycles of 94 °C-30 s, 70–45 °C-20 s (decreasing in 5°increments) and 72 °C-90 s — each of these cycles were repeated 2×; followed by a final cycle — 94 °C-30 s, 40 °C-30 s, 72 °C-45 s, repeated 40×; then held at 72 °C-4min-1×, finishing at 4°-1 min]. Primers used to amplify ND2 were L4221 [5′-AAGGRCCTCCTTGATAGGGA-3′, modified by Macey *et al*. (1998)] & tRNA-trp [5′-CTCCTGCTTAGGGSTTT-GAAGGC-3′, modified by Read *et al*. (2001)]. Targeted fragments were amplified in 40-mL reactions comprising of ~100 ng template DNA, 4 mL 10′ reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTPs, 10 pmol primer and 2 U of Platinum *Taq* polymerase (Life Technologies).

Samples were run out on a 2% agarose gel and cleaned up using a Mo Bio UltraClean DNA Purification Kit (Mo Bio Laboratories, Inc). PCR (~100 ng) product was added to sequence reactions using either DYEnamic ET Terminator (Amersham Pharmacia 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), H4980 (5′-ATTTTTCGTAGTTGGGTTTGRTT-3′, Macey *et al*. 1998) and Myo-L4882 [5′-CMACVTGRCAAAAAY-THGCCCC-3′, modified by Melville *et al*. (2004)] were used for sequencing. Cleaned reactions were resuspended in a loading dye/formamide mix. Sequences were visualized on an ABI 377 Automated Sequencer or an ABI 3010 Capillary sequencer (Applied Biosystems). DNA sequence data were then edited using SEQUENCHER 3.0 (Gene Codes Corporation). Sequences were aligned individually using CLUSTAL_X (Thompson *et al*. 1997). Sequences were translated using the mammalian 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. Distinct haplotype sequences have been lodged on GenBank (Table 2).

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Table 1 Summary of *Crinia georgiana* tissue collection sites, sample sizes and locations in degrees, minutes, seconds. All points were geodetic WGS84

Site	Abbrev.	Sample size	Latitude	Longitude
Moore	MO	4	31°19′32″	115°58′59″
Swan-Avon	SA	4	32°07′57″	116°11′51″
Serpentine	SP	4	32°20′40″	116°05′07″
Murray	MUR	4	32°35′36″	116°00′33″
Harvey-Waroona	HW	4	32°57′36″	116°03′32″
Collie	COL	4	33°31′07″	115°34′27″
Naturaliste Ridge	NR	4	33°57′38″	115°03′21″
Blackwood West	BW	4	34°09′31″	115°17′31″
Blackwood East	BE	3	33°56′57″	116°09′08″
Donnelly Warren	DW	4	34°22′28″	116°09′08″
Shannon-Gardner	SG	4	34°48′23″	116°18′23″
Deep-Frankland	DF	4	34°58′19″	116°43′31″
Kent-Hay	KH	4	34°35′25″	117°18′24″
Kalgan	KAL	3	34°23′59″	118°06′11″
Bremer Bay	BB	4	34°23′55″	119°21′41″
Cape Le Grand NP	CLG	4	33°57′03″	122°09′10″
Mondrain Island	MIS	2	34°07′05″	122°14′35″
Cape Arid NP	CANP	4	33°50′18″	122°59′31″

Phylogenetic analysis

We used phylogenetic analysis techniques in conjunction with sequence divergence estimates and a molecular clock to assess overall phylogenetic structure and timing of major splits. To resolve and assess support for relationships between the major clades and overall phylogenetic structure, maximum-likelihood (ML) analyses of haplotypes were conducted with PAUP*4.0b10 (Swofford 2002). Bayesian Markov chain Monte Carlo (MCMC) phylogenetic analyses were implemented in MRBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). For ML analyses, AIC (Akaike Information Criterion) tests were used to select the best-fit model of evolution from the data and to calculate the nucleotide frequencies, substitution rates, gamma distribution and proportion of invariant sites for the data under the selected model using MODELTEST 3.7 (Posada & Crandall 1998). Branch support for ML trees is provided in the form of bootstrap values calculated from 100 replicates. Starting trees were obtained by stepwise addition and the tree-bisection–reconnection (TBR) method of branch swapping was employed in heuristic searches. Bayesian analyses were conducted using the GTR model with a proportion of invariable sites and the remaining variable sites having

Table 2 ND2 haplotypes within the *Crinia georgiana* phylogeographical data set. The frequency of haplotypes at each collection location are also shown, refer to Table 1 for site name abbreviations. GenBank Accession nos for each haplotype are also shown

Hap. #	Freq.	Site	GenBank#	Hap. #	Freq.	Site	GenBank#
1	2	SA	EF512601	24	1	KH	EF512616
	2	MO		25	1	SG	EF512626
	1	SP		26	1	HW	EF512603
2	2	MO	EF512602		1	KAL	
	1	MUR		27	1	KH	EF512620
	1	SP		28	1	DF	EF512600
3	1	SP	EF512595		1	BE	
4	1	SA	EF512597	29	1	BE	EF512598
5	1	MUR	EF512604	30	1	KH	EF512583
6	1	MUR	EF512594	31	1	DF	EF512623
7	1	HW	EF512612	32	1	DF	EF512581
8	1	HW	EF512613	33	1	SG	EF512619
9	1	SG	EF512586	34	1	KH	EF512589
10	1	BW	EF512592	35	1	DW	EF512607
11	1	NR	EF512618	36	1	SG	EF512584
12	1	NR	EF512628	37	1	NR	EF512588
13	1	SA	EF512622	38	1	NR	EF512599
14	1	BE	EF512621	39	1	DW	EF512609
15	1	BW	EF512615	40	2	BB	EF512624
16	1	MUR	EF512606	41	2	BB	EF512611
17	1	SP	EF512605	42	1	KAL	EF512627
18	1	DW	EF512596	43	1	HW	EF512593
	1	BW		44	2	CLG	EF512610
19	1	DW	EF512591	45	1	CLG	EF512608
20	1	BW	EF512590		1	CANP	
21	4	COL	EF512582	46	3	CANP	EF512614
22	1	KAL	EF512585	47	1	CLG	EF512617
23	1	DF	EF512587	48	1	MIS	EF512625

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a gamma distribution using default priors for MCMC analyses in MRBAYES version 3.1.2. Four independent runs of four chains each were run for 4×10^6 generations sampling every 100 generations, burn-in was set at 40 000 generations. Convergence of posterior probabilities and stationarity of likelihood scores between the two runs was assessed in TRACER version 1.3 (Rambaut & Drummond 2005). Other descriptive statistics such as haplotype diversity (*Hd*) and nucleotide diversity (*p*) were calculated in DNASP version 4.10.3 (Rozas & Rozas 1999).

