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Molecular phylogenetic dating supports an ancient endemic speciation model in Australia's biodiversity hotspot

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Abstract

Southwestern Australia (SWA) is a region of temperate Mediterranean climate isolated by desert from the rest of Australia. Since the Jurassic it has been a geologically stable area that resisted Cenozoic glaciations and today represents an ancient landscape characterized by subdued topography and nutrient-poor soils. Despite these ecological conditions, SWA contains an incredibly rich flora and fauna that includes a great diversity of endemic species and it recently has been identified as a biodiversity hotspot of international significance. Since the early recognition of the high floral diversity in SWA and subsequent recognition of high faunal diversity, much discussion has focused on the origins of this rich endemic biota. Two alternative models have been proposed—the Multiple Invasion Hypothesis and the Endemic Speciation Hypothesis. Multiple tests of these models have variously supported either one, but many of the tests have been poor. Here we use a phylogeny for the myobatrachid frog genus *Heleioporus* to distinguish between these hypotheses. *Heleioporus* comprises six species: five endemic to southwestern Australia with one from eastern Australia. A molecular phylogeny using two mitochondrial genes (*ND2* and 12S rDNA) and one nuclear gene (*rag1*) was used to test alternative theories about the biogeography and the origin of diversity in this genus. Using a relaxed molecular clock, the divergence between the eastern and western species was dated at 25.60 M years, which is considerably older than previously suggested. Our phylogeny of *Heleioporus* is inconsistent with previous biogeographic hypotheses involving repeated invasions from the east to the west and some previous in situ models and instead strongly supports an ancient endemic speciation model. While the split between east and west appears to be contemporaneous with similar splits in *Geocrinia* (Anura) and *Banksia* (Proteaceae) it is much older than splits in a range of other taxa including other anurans.

Keywords: Molecular dating; Biogeography; Speciation; *Heleioporus*; Anura; Frog; Australia

1. Introduction

A central aim of biogeographic studies is to ask when, where and why did lineages radiate (Sanmartin and Ronquist, 2004). Addressing this broad aim requires that we address three more specific issues including the recognition and discovery of phylogenetic patterns (what lineages are out there?), documentation of distributional patterns (where do the lineages occur?), and identification of potential causal processes (how did these lineages evolve?). These questions are particularly pertinent to taxa in regions of the Earth richest in endemic species under threat, biodiversity hotspots (Myers et al., 2000). A comprehension of the roles of history, geography and mechanisms generating diversity are critical to future preservation of these fundamental evolutionary processes.

Southwestern Australia (SWA) is a region of temperate Mediterranean climate isolated by desert from the rest of Australia (Fig. 1). Since the Jurassic it has been a geologically stable area that resisted Cenozoic glaciations, and today it represents an ancient landscape characterized by subdued topography and nutrient-poor soils (Hopper and Gioia, 2004). Despite these ecological conditions, SWA contains an incredibly rich flora and fauna that include a

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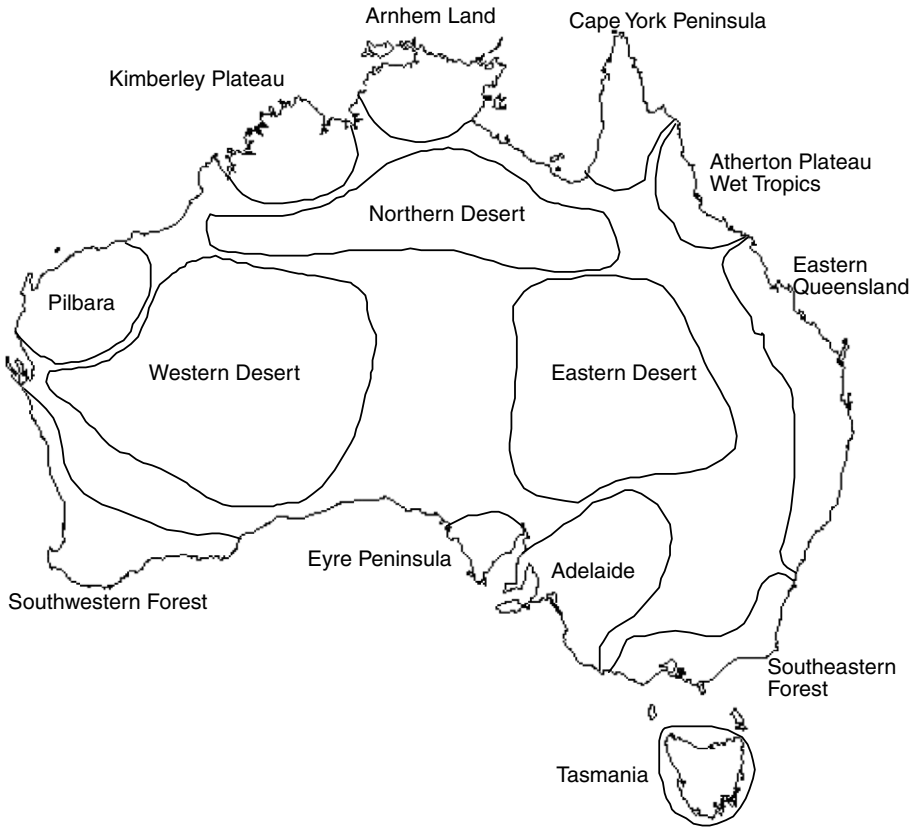

Fig. 1. Map of Australia showing areas of animal endemism (modified from Cracraft, 1991).

huge diversity of endemic species. The high proportion of endemic plant species (79% of all vascular plants in SWA) has led to SWA being named one of the world's 25 biodiversity hotspots (Myers et al., 2000). A significant number of animal species are also endemic, including 13% of the mammals, 26% of the reptiles and over 80% of the amphibians (Myers et al., 2000).

Since the early recognition of the high floral diversity in SWA and subsequent recognition of high faunal diversity, much discussion has focused on the origins of this rich endemic biota. Many authors have noted the climatic and taxonomic affinities between SWA and southeastern Australia (e.g., Hooker, 1860; Diels, 1906; Burbidge, 1960). Together these regions are regarded as the temperate Bassian element of the continent (Burbidge, 1960; Schodde, 1989), but these regions are bisected by the more arid Eyrean element (Fig. 1). Although few species are found in both areas, many genera or related genera are represented in both regions, implying some historical pattern of connectedness. One of the unusual aspects of this distribution is that these radiations typically exhibit greater species-level diversity in SWA relative to the south-east and there are numerous examples from frogs (e.g., Main et al., 1958; Littlejohn, 1981; Roberts and Maxson, 1985), reptiles (e.g., Jennings et al., 2003), birds (e.g., Cracraft, 1986), and plants (e.g., Burbidge, 1960; Nelson, 1974; Crisp et al., 2004).

Models describing the origin of SWA diversity need to explain two possible scenarios—a historical relationship between SWA and other regions of Australia or diversification within SWA. These simple alternative models have been termed the Multiple Invasion Hypothesis (MIH, e.g., Main et al., 1958) and the Endemic Speciation Hypothesis (ESH, White, 1977; Maxson and Roberts, 1984). The MIH suggests that dispersal into the SW has happened multiple times, over long periods of time, resulting in a flora and fauna with complex phylogenetic and biogeographic relationships to the surrounding regions. This hypothesis has been most extensively tested in frogs where diversity, and east–west connectedness in particular, were explained by repeated east–west invasions (Main et al., 1958; Lee, 1967; Main, 1968; Littlejohn, 1981), thus supporting the MIH. However, White (1977) strongly criticized the MIH and suggested the alternative Endemic Speciation Hypothesis, which suggests that clades have radiated within the SW, in some cases after a dispersal event from another region. Recent molecular phylogenetic studies on animal taxa with Bassian distributions have revealed monophyletic western groups, supporting White's hypothesis that endemic speciation has played a major role in promoting SWA diversity (e.g., Barendse, 1984; Maxson and Roberts, 1984; Jennings et al., 2003; Munasinghe et al., 2004; Burns and Crayn, 2006).

The Myobatrachid frog genus *Heleioporus* has featured heavily in both the generation and subsequent tests of these hypotheses (Main et al., 1958; Lee, 1967; Main, 1968; Maxson and Roberts, 1984; Roberts and Maxson, 1985).

Heleioporus comprises six species, five of which are endemic to SWA and one species, *H. australiacus*, is distributed in southeastern Australia (Fig. 2). The first formal hypothesis to account for this pattern was the MIH (Main et al., 1958). Briefly, it was postulated that two source stocks occurred in eastern Australia during the Pleistocene, a wet-adapted Bassian stock and a more arid-adapted Eyrean stock. Frogs of each stock then crossed the Nullabor Plain several times during periods of high rainfall during Pleistocene glaciations and subsequently speciated each time the Nullabor became more arid during interglacials. The final period of high rainfall was not extreme enough to allow the southeastern Bassian frogs to cross the Nullabor, thus accounting for the present distribution of three "Eyrean" species in SWA species (*H. albopunctatus*, *H. psammophilus*, *H.eyrei*) but only two "Bassian" species (*H. barycragus*, *H. inornatus*). In SEA, the Bassian stock survived to the present and is now represented by *H. australiacus*, but the extant distribution required that the Eyrean stock went extinct sometime between the final glacial cycle and the present. From a hypothesis-testing point of view, this MIH scenario predicts the phylogenetic relationships seen in Fig. 3a. Although this scenario was criticized by White (1977), the first detailed test of the MIH for *Heleioporus* was provided by Maxson and Roberts (1984), and a similar test was done with the myobatrachid frog genus *Crinia*, which has a similar

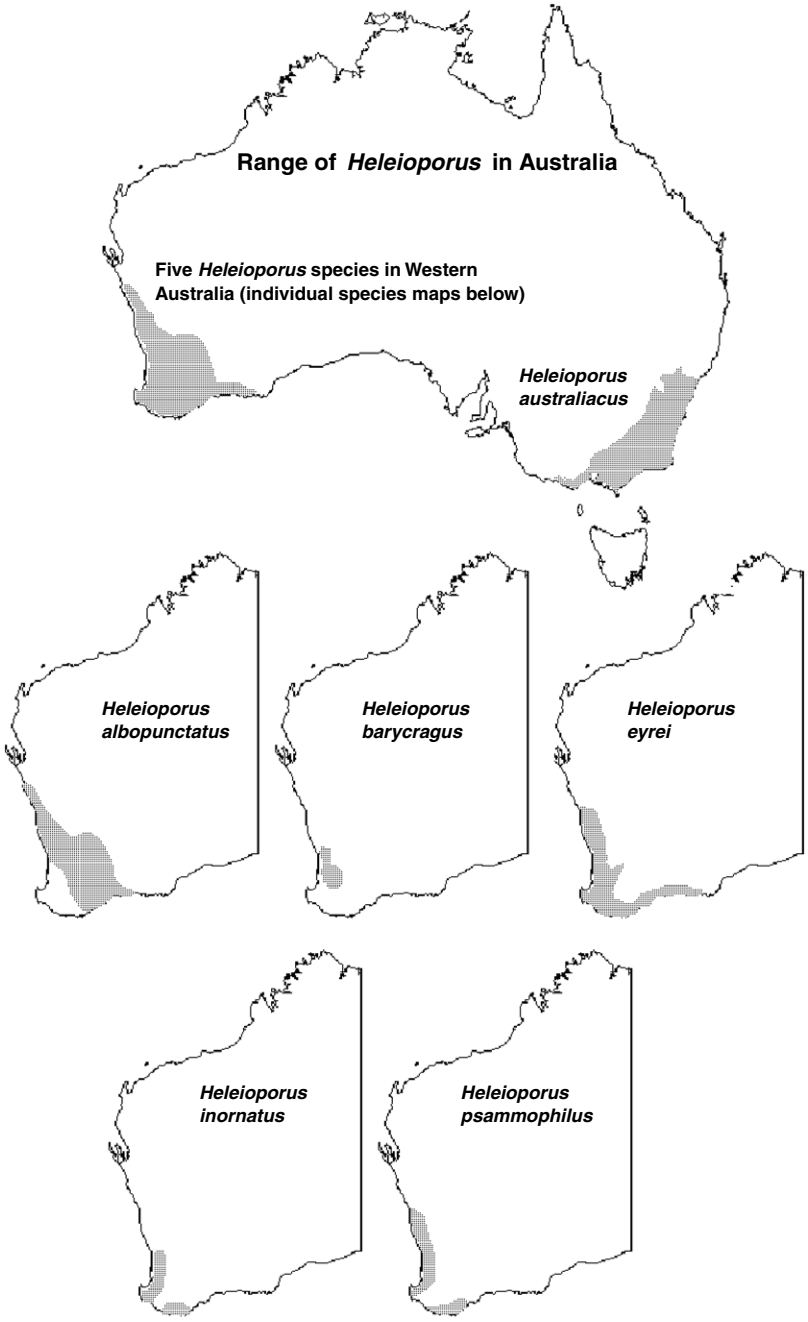

Fig. 2. Map of Australia showing proposed ranges of *Heleioporus* species.

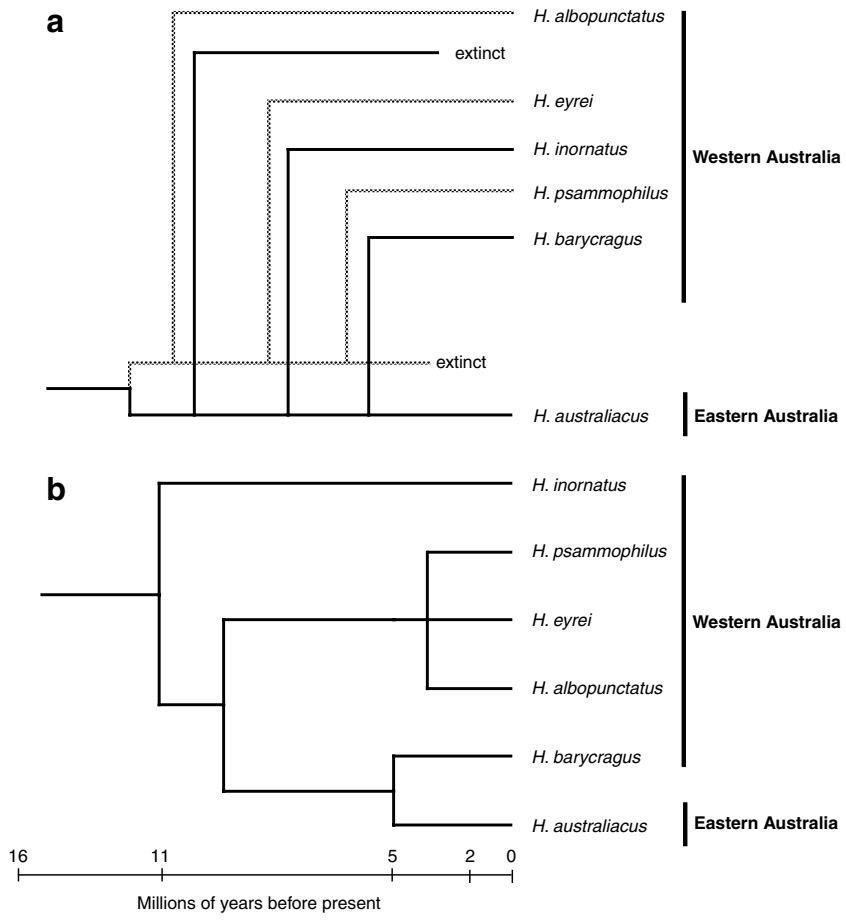

Fig. 3. Alternative hypotheses of *Heleioporus* phylogeny and biogeographical implications. (a) Multiple Invasion Hypothesis (Main et al., 1958)—solid and dashed lines represent independent lineages with a common ancestor. Contemporaneous Pleistocene invasions of each lineage into SW Australia result in two clades, one restricted to SW Australia (Eyrean group; dashed lines) and one with an extant SE representative (Bassian group; solid lines). (b) Endemic Speciation Hypothesis (Maxson and Roberts, 1984)—note Miocene timeframe for east–west split.

distribution in southern Australia (Barendse, 1984). Both studies rejected the MIH in favor of a pattern interpreted as supporting the endemic speciation hypothesis in SWA (Fig. 3b).