The estimate of divergence time between the major *Crinia georgiana* lineages was calculated using the methods outlined in Masta *et al*. 2003). Divergence between major clades was calculated using the formula of Nei and Li for d_A (Nei 1987). The d_A parameter estimates (and SE) were calculated with a Jukes Cantor correction using DNASP version 4.10.8 (Rozas & Rozas 1999). There are no appropriate external calibration points/fossils with which to calibrate a molecular-clock rate for any southwestern 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%/million years, calibrated for ND2 in eleutherodactylid frogs (Crawford 2003). To ensure that the *C. georgiana* ND2 sequences were evolving in a clock-like manner, 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 was any significant difference between the likelihood scores of trees with and without a molecular clock enforced (Felsenstein 1981) in MODELTEST 3.7 (Posada & Crandall 1998).

Phylogeographical analysis

Phylogeographical analysis techniques were used to assess the geographical distribution of genetic structure and to reconstruct the evolutionary history of *C. georgiana*, to identify the impacts, if any, of past climate change. Nested clade phylogeographical analysis (NCPA) provides a test for nonrandom geographical scattering of haplotype groups and a method of inference to distinguish between various historical factors responsible for the associations between gene trees and geography (Templeton 1998). While NCPA has been criticized (Knowles & Maddison 2002), Templeton (2004) defended the use of NCPA as a powerful phylogeographical analysis technique, particularly when all events and processes affecting a species evolutionary history are not known a priori. So NCPA continues to be the most commonly used method for interpreting phylogeographical data.

Unrooted statistical parsimony haplotype networks were created using TCS 1.21 (Clement *et al*. 2000). This network was nested according to the rules outlined in Templeton & Sing (1993), Templeton *et al*. (1995) and Crandall *et al*. (1994). Where interior/tip status was ambiguous the clade/haplotype with the greater outgroup probability was deemed interior (Castelloe & Templeton 1994). Tests for geographical association were carried out on the nested haplotype network in GEODIS version 2.4 (Posada *et al*. 2000). Clades with significant phylogeographical structure, determined by χ^2 contingency tests after 10 000 random permutations, were identified and the significant D_C and D_N values within these clades were then used in conjunction with the latest NCPA inference key (http://darwin.uvigo.es/software/geodis.html) to reconstruct population histories.

Various techniques were used to complement the NCPA analyses. Initially, Tajima's D (D_T) was calculated to ensure sequence data fit the assumption of neutral evolution (Tajima 1989), using DNASP version 4.10.8 (Rozas & Rozas 1999). Where NCPA requires confirmation of 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 under the neutral model using the coalescent simulations permuted 1000 times in DNASP version 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 among sequences and is a powerful test, especially with limited sample sizes (Ramos-Onsins & Rozas 2002). Where secondary contact between distinct haplotype lineages was suspected, the supplementary tests described in Templeton (2001) were carried out. These tests require the calculation of average pairwise distances between the geographical centres of each haplotype/clade found at each sampling site, which is calculated for every nesting level of the cladogram. Secondary contact of divergent lineages can be inferred if haplotypes/clades with divergent geographical centres are found together at one location (Templeton 2001; Templeton 2004).

Population genetic analysis

Population genetic statistics were used to investigate and describe genetic structure between the two major biogeographical regions within the range of *C. georgiana*, and among populations within major phylogenetic lineages identified by phylogeographical and phylogenetic analysis techniques. DNASP version 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 both for the entire species data and within each major lineage. 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).

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Analysis of molecular variance (AMOVA) was calculated in GENALEX version 6 (Peakall & Smouse 2004) with 1000 permutations. Initial AMOVA analyses, using the entire data set, were used to assess the proportion of genetic variation explained by biogeographical regions, within the range of *C. georgiana*, i.e. HRZ vs. SECZ (*sensu* Hopper & Gioia 2004). AMOVA's were also calculated between and among populations across each major lineage to assess genetic variation among populations within each mitochondrial lineage.

Results

Phylogenetic analysis

A 1085-bp fragment of ND2 from 68 individuals revealed 48 haplotypes (Table 2) based on 70 variable sites of which 38 were parsimony informative. Haplotype diversity (*Hd*) was 0.986 ± 0.00003 and nucleotide diversity (*p*) was 0.00967 ± 0.000001. For phylogenetic analysis the TrN + I model of DNA evolution was selected using AIC (Akaike Information Criterion) tests in MODELTEST. The following parameters were enforced in a likelihood analysis with 100 replicates to assess branch support: Base = (0.2995,0.3139, –0.1073), Nst = 6, Rmat = (1.0000, 38.0853, 1.0000, 1.0000, 12.6278), Rates = equal, Pinvar = 0.8487. The phylogenetic tree (showing the ML phylogram topology, Fig. 2) shows two lineages. Lineage 2 has strong support (Bayesian posterior probabilities/ML bootstraps = 100/94) as a monophyletic clade, as do several minor clades within this lineage (clade 1.37–99/85; clade 3.5–100/88; refer to Fig. 3 for clade names). Lineage 2 is largely confined to the southeast coastal zone with only one population further west in the HRZ at the Harvey-Waroona population (HW). Lineage 1 occupies the HRZ. In the Kalgan River population (KAL), a southeast coastal site, two of three frogs also belonged to lineage 1. Lineage 1 lacks bootstrap support as a reciprocally monophyletic clade from Lineage 2 (< 50/< 50); nevertheless separation of the two lineages is supported in a network (see below, Fig. 3), which is generally a more appropriate way to represent intraspecific data with low levels of divergence (Templeton *et al*. 1992). Other clades which receive strong support within lineage 1 coincide with clades 2 (100/95), 2.4 (98/88) and 2.8 (97/81) in Fig. 3.