While the studies by Maxson and Roberts (1984) and Barendse (1984) both supported the ESH, there are two important problems that could not be solved adequately at the time, one of topology and one of the accuracy of molecular dating. The *Crinia* analysis found reciprocally monophyletic western and eastern clades (Barendse, 1984), but in *Heleioporus* the SWA group was paraphyletic, with the eastern species being the sister taxon to *H. barycragus*, explained as a single migration from SWA to SEA around 5 mya (Maxson and Roberts, 1984). This topology does not actually support White's (1977) hypothesis of endemic speciation as he predicted that the western species should form a clade. The problem is that the interpretation of the phylogeny in Fig. 3b as endemic speciation followed by a single west to east migration is only one possible scenario. The tree is also consistent with the concept of the "nested ancestral area" in which species exhibiting the ancestral condition of a character (e.g., the ancestral area) are found embedded within a derived clade (Cook and Crisp, 2005), and thus the topology in Fig. 3b does not exclude the MIH as a possible explanation. In both the MIH and ESH models, different elements of the same biota may have different histories—there may be no single generality.

The second issue is the dating of the split between eastern and western distributed taxa. Maxson and Roberts (1984) hypothesized a single migration from SWA to SEA approximately 5 mya. This was far younger than any other postulated trans-Nullabor divergence for frogs (28–10 mya, Roberts and Maxson, 1985) and it coincides with a period of extreme aridity at the end of the Miocene. The other issue is that the phylogenetic estimate of Maxson and Roberts (1984) was a phenetic tree based on limited albumin immunological distance data and a non-specific, linear molecular clock. Nonetheless, more recent studies based on a diverse set of taxa have suggested that the 5 mya date is plausible. The results of dating analyses and molecular phylogenies of plant and animal genera suggest that different elements of the biota colonized SWA at different times throughout the Late Oligocene to Late Miocene (28–5 mya)

and subsequently radiated within SWA (Roberts and Maxson, 1985; Munasinghe et al., 2004; Jennings et al., 2003; Crisp et al., 2004). Some genera appear to have colonized SWA multiple times throughout the Early to Mid Miocene (23–10 mya), radiating within SWA on each occasion (Crisp et al., 2004).

Clearly, there are here unresolved issues here and a more sophisticated phylogenetic estimate and more robust phylogenetic dating techniques can provide clarity to the issues that we have outlined. Here we use DNA sequence data from two regions of the mitochondrial genome (12S rDNA and ND2 genes) to produce a robust phylogenetic hypothesis for the frog genus *Heleioporus*. We then use these data to test the relationships predicted by Main et al. (1958) and Maxson and Roberts (1984). In addition, we added nuclear and mitochondrial sequence data to a diverse set of related taxa to date the trans-Nullabor split in this genus to assess the reliability of previous Pleistocene and Pliocene estimates.

2. Materials and methods

2.1. Taxon selection

We were interested in generating a phylogeny for *Heleioporus* as well as using molecular dating techniques to date key nodes that were relevant to testing the alternative biogeographic hypotheses. Therefore, our sampling was done at two levels. For the *Heleioporus* phylogeny we included multiple individuals (3–13) from all six species and we tried to include samples from as much of each species' range as possible (Table 1). The closely related genera *Neobatrachus* and *Notaden* are thought to be sister taxa to *Heleioporus* by Farris et al. (1982) and Maxson (1992), so we used multiple species from these genera as outgroups in a phylogenetic analysis of *Heleioporus* (Table 1).

To estimate divergence times within *Heleioporus* we needed a calibration point of known or estimated age, and this determined which additional species we needed to sample. Biju and Bossuyt (2003) dated the node separating the Families Heleophrynidae and Myobatrachidae at 150 mya. To use this calibration date we included taxa within the subfamilies Myobatrachinae (*Pseudophryne dendyi*, *Uperoleia borealis*), Limnodynastinae (*Lechriodus flecheri*, *Limnodynastes tasmaniensis*) and family Heleophrynidae (*Heleophryne natalensis*, *H. purceili*) and combined these data with exemplars from each *Heleioporus* species (Table 1). This sampling allowed us to construct a tree that included both the Heleophrynidae/Myobatrachidae node and the node separating *H. australiacus* from the western group to estimate divergence times within *Heleioporus*. A representative of the genus *Bufo* was used as an outgroup in this analysis.

2.2. Data collection

DNA was extracted from liver or muscle samples using a modified CTAB protocol, suspended in TE buffer and stored at 4 °C. For this study we targeted the ND2 and 12S genes as these have provided good resolution at this level in other Australian anurans (e.g., Read et al., 2001). The rag1 nuclear gene was sequenced to construct the dating tree as in recent studies it showed the best resolution at this level compared to alternative nuclear genes (Biju and Bossuyt, 2003, supplementary material).

Target DNA was amplified using a modified version of the stepdown PCR profile employed by Keogh et al. (2000). The following primers were used to amplify and to sequence *ND2*: L4221 (forward; Macey et al., 1998) and tRNA_Trp (reverse; Read et al., 2001); 12S rRNA: tPhe (forward; Wiens et al., 1999) and H3296 (reverse; Richards and Moore, 1996); rag1: Mart.FL1 and Amp.R1 (Hoegg et al., 2004). Target fragments were amplified in 40 μL reactions, which comprised the following: ~50 ng template DNA, 4 μL 10× reaction buffer, 3 mM $MgCl_2$, 0.5 mM dNTPs, 10 pmol each primer and 1 unit Platinum *Taq* DNA polymerase. Amplification products were purified on 2% agarose gels and target fragments excised. Products were extracted from agarose using an UltraClean™ 15 kit (Mo Bio Laboratories, Inc., Solana Beach CA), resuspended in 20 μL deionised water and stored at −20 °C.

Sequencing of purified PCR products was performed directly using the corresponding PCR primers. DNA sequences of forward and reverse strands were obtained using the ABI Prism BigDye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA). Reactions comprised the following: ~30 ng purified PCR product template, 1 mL BigDye Terminator Ready Reaction Mix, 4.5 mL 5× sequencing buffer, 3.2 pmol of primer, and made up to 20 mL with deionized water. Sequencing reactions were visualised using an ABI 3100 Automated capillary sequencer. The 12S rDNA fragment was approximately 1190 bp long and included approximately 960 bp of 12S rDNA, $tRNA^{VAL}$, and 250 bp 16S rDNA. The *ND2* fragment was approximately 1200 bp long, which included the entire $tRNA^{MET}$ and ND2 gene sequence, and partial sequence for $tRNA^{TRP}$ and $tRNA^{ILE}$. The *rag1* fragment was approximately 820 bp long.

2.3. Data analysis and data set construction

DNA sequence data were edited using Sequencher 3.0 (© Gene Codes Corporation, Ann Arbor, MI). MtDNA sequences were concluded to be mitochondrial rather than nuclear in origin on the basis of similarity with previously published and publicly available sequence data and translatability of the coding regions. *Rag1* sequences were checked for accuracy using the same criteria. Nuclear paralogues of both the 12S rDNA fragment and *ND2* regions were amplified from sample AU1 (Table 1). These were detected by gross length and sequence discrepancies. Sequences from this animal were excluded from all subsequent analyses.

Sequence fragments were aligned separately using ClustalX (Thompson et al., 1997). Default settings were used

Table 1
Locality and voucher information for all individuals used in this study

Species	Locality[a]	Museum No.[b]	Voucher No.[c]	Note[d]
Ingroup				
Heleioporus australiacus				
AU1	Narooma, NSW	ABTC66413		
AU2	Narooma, NSW	ABTC26468		ab
AU3	Helensburg, NSW	ABTC17806	SAMAR45377	ab
AU4	Watagan Mtns, NSW	ABTC7143		ab
AU5	Ku-ring-gai Chase, NSW			abc
Heleioporus barycragus				
BA1	Darlington, WA	ABTC62829	WAMR115981	ab
BA2	Beechina WA	ABTC62831	WAMR115982	ab
BA3	9k SE Chidlow WA	ABTC62833	WAMR115984	abc
Heleioporus albopunctatus				
AL1	Lort River, WA	ABTC15776	SAMAR43004	a
AL2	Quairading WA	ABTC16163	SAMAR39230	a
AL3	Geraldton, WA	114518		a
AL4	1 km N Highbury, WA	116212		a
AL5	8 km S Eneabba, WA	131876		a
AL6	14 km W Brookton, WA	140598		a
AL7	14 km W Brookton, WA	140599		ac
AL8	9 km SE Chidlow, WA	ABTC62837	WAMR115988	a
AL9	9 km SE Chidlow, WA	ABTC62838	WAMR115989	a
AL10	9 km ESE Chidlow, WA	ABTC62892	WAMR116285	a
Heleioporus inornatus				
IN1	11 km E Kalamunda, WA	ABTC62798	WAMR113748	a
IN2	1.5 km SW Meelup, WA	90146		a
IN3	12 km NE Jarrahdale, WA	113831		ac
IN4	Bruce Road, WA	116377		a
IN5	Batalling NR, WA	137365		a
IN6	Batalling NR, WA	132920		a
Heleioporus eyrei				
EY1	7 km NW Margaret River, WA	90126		a
EY2	19 km E Black Point, WA	90138		a
EY3	Kenwick Botany Reserve, WA	104383		a
EY4	Bold Park, WA	106148		ac
EY5	10.5 km SE Chidlow, WA	116193		a
EY6	Talbot Rd, Swan View, WA	116195		a
EY7	15 km WNW Cataby, WA	140492		a
EY8	8 km NW Albany, WA	ABTC62853	WAMR106103	a
Heleioporus psammophilus				
PS1	5 km SSW Mt Chudalup, WA	90166		a
PS2	12 km WSW Bolgart, WA	127422		a
PS3	Swan View, WA	ABTC62849	WAMR116072	a
PS4	22 km E Walpole, WA	ABTC62852	WAMR116102	a
PS5	12 km ESE Chidlow, WA	ABTC62888	WAMR116260	ac
PS6	145 km E Ravensthorpe, WA	ABTC15890	SAMAR40353	a
PS7	28 km E Northcliffe, WA	ABTC28321		a
PS8	1 km W Lort River, WA	ABTC28323		a
PS9	5 km SSW Mt. Chudalup,WA	90159		a
PS10	8 km W Esperance, WA	127405		a
PS11	Kalbarri, WA	129795		a
PS12	25 km NNE Bow Bridge, WA	140651		a
PS13	107 km W Lort River, WA	ABTC15771	SAMAR43003	a
Outgroups				
*Neobatrachus pictus*1	Kangaroo Island, SA	ABTC33563	SAMAR37364	a
*Neobatrachus pictus*2	11 km N Magrath Flat, SA	ABTC34317	SAMAR39496	a
Neobatrachus pelabatoides	Nokaning,WA	ABTC33318		a
Neobatrachus albipes	30 km W Coolgardie, WA	ABTC15802		a

Table 1 (*continued*)

Species	Locality[a]	Museum No.[b]	Voucher No.[c]	Note[d]
Neobatrachus centralis	35 km N Miandana WH, WA	ABTC38623	SAMAR51307	a
Neobatrachus kunapalari	67 km S Moorine Rock, WA	113538		abc
Notaden bennetti	31.5 km E Nyngan, NSW	ABTC17767	SAMAR45366	abc
Adelotus brevis	Lionsville Rd, Washpool Ck, NSW	ABTC24210		bc
Limnodynastes tasmaniensis	Kangaroo Island, SA	ABTC3344	5AMAR37346	bc
Lechriodus fletcheri	Lamington NP, QLD	ABTC25947		c
Uperoleia borealis	Ivanhoe Station, WA	129205		c
Pseudophryne dendyi	25 km S Delegate, VIC	ABTC40926		c
Heleophryne natalensis	Tugela, KZN, S. Africa			c
Heleophryne purcelli–(12S rRNA GenBank No. AY364356; rag-1 GenBank No. AY364221)	South Africa			cd
Bufo marinus	North Queensland			c

[a] NSW, New South Wales; SA, South Australia; QLD, Queensland; VIC, Victoria; WA, Western Australia.
[b] ABTC: Australian Biological Tissue Collection, South Australian Museum.
[c] SAM: South Australian Museum; WAM: Western Australian Museum.
[d] (a) Sample used in generating *Heleioporus* phylogeny; (b) sample used in SH topology tests; (c) sample used in tree for dating estimate; (d) sequence information from GenBank, originally from Biju and Bossuyt (2003).

for all parameters. The multiple alignments were checked by eye, and all ambiguities and variable characters compared with the original sequences to reduce the possibility of computer or editing error. The 12SrRNA and *tRNA* alignments were refined by eye. The *Xenopus laevis* secondary structure maps (Cannone et al., 2002) were used as a template to determine positional homology and to designate stem and loop positions. Aligned sequences were concatenated and split into five partitions: first, second and third codon position (coding sequences), paired or unpaired (corresponding to the stems and loops in the non-coding sequences). Several regions of the non-coding sequence could not be unambiguously aligned with confidence and were deleted from all the final analyses. The alignments produced from this study will be made available on TreeBASE (http://www.treebase.org/treebase). Base composition differences among taxa were tested for each gene as implemented in PAUP*v4.0b10 (Swofford, 2002).

Two alignments were compiled to address the different questions: (a) *ND2* and non-coding mtDNA sequences for all *Heleioporus* samples with *Neobatrachus* and *Notaden* as outgroups, and (b) Non-coding mtDNA and *rag-1* sequences for myobatrachid and heleophrynid taxa with *Bufo marinus* as an outgroup. Alignment (a) was used to test alternative hypotheses of *Heleioporus* phylogenetic relationships, and alignment (b) was used to date the east–west split in *Heleioporus*.

2.4. Phylogenetic analysis

Congruence among partitions within each alignment was tested by the partition homogeneity test implemented in PAUP*. Partitions in both alignments were tested for substitution saturation by plotting pairwise transition:transversion ratios against uncorrected genetic distance.

Both alignments were analyzed under the same conditions. To counter the possible bias or confounding effects of different models and phylogenetic methods, trees were constructed using neighbour-joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference. The trees generated by the different methods were checked for congruence.

Neighbour-joining was performed in PAUP* 4.0b10 (Swofford, 2002) with distance corrections using a simple (K2P; Kimura, 1980) and a complex (GTR+I+Γ) model obtained using ModelTest 3.06 (Posada and Crandall, 1998). MP and ML heuristic searches were performed using 10 random-stepwise addition replicates with tree-bisection-reconnection branch swapping. All characters were treated as unordered and weighted equally. Gaps were treated as missing data. The best-fitting models of sequence evolution for the ML models were determined by the AIC in ModelTest 3.06 (Posada and Crandall, 1998). Node support was assessed using the nonparametric bootstrap technique with 1000 MP replicates and 100 ML replicates, with 70%

support considered well supported (Hillis and Bull, 1993; Efron et al., 1996).