Pairwise differences in haplotypes between the two major lineages ranged between 1.29% and 2.49% (uncorrected *p*). The score of the likelihood tree without enforcing a molecular clock was – In L = 2090.89, the score of the tree with a molecular clock enforced was – In L = 2116.49. The likelihood-ratio test showed that sequences did not depart from a clock-like model of evolution (n.s.; *P* = 0.276). The number of nucleotide substitutions (d_A) between Lineages 1 & 2 was 0.01426, which gives a divergence time of ~1.49MBP (± 2SE of 226 000Y). The first lineage encompasses the majority of the species range, covering the western and southwestern populations and encompassing the entire southwest forest system. Sequence divergences range from 0.09–1.01% within the southwest forest clade. The second lineage comprises all populations on the south coast east of Albany. The HW and KAL populations had only one individual of four and three, respectively, from this second lineage. Sequence divergences within the southeast coastal clade ranged from 0.09–0.92%.

Phylogeographical analysis

Tajima's *D* showed that the *Crinia georgiana* mtDNA data set was consistent with neutral evolution (D_T = –1.119; $P > 0.05$–n.s.). All 48 haplotypes were joined with a 95% probability of parsimonious connection in TCS 1.21. The total cladogram was nested at the 5-step level, with a maximum of 14 mutation steps between any two haplotypes (Fig. 3). The GEODIS output showed several clades within the nested *C. georgiana* haplotype network with significant phylogeographical structure from which biogeographical inferences could be made (significant χ^2 *P* value: Table 3). For clade 2.2, we inferred past gradual range expansion followed by fragmentation from the northwestern HRZ (MO, SA, SP, MUR & HW) to some south coast forest populations (DF, KH & KAL). Independent tests for demographic expansion show evidence for range expansion in clade 1.10 (R_2 = 0.1241; $P \leq 0.05$), but not for any other clade within the nested group (R_2 = 0.364; $P > 0.05$–1.22 and R_2 = 0.379–1.11; $P > 0.05$–n.s, R_2 could not be calculated for other clades in the nested group as there were only single haplotypes in these clades). There is a significant geographical signal within clade 2.6 but inadequate geographical sampling prevents any viable inference of history.

Significant phylogeographical structure was detected within clade 3.1. Clades 2.1 (SG, BW & NR), 2.5 (BE & BW) and 2.6 (DW, BW & COL) have ranges that mostly do not overlap with the rest of the clades in the nested group. Clades 2.1, 2.5 and 2.6 are also separated from the central ancestral clade by a series of missing haplotypes. Range expansion was detected in clades 2.2 (R_2 = 0.0707; $P < 0.001$) and 2.9 (R_2 = 0.0843; $P < 0.001$), but not other clades. This suggests gradual range expansion into southwest coastal areas from the northern high rainfall region followed by fragmentation. The supplementary testing for secondary contact shows moderate distance values for the HW, DF, KH and KAL sites at the 2-step level probably reflecting the presence of both clades 2.2 and 2.9 at these sites (Fig. 4). While clades 3.2 and 2.8 show no significant phylogeographical structure using NCPA, the high support for clade 2.8 would further add to this inference of range expansion into southwest coastal areas followed by fragmentation.

Lineage 2 (clade 4.2), or the SECZ lineage, is characterized by local population structure and several allopatric fragmentation events. We inferred fragmentation amongst

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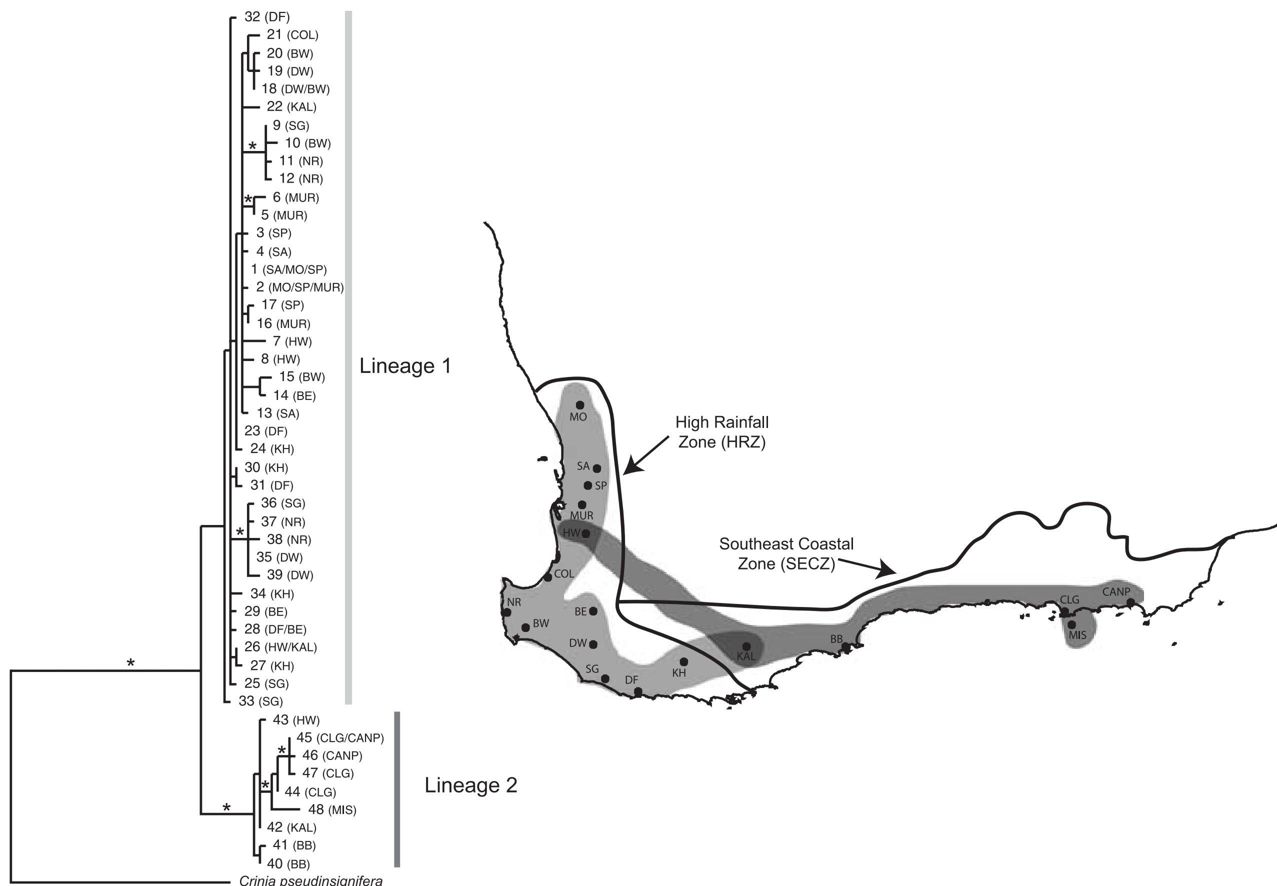