Bayesian phylogenetic analyses of both data sets were performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001). One advantage of current Bayesian inference software over maximum likelihood approaches is the ability to use mixed-model analyses. That is, it allows user-specified data partitions to evolve under separate independent models. This was considered particularly desirable for the analysis of alignment (b) because recent simulation studies have shown that ML may incorrectly estimate branch lengths when two data partitions that have evolved heterogeneously are analyzed under a single evolutionary model (Kolaczkowski and Thornton, 2004). This problem could affect subsequent estimates of divergence dates. Therefore, each alignment was partitioned by gene and partitions analyzed simultaneously. The substitution models used for each partition were determined by the AIC in ModelTest 3.06, although the parameter values were free to vary. The Markov chain Monte Carlo process was set so that one cold chain and three heated chains ran simultaneously for 2,000,000 generations. Trees were sampled every 100th generation for a total of 20,001 trees. The chains (i.e., the log likelihood sum) reached apparent stationarity around the 10,000th generation, and the first 100,000 generations (i.e., the first 1000 trees) were deleted as the "burn in" of the chain. The remaining 19,001 trees were used to construct a majority-rule consensus tree in MrBayes. Support for each node was assessed using posterior probabilities, and branch lengths were obtained using the "sumt" option of MrBayes. Branch lengths were estimated as the mean of the posterior distribution of this set. Although this leads to the branch lengths being fixed for further analyses, we felt that the removal of saturated data and the use of Bayesian analysis to avoid model mis-specification would provide a reasonable estimate of branch lengths as long as the Markov chain had reached stationarity. The results of five replicate runs were analyzed by eye to assess repeatability as another measure that the chains had reached stationarity.

2.5. Topology testing

Statistical testing of alternative tree topologies was conducted using Shimodaira-Hasegawa tests (SH-test; Shimodaira and Hasegawa, 1999). The phylogenetic hypotheses of Maxson and Roberts (1984) and Main et al. (1958) predict very similar unrooted topologies and differ only in the resolution within the Eyrean group and the placement of the outgroup node (Fig. 3). In light of this, all possible outgroup placements of the unrooted tree that maintained species monophyly were tested (see Fig. 4b). A reduced taxon data set of three or four exemplars per species and four outgroup taxa was used to speed computation time with no loss of generality (see Table 1 for included taxa). Model selection and ML analyses were performed as above. One tree (T1) was generated under no constraints and eight alternatives (T2–T9) generated by constraining the position

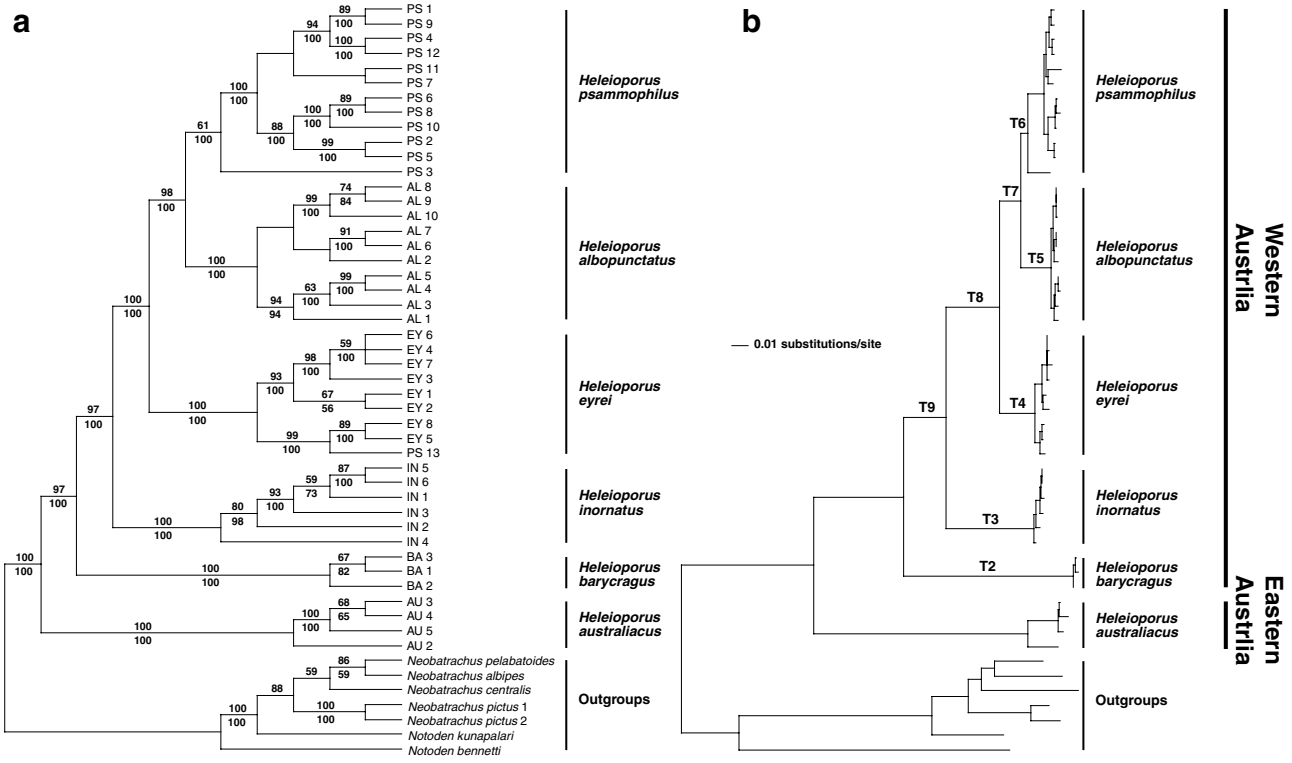

Fig. 4. Phylogeny of *Heleioporus* based on combined analysis of 12S rRNA and ND2 mtDNA gene sequences. (a) Cladogram showing MP bootstraps above the branch and Bayesian posterior probabilities below. (b) ML phylogram showing alternative root positions tested by SH-tests.

of the outgroup node to a particular branch. The positions of the taxa on either side of the root were unconstrained. The alternative biogeographic hypotheses were congruent with T3 (MR) and T8 (Main). SH tests were performed using full optimization in PAUP* 4.0b10 (Swofford, 2002).

2.6. Estimating divergence times

The most common method of determining divergence times has been by invoking a molecular clock (Zuckerkandl and Pauling, 1965). Empirical studies have shown that departures from clock-like evolution are abundant (e.g., Li, 1993; Bromham and Penny, 2003) and methods have been developed to cope with data that do not evolve in a clock-like fashion (e.g., Sanderson, 1997, 2003; Thorne et al., 1998). The combined data set in alignment (b) was tested for violation of clock-like behavior using likelihood ratio tests (Felsenstein, 1981). Although the result of this test showed that these data were consistent with a model of rate constancy ($p = 0.07$), the fact that the data appeared to approach non-uniform rate was of concern. Therefore, divergence times were estimated using penalized likelihood as implemented in the program r8s version 1.5 (Sanderson, 2003), which estimates absolute substitution rates and divergence times for a given tree under a relaxed molecular clock. This is achieved by smoothing changes in substitution rates across the tree under a user-specified smoothing parameter. High values of this parameter (i.e., >1000) strongly penalize large changes across branches and essentially lead to clock-like conditions, whereas lower values are optimal for data severely violating the assumption of rate constancy. Thus, optimizing the smoothing parameter using an internal cross-validation procedure enabled the relative level of departure from rate-constancy to be determined empirically.

The Bayesian phylogeny and associated mean estimates of branch lengths generated from the analysis of combined alignment (b) was used as the input tree in r8s. The optimal smoothing parameter, ranging from 10^0 to 10^8, was selected prior to the dating by cross-validation. Absolute rates across the tree were calculated using the Truncated-Newton algorithm and started ten times to avoid local optima. The tree was calibrated using the estimated divergence of the Heleophrynidae from the Myobatrachidae at 150 mya, with a range of 109–198 mya (95% credibility interval; Biju and Bossuyt, 2003). A point estimate of divergence times for each node was derived by fixing the date of the Heleophrynidae/Myobatrachidae node at 150 mya. A range for the estimate was derived to incorporate uncertainty in the calibration point and the rate smoothing process. Firstly, the upper and lower bounds of the 95% credible interval were used to incorporate uncertainty in the fixed date estimate. Secondly, to incorporate the variation within the r8s algorithm ranges were calculated using the internal confidence method (Sanderson, 2003; Cutler, 2000). This method provides upper and lower age estimates for each node based on the calibration age and the shape of the likelihood parameter space around the maximum likelihood estimate with limits defined by a parameter *s*. In this analysis *s* was set to 4.0. This was applied to the upper and lower values of the 95% credible set when used as calibration dates to ensure that a conservative estimate was obtained.

3. Results

3.1. Phylogeny of Heleioporus

After removal of hypervariable regions and ambiguously aligned positions the combined dataset comprised 2221 nucleotides from protein coding (*ND2* 1053 bp) and non-coding (12SrRNA and *tRNA*s 1192bp) mtDNA. Of these, 155 nucleotides were uninformative and 731 sites were parsimony informative. For *ND2* 76 sites were uninformative and 476 base pairs were parsimony informative. For the non-coding rDNA 79 sites were uninformative and 225 base pairs parsimony informative. A partition homogeneity test confirmed homogeneity of the partitions ($p > 0.99$), and no base composition bias was evident in the data ($p > 0.99$). Therefore, all analyses were performed on the combined alignment.

Inferred phylogenies from NJ, MP, ML and Bayesian analyses of the combined data set all produced the same overall topology (Fig. 4a). This topology was resilient to different analytical methods, optimality criteria, and sub-optimal substitution models (data not shown). High levels of support were found for the monophyly of each species except *H. psammophilus* (MP bootstrap = 61% Posterior Probability 1.00). Clade support was always high for the between-species relationships (bootstraps >95%, posterior probabilities >0.95), although there was variation in support for some intra-specific relationships. The optimal topology contained reciprocally monophyletic western and eastern clades. Within the western group the "Eyrean" group of species (*H. albopunctatus*, *H. psammophilus*, *H. eyrei*) was always found to be monophyletic, and the "Bassian" group of species (*H. australiacus*, *H. barycragus*, *H. inornatus*) (Main et al., 1958) is paraphyletic even when *H. australiacus* is not considered.

3.2. Topology testing

The best tree from the analysis of the reduced dataset was congruent with that in Fig. 4. The results of the SH-tests can be seen in Table 2. All alternative root placements were conclusively rejected ($p < 0.014$ in all cases). In particular, the root placement consistent with the tree of Maxson and Roberts (1984, T3) was rejected ($p = 0.011$) and that of Main et al. (1958, T8) rejected ($p = 0.014$).

3.3. Divergence time estimates

After removal of hypervariable regions the data matrix for the deep phylogeny comprised 2111 characters from *rag1* and non-coding mtDNA genes. The unpaired sites of

Table 2
Results of Shimodaira-Hasegawa tests of alternative root placements and topologies proposed by Main (1968), Main et al. (1958), Lee (1967) (T8) and Maxson and Roberts (1984, T3)

Alternative topology	$-\ln L$	Difference in $-\ln L$	p-value
Optimal tree (T1)	9814.51		
T2	9837.41	22.90	0.013
T3	9842.69	28.18	0.011
T4	9854.29	39.78	0.004
T5	9855.49	40.98	0.004
T6	9856.63	42.12	0.002
T7	9854.20	39.69	0.004
T8	9842.23	27.72	0.014
T9	9837.59	23.08	0.013

See Fig. 3 for locations of root placements. All p-values are significant (<0.05) indicating that the alternative topologies are significantly different from the maximum likelihood tree.

the non-coding mtDNA sequences showed evidence of saturation when transition:transversion ratios were plotted against sequence divergence, and were discarded. The final matrix comprised 1403 characters (including 570 base pairs of non-coding mtDNA sequence and 833 base pairs of *rag1* sequence) of which 533 base pairs were variable and 369 were parsimony informative. No base composition bias was evident in the data ($p > 0.99$) and a partition homogeneity test confirmed homogeneity of the partitions ($p > 0.12$). Further investigation of this result by conducting separate Bayesian and MP bootstrap analyses for each gene revealed that no incongruent clades were well supported and the combined alignment was used in further analyses.

The analysse based on the combined dataset using MP, ML and Bayesian Inference all produced the same topology (Fig. 5). All analyses support the monophyly of the Myobatr-

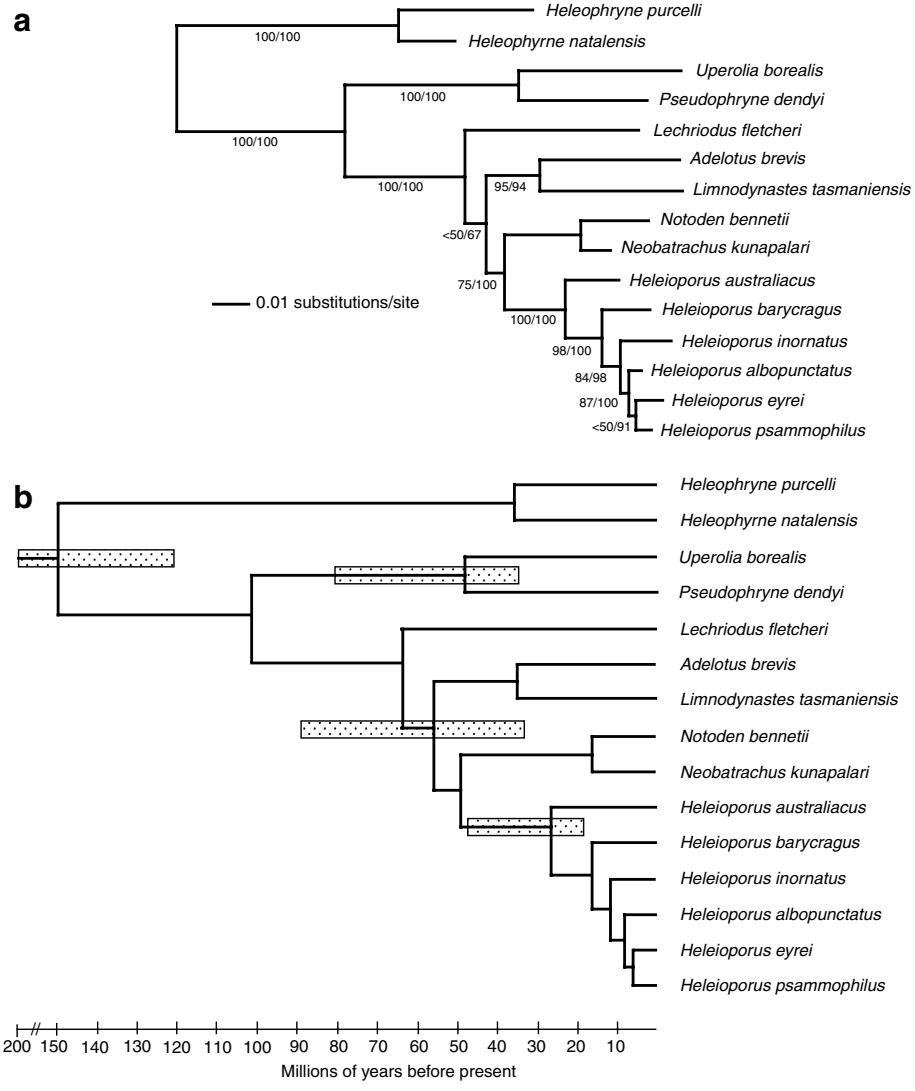

Fig. 5. (a) Bayesian estimate of Myobatrachidae phylogeny and branch lengths based on 12S rDNA and *rag1* sequences used in divergence time analysis. Numbers at nodes indicate MP bootstrap and posterior probabilities respectively. (b) Chronogram based on penalized likelihood molecular dating analysis of the 12S and *rag1* sequences. Age estimates based on fixing root age at 150 mya. Shaded bars indicate the upper and lower bounds of each relevant divergence date estimate (see text for details).