Fig. 2 Maximum-likelihood phylogram of 48 *Crinia georgiana* ND2 haplotypes showing two major lineages with *Crinia pseudinsignifera* as an outgroup. Bootstraps were calculated from 100 replicates and Bayesian posterior probabilities from 4 million MCMC generations. ML bootstrap values for clades above 70 are represented by * (refer to text for exact values). TrN + I + G model of DNA evolution was enforced in maximum-likelihood analyses as suggested by AIC tests in MODELTEST 3.7. Map of southwestern Australia is inset with shaded areas representing the range of the two major lineages, for site name references see Table 1. Map also shows the distribution of the two biogeographical zones in the range of *C. georgiana*: the High Rainfall Zone and the Southeast Coastal Zone (cf. Hopper & Gioia 2004).

Table 3 Biogeographical inferences for nested *Crinia georgiana* clades with significant phylogeographical structure, specified by a χ^2 nested contingency test. *P* values are calculated from 10 000 random permutations and are considered significant if permuted expected χ^2 values greater than or equal to the observed

Nested clade	χ^2 Permuted *P* value	Chain of inference	Inferred process
2.2	< 0.001	1-2-3-5-15-PF &/or LDC-21	PGRE w/F
2.6	0.029	1-19-20	IGS
3.1	< 0.001	1-2-11-RE-12–13-LDC w/PF or PF w/RE-21	PGRE w/F
3.5	0.022	1–19	AF
4.2	< 0.001	1–19	AF
Total Cladogram	< 0.001	1-2-11-RE-12	CRE or PF w/CRE*

PF, past fragmentation; LDC, long distance colonization; RE, range expansion; CRE, contiguous range expansion; PGRE, past gradual range expansion; AF, allopatric fragmentation; F, fragmentation; IGS, inadequate geographical sampling; w/,with. * Inference of PF w/CRE is adopted as the appropriate inference despite simple CRE being inferred by the NCPA inference key.

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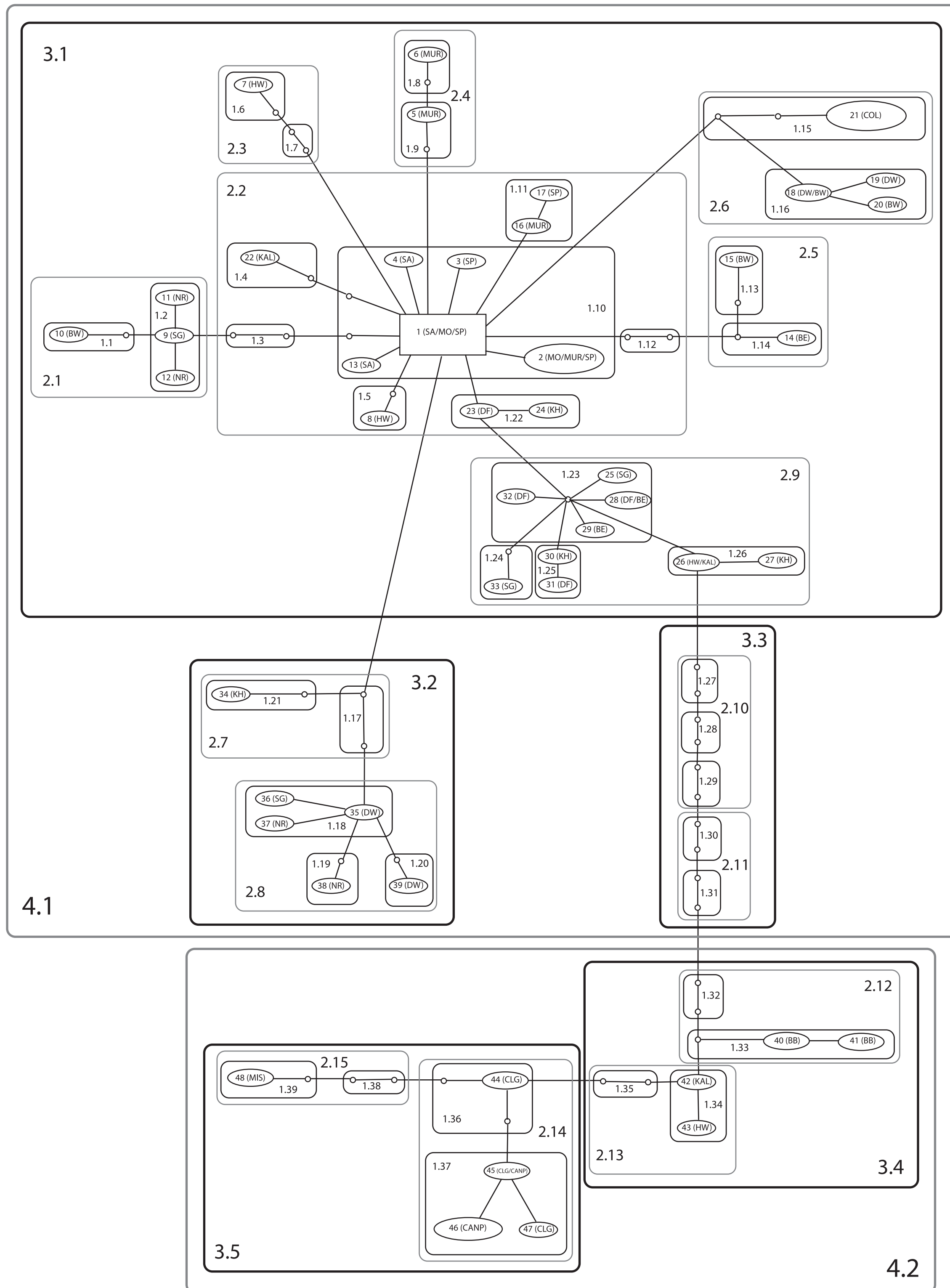

Fig. 3 Haplotype network for all 48 *Crinia georgiana* ND2 haplotypes created in TCS 1.21. Each line represents a single mutational change. Ellipse size is proportional to haplotype frequency with small open circles representing missing haplotypes and the square representing the ancestral haplotype as inferred by TCS using outgroup weights. All connections, up to 14 steps, are within the 95% confidence limits of a parsimonious connection. Clades were nested using rules outlined in (Templeton *et al*. 1987; Crandall 1994; Templeton *et al*. 1995).

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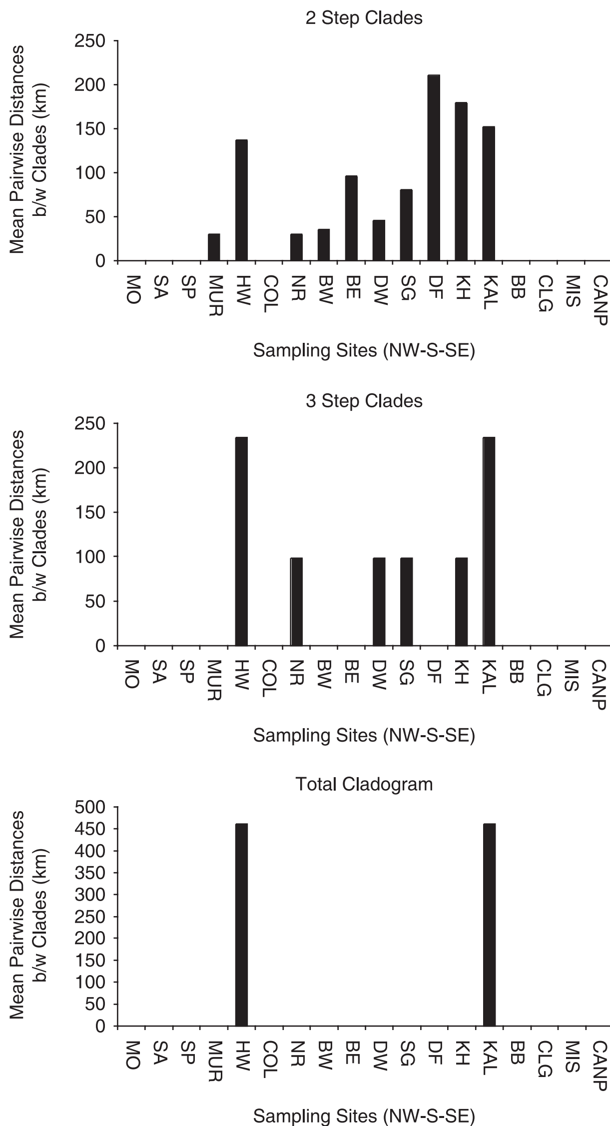

Fig. 4 At many locations two or more clades co-exist leading to an inference of secondary contact. This figure plots mean pairwise distances (km) between the geographical centres of *Crinia georgiana* clades found at each sampling location at the 2 step, 3 step and Total cladogram nesting levels. Only clade levels where an inference of secondary contact was possible are shown. Sites where geographically divergent clades (i.e. high distance values) are present relative to the distribution of the species represent sites of secondary contact between divergent lineages. Sites are ordered north to south then west to east (Fig. 1, Table 1). For principles and methodology behind this supplementary test for NCPA see Templeton (2001).

far southeast coastal zone populations (CLG + CANP & MIS) associated with the separation of Mondrain Island from the coast by rising sea levels (clade 3.5, Table 3). Fragmentation is also inferred in clade 4.2 between the far southeast coastal populations from the Esperance region (CLG, CANP & MIS) and the haplotypes from the western portion of the range of lineage 2 (BB, KAL and HW populations). At the total cladogram level we made an overall inference of contiguous range expansion. Figure 4 shows evidence for secondary contact between these two discrete mitochondrial lineages in the HW and KAL populations. Clade 4.1 shows evidence of rangeexpansion ($R_2 = 0.0408$; $P < 0.01$). Clade 4.2 does not show evidence of range expansion (n.s.; $P > 0.05$).

Population genetic analysis

Analyses of molecular variance across the whole *C. georgiana* data set sought to determine the proportion of genetic variance attributed to Hopper & Gioia's (2004) HRZ & SECZ biogeographical regions. Further AMOVA analyses assessed the amount of genetic variance among and within the populations within each of the discrete lineages (Fig. 2) within the *C. georgiana* data set. As populations of single individual cannot be incorporated, for these population analyses the single individuals from populations KAL & HW that fell out with Lineage 2 were grouped as a single genetic population unit. This was justified by principal components analysis, performed in GENALEX version 6 with 1000 permutational steps (Peakall & Smouse 2004), which indicated that these individuals were from the same genetic population (results not shown). The network created for NCPA also supports this. Table 4 is a summary table of population genetic analyses. AMOVA results across the entire species range show that 64% of the genetic variation is accounted for by differences between the HRZ & SECZ. When calculated for the entire *C. georgiana* ND2 data set Snn (0.322; $P > 0.001$) suggests that total population differentiation is extremely low. AMOVA concurs with low overall levels of population structure, with more genetic variation accounted for by individuals within populations (22%) than between (14%). Low differentiation levels overall are probably reflective of the high levels of dispersal within the majority of the species range, covered by Lineage 1. Lineage 1, mainly confined to the HRZ, also exhibits extremely low population differentiation (Snn = 0.120; $P > 0.001$), and this is reflected in the AMOVA results, which show that the majority of genetic variation is among individuals within populations (76%) rather than between populations (24%). Lineage 2 on the other hand displays the opposite trend with highly differentiated populations (Snn = 0.844; $P > 0.001$), which also accounts for 85% of the genetic variation within this lineage.