Table 3
Divergence times estimated via r8s analysis

Divergence	Estimated age (mya)	Range
H. australiacus from Western clade	25.60	17.52–42.30
H. barycragus from Western clade	18.35	11.28–33.70
H. inornatus from Western clade	11.81	7.09–20.37
Pseudophryne–Uperoleia	45.41	27.32–72.47
Limnodynastes–Heleioporus	52.08	38.54–83.05
Myobatrachinae–Limnodynastinae	101.88	69.04–144.12

Smoothing parameter was set to 100 and ranges calculated with $s = 4.0$ (Sanderson, 2003).

achidae, Myobatrachinae and Limnodynastinae and relationships within clades are comparable to other recent data sets. The relationship of *Notaden* and *Neobatrachus* as a monophyletic, sister group to *Heleioporus* was supported. The relationships within *Heleioporus* were consistent with the results of analysis (a) except for the placement of *H. eyrei* as the sister taxon to *H. psammophilus*. Although weakly supported in some areas, the Bayesian consensus tree was used for estimating divergence times on the grounds that it was broadly consistent with previously published generic relationships (e.g., Farris et al., 1982; Hutchinson and Maxson, 1987; Maxson, 1992) and the fact that all analytical methods produced the same topology increased confidence that a reasonable solution had been found.

The results of molecular dating analyses using the clock-independent approach are shown in Table 3. The cross-validation procedure determined an optimal smoothing parameter value of 100, indicating that these data depart somewhat from clock-like evolution. Analyses with larger smoothing parameters (up to 10^8) resulted in very similar divergence date estimates to those using the optimal smoothing parameter. Uncertainty associated with the calibration age produced large ranges for all estimated divergence times (Table 3). The results suggest that the eastern and western *Heleioporus* separated in the Mid-Late Oligocene ca 25 mya and that no significant genetic exchange has occurred since this time.

4. Discussion

This study has produced a robust molecular phylogeny for the myobatrachid frog genus *Heleioporus*. This result has been used to test hypotheses of the pattern and timing of events in southern Australian frog biogeography. The results have highlighted substantial differences from details of previous biogeographical hypotheses and have estimated the divergence of eastern and western species to be considerably older than previously thought. The following discussion highlights the main features relating to the phylogeny of *Heleioporus* with specific reference to the biogeographical implications of the findings.

4.1. Phylogeny of Heleioporus

The phylogenetic analyses in this study found high support for the monophyly of all six *Heleioporus* species with the exception of *H. psammophilus*. *H. psammophilus* received high posterior probability values in all analyses but low bootstrap values in parsimony analyses (Fig. 4a). The cause of this result was traced to the single individual PS3. Removal of this animal produced 100% bootstrap support for this species in all analyses. However re-examination of the sequence for this individual provided no reason to reject the veracity of the phylogenetic estimate, and it was retained in the analysis. Bayesian posterior probabilities have been shown to provide unwarranted support for short internodes where parsimony bootstraps provide more conservative estimates and could be the source of the apparent conflict on this branch (Alfaro et al., 2003; Douady et al., 2003). This sample came from an animal from Swan View, the sole location sampled for this species that is on the coastal plain west of the Darling Escarpment. This escarpment has been shown to be ecologically and geologically distinct (Seddon, 1972; Kendrick et al., 1991) and defines the distributional boundaries of the frogs *H. barycragus*, *H. inornatus* (Tyler et al., 1994), *Crinia insignifera* and *C. pseudinsignifera* (Blackwell and Bull, 1978). A recent study of genetic differentiation within *H. psammophilus* supports the notion that the Darling Escarpment is at least a partial barrier to dispersal for this species (Berry, 2001), a conclusion supported by our results. Therefore we conclude that this result is real and probably reflects true underlying phylogeographic structure in this species that requires further investigation.

The other curious result found here is the placement of sample PS13, which was identified in museum records as *H. psammophilus* but appears in the well-supported *H. eyrei* clade. There are two plausible explanations for this result. Morphologically *H. psammophilus* and *H. eyrei* are virtually identical and can be distinguished reliably only by the male call (Cogger, 2000). It is entirely plausible that this animal was mis-identified and is indeed *H. eyrei*, but examining the museum specimen did not clarify this. The second explanation is that *H. psammophilus* and *H. eyrei* could hybridize in the wild. These species are sympatric for much of their range and are often found breeding at the same sites contemporaneously (Lee, 1967). In addition, *in vitro* experiments have shown the potential of female *H. eyrei* and male *H. psammophilus* species to hybridize under laboratory conditions and to produce viable tadpoles, albeit with reduced hatching success (Lee, 1967). Therefore, it is possible that hybridization has occurred, although the finding of otherwise reciprocally monophyletic groups in these highly sympatric species indicates that it is relatively uncommon if it occurs at all. In either case, these discrepancies have no effect on the focus of this paper, and further studies are required to assess the phylogeography and taxonomic status of these two anomalous individuals.

Divergences within species were much smaller than between species (Fig. 4b), implying that recent events have shaped the phylogeographic history of each species. One notable exception was the divergence within the *H. australiacus* clade (Fig. 4b). The results show a relatively deep

phylogenetic break between northern (samples AU3–AU5) and southern (AU2) *H. australiacus*. These samples were collected on either side of a break in the range of *H. australiacus* that corresponds to a change in environmental conditions (Penman et al., 2005). These populations show levels of genetic distance in ND2 and 12S genes similar to that seen between *Geocrinia laevis* and *Geocrinia victoriana* in eastern Australia (Read et al., 2001), and might represent different species. Although mtDNA divergence is an important piece of evidence, it should not be considered the only criterion on which taxonomic decisions are based, and a more fine-scale study of these populations would be required before taxonomic revisions should be made.

4.2. Order of branching events

The dominant feature of the *Heleioporus* phylogeny is the finding of reciprocally monophyletic eastern and western groups. This result supports the Endemic Speciation Hypothesis in the form advocated by White (1977), but the phylogenetic relationships predicted by the Multiple Invasion Hypothesis and that predicted by Maxson and Roberts (1984) were rejected (Table 2). In particular the prediction of *H. australiacus* as the sister taxon of *H. barycragus* common to both previous hypotheses was rejected, although the monophyly of the "Eyrean" group of species was confirmed.

The inferred position of the root has a major impact on subsequent hypotheses regarding biogeographic and evolutionary scenarios in this group. Although SH-tests indicated that the root position in Fig. 4a is preferred over alternative positions, it is possible that systematic biases have influenced the tree-scoring process. The outgroup criterion was used to root the *Heleioporus* phylogeny with outgroups chosen based on previous hypotheses of myobatrachid phylogeny (Heyer and Liem, 1976; Farris et al., 1982). The outgroup criterion has been shown to be effective and accurate under most conditions, although it can be inaccurate when ingroup and outgroup taxa are too divergent from each other (Wheeler, 1990; Swofford et al., 1996; Huelsenbeck et al., 2002). In such situations the root can be essentially random on the ingroup topology (Huelsenbeck et al., 2002) or be systematically attracted to the longest ingroup branch (Wheeler, 1990; Stiller and Hall, 1999; Susko et al., 2005). Detecting these effects can be problematic, as often the longest ingroup branch is likely to be the correct position of the root (Swofford et al., 1996). We feel that such biases are unlikely to be causing incorrect rooting of the *Heleioporus* tree because base composition bias and substitution saturation of ingroup and outgroup sequences was minimal, and analyses with gene partitions separately or combined under different methods gave the same result (results not shown). In addition, the genetic distances between outgroup and ingroup sequences were considerably less than expected if the sequences were random. Adding extra outgroups during the topology-testing and dating analyses, as well as adding a nuclear gene for the estimation of divergence times produced an identical root placement.