Discussion

The mtDNA sequence data show two haplotype lineages within *Crinia georgiana* (Fig. 2) with 1.29%–2.49% sequence divergence, strong bootstrap support and an estimated divergence date of 1.49 million years ago (Ma) (± 226 000

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Table 4 Summary table of population genetic statistics for *Crinia georgiana* as a whole in addition to results from the two major mitochondrial lineages identified in phylogenetic and phylogeographical analysis. Analysis of molecular variance (AMOVA) results are presented for the whole species dividing up the distribution into two regions [High Rainfall (HR) Zone and Southeastern Coastal (SEC) Zone, *sensu* Hopper & Gioia 2004 and among populations within each major lineage. Hudson's Snn 'nearest neighbour'statistic is also presented as a measure of genetic differentiation among populations across the species and within major lineages. *P* values for each of these analyses were calculated via 1000 permutations. ***$P \leq 0.001$

	Population genetics analysis summary results table							
Source	d.f.	SS	MS	Est. Var.	%	Stat	Value	Hudson's Snn
Whole Species								0.322***
HR Zone Vs SEC Zone	1	150.853	150.853	5.666	64%	ϕ_{RT}	0.643***	
Pop's/region	16	104.088	6.506	1.211	14%	ϕ_{PR}	0.385***	
Indiv./Within Pop's	50	96.500	1.930	1.930	22%	ϕ_{PT}	0.781***	
Lineage 1								0.120***
Among Pops.	13	52.090	4.007	0.580	24%			
Within Pops.	38	70.583	1.857	1.857	76%	ϕ_{PT}	0.238***	
Lineage 2								0.844***
Among Pops.	4	34.813	8.703	2.640	85%			
Within Pops.	11	5.000	0.455	0.455	15%	ϕ_{PT}	0.853***	

years), or around the Plio-Pleistocene border (~1.64 Ma). Given an initial lineage split at the Plio-Pleistocene border and the minimum age of isolation of offshore Islands throughout the southwest ~5000 years ago, subsequent phylogeographical structure within each lineage appears to primarily be related to climatic fluctuations throughout the Pleistocene. Following initial fragmentation both lineages have expanded through inland regions, coming into secondary contact at two sites, in the central western forest (Harvey-Waroona) and at the meeting of the high rainfall and southeast coastal zones (Kalgan River, Figs 2, 4 and 5a). There is evidence of repeated cycles of fragmentation followed by range expansion within Lineage 1, the haplotype lineage largely confined to the HRZ (Figs 2 and 5b). Higher levels of genetic structure and signals of allopatric fragmentation characterize lineage 2 (Fig. 5b, c), which is largely confined to the more arid SECZ with some obvious patterns of differentiation on offshore island populations isolated by sea level rises most recently after the last glacial maximum (Mondrain Island, Fig. 5c).

Biogeography of Crinia georgiana *and southwestern Australia*

The main biogeographical hypothesis adopted for *C. georgiana* is that the species appears to be a formerly widespread lineage fragmented into two lineages, between the HRZ and SECZ biogeographical regions, each of which then expanded to come into secondary contact at several sites. Divergence estimates suggest the separation of the two lineages occurred around the beginning of the Plio-Pleistocene border glacial cycles, with each lineage subsequently expanding through inland areas during wetter interglacial periods. An inference of contiguous range expansion was originally given by NCPA, with no inference of fragmentation despite 14 mutational steps separating the two major lineages. Additionally, range expansion is not detected for Lineage 2, but is for Lineage 1. Subsequent contraction and fragmentation within lineages may account for the incorrect inference, alternatively expansion may have been very recent and rapid leading to a lack of signal and may explain both these phenomena (Masta *et al*. 2003). The occurrence of divergent lineages with different geographical centres and largely nonoverlapping distributions at the Harvey/Waroona and at the Kalgan sampling sites is consistent with fragmentation followed by range expansion and subsequent population mixing. Sampling from populations in intermediate inland areas between the KAL and HW sites and larger sample sizes, may have yielded more accurate inferences. Additionally, molecular-clock estimates are fraught with difficulties (Rambaut & Bromham 1998; Gillooly *et al*. 2004), the date obtained of ~1.49 Ma provides an estimate that is consistent estimated climate change in Australia (Galloway & Kemp 1981; Kendrick *et al*. 1991) and tightly links with the onset of 100 000 years glacial cycling at 1.5 Ma (Rutherford & D'Hondt 2000), and with dramatic changes seen in other southwestern Australian biota (Hopper 1979; Rabosky *et al*. 2004).

The Plio-Pleistocene border (1.64 Ma) was a time of immense climatic change in Australia followed by arid pulses increasing in frequency and intensity during glacial maxima (Bowler 1976; Kershaw *et al*. 1991; Macphail 1997). High seas and wet humid conditions are indicated at the Plio-Pleistocene border, followed by a rapid regression and reversion back to arid conditions first seen in the late Miocene (Galloway & Kemp 1981; Kendrick *et al*. 1991). A significant drop in rainfall has been inferred for the southwest at the Plio-Pleistocene border, falling to below

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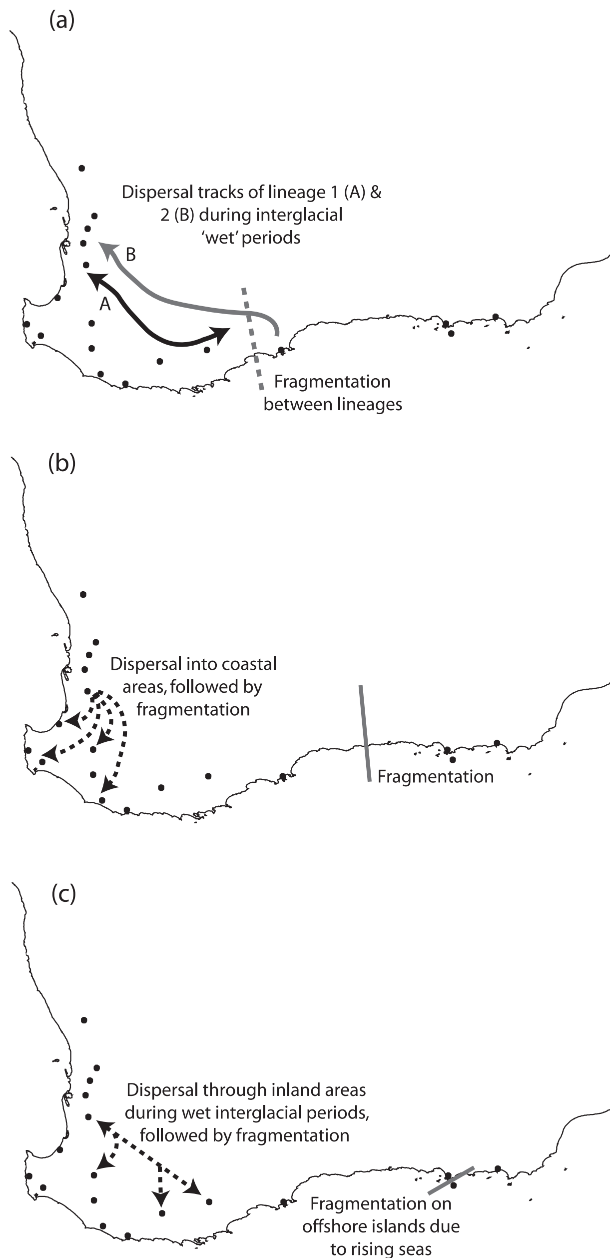