Recent genetic studies of a number of taxa have challenged the long-held perception that endemic speciation in WA is implausible. For anurans, the discovery of high intra-specific genetic divergence between contemporary frog populations in the southwest has demonstrated the potential for genetic sub-division and population isolation in this area (Driscoll, 1998). Phylogenetic analyses in other taxa have found monophyletic western and eastern groups which have corroborated the endemic speciation hypothesis, e.g., *Crinia* frogs (Barendse, 1984); pygopodid lizards (Jennings et al., 2003); crayfish (Munasinghe et al., 2004) and the discovery of Miocene radiations of plant taxa within SWA reinforces the hypothesis that endemic speciation is a real phenomenon (Crisp et al., 2004). The climatic isolation of southwest WA from the rest of Australia is thought to be responsible for the high levels of endemism in the southwestern flora and less vagile fauna (Hopper et al., 1990; Harvey, 2002) and has led to the recognition of southwestern Australia as a global biodiversity hotspot (Myers et al., 2000). The findings of this study show that this pattern also holds true for *Heleioporus* and that the hypothesis of speciation within the southwest and southeast is sufficient to explain the distribution of extant species. Consequently, these results call for modifying our understanding of the sequence of events within this group with the trans-Nullabor split occurring earlier than any of the speciation events within Western Australia.

4.3. Timing of divergences

Molecular dating with non-uniform evolutionary rates is still in its infancy. The results of molecular dating have become more accepted in recent years yet should still be evaluated critically. Independent estimation of the divergence times proposed here is difficult due to the poor fossil record of the Limnodynastinae. The accuracy of divergence time estimation from molecular sequence data depends on the accuracy of a number of factors including the model of evolution, the reconstructed topology and branch lengths, the calibration point estimate and the rate-smoothing process.

It is difficult to evaluate the accuracy of the phylogeny used to obtain the date estimates due to the poor state of Myobatrachid systematics (Farris et al., 1982; Roberts and Watson, 1993). Therefore, in this study the removal of potentially misleading data (i.e., substitution-saturated sequences) and use of appropriate models and techniques was the most satisfactory way to justify the choice of phylogeny and associated branch lengths. Bayesian inference of phylogeny allowed uncertainty in the model parameters to be incorporated into the phylogenetic reconstruction and allowed the different partitions of the combined data to be modelled independently of each other potentially leading to more accurate reconstruction (Yang and Yoder, 2003; Nylander et al., 2004). In addition, modelling data

partitions independently potentially resulted in more accurate branch length reconstruction, as combining heterogeneously evolving sequences under a single model can lead to an "averaging" of branch lengths under the criterion of maximum likelihood (Kolaczkowski and Thornton, 2004). Despite this, all analytical methods found low nodal support and short branch lengths for the basal region of the Limnodynastinae (Fig. 5). This result is consistent with rapid cladogenesis within this group and may be partly responsible for the difficulty in producing a well supported morphologically based phylogeny (Heyer and Liem, 1976; Farris et al., 1982). Here we base our discussion on the results dependent on the tree in Fig. 5. The results of dating analyses performed using alternative sub-optimal resolutions of the basal nodes were not significantly different from the results in Table 3.

The divergence time estimate of *H. australiacus* from the western clade at 25.60 mya (17.52–42.40 mya) is considerably older than the 5 mya estimate in Maxson and Roberts (1984). The range of this estimate was designed to be liberal and to incorporate a large part of the uncertainty around the dating process. Despite this, the range of dates for the basal split in *Heleioporus* excludes both previous estimates of Pleistocene origin (Main et al., 1958) or 5 mya (Maxson and Roberts, 1984). This finding rejects the hypothesis that this group was involved in Late Miocene or subsequent interchange of mesic biotas of the southeast and southwest. The results suggest that the eastern and western lineages of *Heleioporus* separated in the Mid-Late Oligocene and that no significant genetic exchange has occurred since this time.

Empirical estimates of divergence times between taxa in the mesic temperate biotas of the southeast and southwest are relatively few. The recent addition of non-clock dating methods has increased the number of molecular studies addressing this issue of timing splits between the southeast and southwest temperate biomes. The dates from these studies are varied, but many appear to suggest splits during the Miocene (20–5 mya), with most concentrating around the 12–20 mya range. Using an albumin molecular clock, Roberts and Maxson (1985) found a number of frog species pairs originating in the Miocene between 5 and 17 mya and suggested that repeated marine incursions into the Eucla basin over the last 23 my could have split previously continuously distributed populations into eastern and western species. Major east–west splits of Miocene age have also been postulated for crayfish (10–19 mya, Munasinghe et al., 2004), and within two pygopodid lizard genera (17–23 and 12–16 mya, Jennings et al., 2003), while multiple episodes of east–west contact and separation throughout the Oligocene to Mid Miocene have been suggested for a number of plant families (Crisp et al., 2004).

The last 30 my has seen major climatic changes in Australia. The late Eocene and Early Oligocene (ca. 30–25 mya) saw the final rifting of continental Australia from Antarctica and the opening of the Southern Ocean as Australia moved northwards (Frakes, 1999). This major geological event precipitated a period of rapid cooling across the continent from ca. 28–20 mya. In southwest and southeast Australia this rifting also triggered a climatic change from an aseasonal-wet biome to a more seasonal temperate one. The Miocene (23–5 mya) saw a gradual increase in aridity across central and southern Australia and possibly bursts of very arid conditions (Frakes, 1999). It is probable that southwestern Australia has been isolated for at least the last 30 mya, both by the increasing aridification of central Australia throughout the Miocene and the formation of the Nullabor Plain by repeated marine incursions (41–30 mya and 23–20 mya, Hopper and Gioia, 2004). In combination these potentially provided either marine or edaphic barriers to dispersal from across southern Australia throughout the last 25 my. Although the range of our date estimate is such that we cannot realistically rule out an early Miocene divergence in *Heleioporus*, it seems more reasonable that the separation is of Oligocene age. The estimated divergence at ca. 25 mya notably coincides with two other estimates. Using the albumin clock Roberts and Maxson (1985) estimated the divergence date of one pair of frog sister species, *Geocrinia leai* and *G. victoriana* at around 25–27 mya. In addition, using a similar dating method to that employed in our current paper, Crisp et al. (2004) showed that the last trans-Nullabor split within the *Banksia* genus occurred around 25–30 mya. Other plant taxa studied including *Eucalyptus* and *Acacia* show evidence of multiple episodes of trans-Nullabor biotic exchange throughout the Miocene (Crisp et al., 2004). These latter taxa are ecologically more adapted to arid conditions than *Banksia*, and are all currently distributed across the arid northern boundary of the Nullabor, whereas *Banksia* is restricted to the wetter extremes of SWA and SEA similar to *Heleioporus*. *Banksia* exhibits similar ecological tolerances to *Heleioporus*, suggesting that the similar distribution of these taxa could have a common cause. The onset of aridity across southern Australia at around 30–25 mya seems to be a good candidate for the initial separation and maintenance of independent evolutionary lineages in SWA and SEA in both *Banksia* and *Heleioporus*, but it has proven to be less of a barrier to more arid-adapted taxa such as *Eucalypus* and *Acacia*. Subsequent studies of ecologically distinct genera with trans-Nullabor distributions should focus on the divergence dates.

This study has focused on the genus *Heleioporus* because this genus has been influential in formulating and testing theories of southern Australian biogeography. However, a number of other myobatrachid genera exhibit similar distributions in southeast and southwest Australia (Littlejohn, 1981; Roberts and Maxson, 1985) and exploring the pattern and timing of divergences in these taxa will be an important step towards increasing our understanding of the biogeography of southern Australia. This would be an effective strategy even in the absence of accurate absolute calibration points, as the relative timing of divergences and radiations would be of great interest to developing hypotheses of southwest Australian biogeography.

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