Fig. 5 Biogeographical hypotheses regarding the two major lineages within *Crinia georgiana* and their responses to Plio-Pleistocene climatic fluctuations. Figure 5a presents the biogeographical hypothesis of initial fragmentation of the two major *C. georgiana* lineages caused by arid conditions followed by recent dispersal across inland regions during wetter interglacial periods. Figure 5b Shows fragmentation of southeast coastal populations and a restriction of dispersal from the north into southwest coastal populations effected by increasing aridity (the latter may also be compounded by increasing salinity of coastal wetlands during interglacials). Figure 5c shows phylogeographical hypothesis regarding the response of the two major *C. georgiana* lineages to interglacial wet periods, where dispersal is likely to be established across inland areas (followed fragmentation by aridity) and populations become isolated on offshore islands by rising sea levels. •, sampled populations.

600 mm for the first time on the southeastern edge of the southwest land division (Macphail 1997). Palynological evidence also shows rainfall decreasing at both the northwest (< 200 mm) and southeastern margins (200–400 mm) of the higher rainfall zone (Macphail 1997; Dodson & Macphail 2004) (cf. a current level of 300 + mm, up to 600 mm in the Esperance region; Bureau of Meteorology, www.bom.gov.au/). Divergence estimates suggest the separation between *C. georgiana* lineages occurred around the Plio-Pleistocene border. Climates today are similar to interglacial wet periods throughout the Quaternary, however, currently it is still slightly drier and less humid than many of the 'wetter' interglacial periods (Dodson & Ramrath 2001). Hypotheses closely linking the historical biogeography of *C. georgiana* with climate and associated rainfall fluctuations as the species relies on seasonably predictable rainfall for successful recruitment (Dziminski & Roberts 2006), therefore any significant change in rainfall levels and predictability, as has been the case with severe arid pulses, are certain to disrupt the breeding cycle of this species.

Complex interactions between a changing climate and sea levels has lead to the diversity observed within the southwestern Australian plant communities, primarily in the changeable Plio-Pleistocene era. Dramatic fluctuations in rainfall within Hopper's (1979) transitional rainfall zone have not only shaped to biogeography of endemic plants, but have also impacted on endemic fauna. Plant distributions show a similar pattern to that seen within *C. georgiana*, with sister species affiliations or disjunct distributions between the HRZ and scattered throughout the wetter pockets along the SECZ (Hopper 1979; Hopper & Gioia 2004). Hopper & Gioia's (2004) southeastern coastal and the high rainfall biogeographical zones also distinguish much of the genetic diversity within *C. georgiana* (Table 4). A scenario where populations are fragmented into high rainfall and southeast coast lineages followed by expansion during interglacial periods may also explain the distribution patterns of *Litoria moorei* and *Litoria cyclorynchus*, a pair of recently diverged anuran species (Roberts & Maxson 1988; Cale 1991; Burns & Crayn 2006) which hybridize in this border region (Cale 1991). With climate change rainfall patterns within the southwest are beginning to shift and will get worse in the future, a trend of less rainfall during the formerly predictably wet Autumn/Winter period to more rain in the formerly dry Summer period is predicted to continue and intensify (Hughes 2003). Given a history so closely tied to climate there is concern for the ability of this species, *C. georgiana*, and other southwestern Australian endemics to cope with future climate change. Furthermore, should species be able to cope with the change in rainfall seasonality and rainfall levels return to normal, the combined effects of salinity and habitat destruction may alter the ability of biota to move through historical inland dispersal tracks.

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Phylogeographical and population genetic patterns

The phylogeographical history of the two major *C. georgiana* lineages differ markedly, with lineage 1, which is largely confined to the HRZ, showing repeated episodes of range expansion with subsequent fragmentation in southwest coastal and inland areas. Range expansion followed by fragmentation occurred between northern and southwest coastal areas and between the southeastern and northern-forested areas of the HRZ within this lineage (Fig. 5b, c). Population structure within the forest system (lineage 1) was low, this is also consistent with the repeated dispersal inferences of NCPA and confirms the results of the only previously available genetic data (from allozyme studies) for *C. georgiana* (F_{ST} = 0.066-over 237 km) (McDonald 1998). Similar levels of population structure are seen in other widespread southwestern amphibian taxa (F_{ST} = 0.087 over 100 km *Heleioporus albopunctatus;* Davis & Roberts 2005); F_{ST} = 0.088 over 80 km *Heleioporus psammophilus* (Berry 2001). Lineage 2 occupying the arid SECZ (lineage 2) appears to be characterized by several instances of allopatric fragmentation. This is reflected in the results of population genetic analyses on this lineage confirming higher levels of population genetic structure in the southeast coastal zone.

Biogeographical hypotheses within each lineage, following a split around the Plio-Pleistocene border, are consistent with a fluctuating climate throughout the Pleistocene. Arid maxima are associated with significant drops in rainfall (Macphail 1997) and climatic fluctuations in general have been associated with dramatic changes in rainfall throughout the more inland regions of the southwest (Hopper & Gioia 2004). Arid pulses are likely to have led to restricted dispersal between the wetter northern and southeastern coastal regions of the HRZ within lineage 1. Aridity would cause this primarily by leading to a contraction of the species range to coastal areas and further by restricting dispersal between the wetter refugial areas along the coast. Strong signatures of range expansion from the northern and southeastern coastal regions of the HRZ indicate that these regions have acted as primary refugial areas for *C. georgiana* lineage 1 haplotypes during arid maxima (Lessa *et al*. 2003). Northern and southern refugial areas are also suggested for plant taxa, with a common congruent biogeographical track observed between northern and southern regions of the HRZ (Hopper & Gioia 2004). Palynological evidence shows northern highland regions remaining relatively wet even during arid maxima because of the relief of the Darling Ranges (Macphail 1997). A southern forest refugial area is also well corroborated by the presence of several relictual plants and animals with Gondwanan affinities (Hopper *et al*. 1996; Roberts *et al*. 1997).

Interglacial wet periods appear to have allowed repeated range expansion throughout the interior regions of the southwest. Wet interglacial periods have been noted as times when the HRZ extended far into the currently semi-arid regions (Hopper & Gioia 2004) and this would have allowed *C. georgiana's* range to expand into inland areas, where the combined effects of adult movement between catchments during rain and tadpole movement across catchments during flooding may have resulted in the current dispersal patterns observed (Fig. 5c). In inland regions of the southwest many of the upper catchments of rivers draining towards the coast come into close contact (Fig. 1). While these upper regions of catchments are currently thought of as more palaeodrainage systems (Beard 1999), significant increases in rainfall during wetter interglacials would have expanded suitable available breeding habitat for the species throughout inland reaches of the southwest.

Dramatic sea level fluctuations were also associated with climatic fluctuations of the Pleistocene, with high sea-level stands during interglacial maxima and low sea-level stands corresponding to glacial/arid maxima (Galloway & Kemp 1981). Cenozoic transgressions during high sea-level stands have been shown to have consistently affected the area east of Augusta and Geographe Bay areas in the extreme southwest, in addition to vast sections of the western coastline (Sircombe & Freeman 1999). Subsequently, southwestern coastal plain vegetation communities did not fully develop to their current positions until the mid-late Pleistocene (Kendrick *et al*. 1991). During lower sea levels the species could move into and occupy newly available coastal habitats on the Swan Coastal Plain and extreme southwest corner. Higher sea levels than present are known to have lead to severe and rapid change in coastal plant communities (Sircombe & Freeman 1999; Hageman *et al*. 2003) and coastal wetlands and estuarine systems (Hodgkin & Hesp 1998). These processes are very likely to have lead to the restricted gene flow between coastal populations (lineage 1, Fig. 5b) and isolated populations on offshore islands (lineage 2, Fig. 5c). Arid cycles were also noted to impact on the extreme southwestern flora and fauna (Dortch 2004), therefore the combined effects of dramatic sea level and salinity changes and pulses of aridity are likely to be responsible for the signal of restricted gene flow among coastal populations and between coastal populations and those in more stable refugial areas (Fig. 5b).

Predominant inferences within lineage 2 are of fragmentation of populations in the Esperance region from populations further west within the range of lineage 2. This is most likely to be due to the increasingly frequent and intense arid pulses of the Pleistocene (Fig. 5b). *Crinia georgiana* has never been collected in the area between these two regions and has been noted as extremely rare in the Fitzgerald region (Chapman & Newbey 1995), 30–40 km east of Bremer Bay. Rainfall maps (Hopper & Gioia 2004)

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show that between these regions rainfall declines to below 600 mm, which appears to be the limit of the species' distribution from known records. Dispersal may have occurred through now flooded coastal habitats during low sea levels along the southeastern coastline to be fragmented by rapidly rising seas throughout the late Pleistocene (Hageman *et al*. 2003), as has been the case with populations known from offshore Islands. Alternatively, the area between Esperance and Bremer Bay may still have been extremely arid during recent interglacials. Ever increasing aridity would therefore prevent significant dispersal of *C. georgiana* through newly created coastal habitats, resulting in a pattern of isolated refugial populations often seen in the plants of this region (Wright & Ladiges 1997; Hopper & Gioia 2004). Hence it is likely that the combined influence of sea level and climatic fluctuations have contributed to the fragmentation of the Esperance populations from the rest of lineage 2.

Conclusions

On the basis of this study, we propose the following scenario to explain the current haplotype distributions of *Crinia georgiana*. The clear phylogenetic break within *C. georgiana*, which separates lineages from the HRZ (lineage 1) & SECZ (lineage 2), resulted from aridification around the Plio-Pleistocene border. This fragmented populations from the HRZ and those from the southeastern coast, isolating the latter into more mesic pockets along the predominately arid and hostile southeastern coast. With ameliorating conditions during Pleistocene interglacials the two now-divergent lineages expanded through inland areas to reclaim much of the species' former range. Subsequent intense Pleistocene aridification cycling would then have resulted in repeated fragmentation within both lineages. Refugia existed in the northern and southeastern portions of the HRZ (lineage 1) and the species has persisted in the Bremer Bay-Fitzgerald River, and Esperance regions along the semi-arid southeast coast (lineage 2). Wetter interglacial climates during the Quaternary allowed for repeated dispersal through inland areas between refugial areas within the HRZ. The compounded effects of high seas, leading to isolation of *C. georgiana* populations on offshore Islands off the southeastern coast, and arid conditions probably affected fragmentation of southwest coastal populations of the HRZ lineage. Together these results imply a remarkably similar biogeographical history to that seen in relictual plants and other endemic frogs of southwestern Australia, confirming the biogeographical zones outlined by Hopper & Gioia (2004). Given these historical patterns and the human mediated modification of habitats throughout inland regions, there is some concern for the evolutionary potential of the species in light of predicted climate change.

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This study forms part of the PhD thesis of Danielle Edwards as a member of the laboratory of Prof. Dale Roberts, with molecular work conducted in Assoc. Prof. Scott Keogh's laboratory. Danielle Edwards is interested in the biogeography and population genetics of herpetofauna, and is currently a Postdoctoral Associate in the laboratory of Assoc. Prof. Scott Keogh. Dale Roberts is interested in the biogeography of, and sexual selection in Australian frogs. Scott Keogh is interested in the biogeography and systematics of a range of herpetofauna.

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