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Aridification drove repeated episodes of diversification between Australian biomes: Evidence from a multi-locus phylogeny of Australian toadlets (*Uperoleia*: Myobatrachidae)

Renee A. Catullo [a,b,*], J. Scott Keogh [a]

[a] Evolution, Ecology & Genetics, Research School of Biology, The Australian National University, Canberra, Australia
[b] CSIRO Land & Water, GPO Box 1700, Canberra, ACT 2601, Australia

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ABSTRACT

Australia is a large and complex landmass that comprises diverse biomes ranging from tropical rainforests to harsh deserts. While Australian biotic diversity has evolved in response to landscape and climate changes, evidence of Miocene or later biome shifts are few. The Australo-Papuan endemic frog genus *Uperoleia* is widely distributed across mesic, monsoonal tropic and arid regions of Australia. Thus, it represents an ideal system to evaluate biome shifts as they relate to known landscape and climate history. We comprehensively sampled the distributional range of 25 described *Uperoleia* species and generated a detailed molecular phylogeny for the genus based on one mitochondrial and five nuclear loci. Our results support a single origin of monsoonal tropic taxa, followed by diversification within the region under the influence of the Australian monsoon. Molecular dating analyses suggest the major divergence between eastern mesic and monsoonal species occurred in the Miocene approximately 17 million years ago, with repeated evolution of species from monsoonal biomes to arid or mesic biomes in the later Miocene, early Pliocene and at the beginning of the Pleistocene. Our detailed sampling helps to clarify the true distributions of species and contributes to on-going work to improve the taxonomy of the genus. Topological differences between nuclear and mitochondrial phylogenies within major clades suggest a history of mitochondrial introgression and capture, and reduce the ability to resolve close interspecific relationships.

1. Introduction

Biomes are communities of flora and fauna occupying a major habitat defined by broadly similar climate or landscape. Although terrestrial species are usually confined to one particular biome, the distribution of closely related species across biomes can reveal patterns of speciation as they relate to biome history (Coyne and Orr, 2004). Within biomes, phylogeographic studies have shown that past climatic fluctuations have driven diversification. In the Northern Hemisphere, Pleistocene glaciation drove lineages to refugia, where they subsequently diverged through allopatry and drift, or through adaptation to available environmental niches (Hewitt, 2004). In Australia, although there was little glaciation, extreme aridification linked to Pleistocene cycles also drove lineages into refugia (Catullo et al., 2013; Garrick et al., 2012; Pepper et al., 2011a), with similar patterns of subsequent diversification. Australia also developed extremely xeric biomes into which many species diversified, often during the less xeric interglacial periods (Chapple et al., 2011; Rabosky et al., 2007).

Modern-day Australia is divided into three major biome types – the monsoonal tropics, the arid zone, and the winter-rainfall mesic regions in the southwest and east (Fig. 1). The monsoonal region of Northern Australia (Fig. 1, green) is defined by having a summer wet season associated with cyclonic rainfall, and a dry winter season (Bowman et al., 2010). Although the monsoonal tropics have been defined as mesic in terms of total rainfall (Fujita et al., 2010), this biome differs substantially from other Australian mesic regions, which have winter rainfall. Australia has two independent winter-rainfall mesic regions (Fig. 1, purple & light gray). The eastern mesic zone has been defined in various ways; we follow Byrne et al. (2008) and Fujita et al. (2010) in including the semi-arid Murray–Darling basin, which receives substantially more rainfall than does the arid zone (Smith and Chandler, 2010). The south-western mesic zone is separated from the eastern mesic zone by the arid

* Corresponding author. Current address: CSIRO Land & Water, GPO Box 1700, Canberra, ACT 2601, Australia.
E-mail address: renee.catullo@anu.edu.au (R.A. Catullo).

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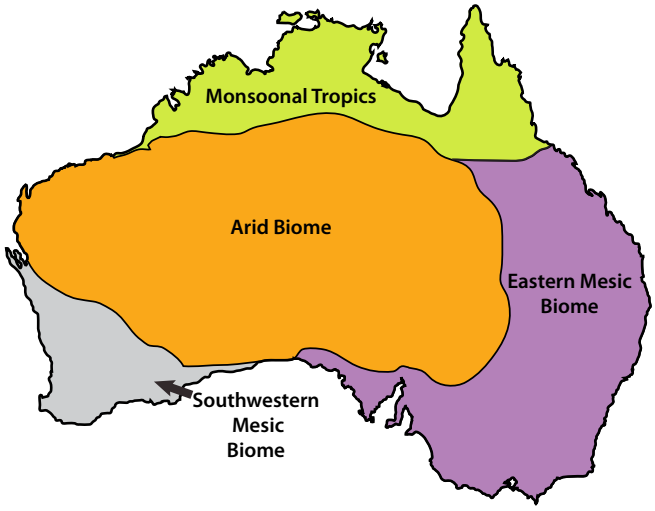

Fig. 1. Major biomes of the Australian mainland (colors). Modified from Byrne et al. (2008), Catullo et al. (2013), Chapple et al. (2011), Fujita et al. (2010), Potter et al. (2012).

deserts of Australia. The arid zone (Fig. 1, orange) is a massive xeric region occupying approximately 70% of the Australian landmass, and is characterized by intense aridity and highly variable rainfall (Byrne et al., 2008).

Recent reviews of historical biogeography within each of the biomes have been completed. Patterns of diversification within the arid zone have been extensively reviewed in Byrne et al. (2008) and Pepper et al. (2011a,b), with these studies demonstrating that mountains likely acted as refugia during hyper-arid climate cycles, playing a role similar to non-glaciated regions of the Northern Hemisphere in the generation of diversity. A general overview of diversification in mesic regions of Australia is provided in Byrne et al. (2011), while repeated patterns of diversification in the eastern mesic zone are reviewed in Chapple et al. (2011). The first reviews of broad-scale biogeographic structure in the Monsoonal Tropics were on *Heteronotia* geckos (Fujita et al., 2010), saxicolous rock wallabies (Potter et al., 2012) and alluvial *Uperoleia* frogs (Catullo et al., 2013). However, well-sampled studies of the patterns of diversification between biomes are rare.

Various lines of evidence suggest that Australian mesic environments are the ancestral environments for most Australian biota (reviewed in Byrne et al., 2011; Chapple et al., 2011). In the last 20 million years, Asian-origin taxa became a significant component of the monsoonal tropics region, likely through the increasing proximity of the Australian continent (reviewed in Bowman et al., 2010; Crisp and Cook, 2013). Studies of diversification of arid zone taxa have supported two patterns: a single, old origin of arid taxa followed by diversification, or recent and repeated evolution of arid tolerance (reviewed in Byrne et al., 2008). Fujita et al. (2010) reported multiple transitions between monsoonal and arid lineages in the gecko *Heteronotia binoei*, although lineages were not always found exclusively in a single biome. This study reconstructed a monsoonal origin for arid lineages, with transitions linked to cycles of aridification in the Pliocene and Pleistocene. *Heteronotia* entered Australia from Asia in the Miocene and does not occupy the mesic regions of Australia; thus, this study was unable to address the questions of relationships between all major Australian biomes, and was limited to the last 5 million years.

In this study we examine patterns of between-biome diversification in the frog genus *Uperoleia* (family Myobatrachidae). The Myobatrachidae has an ancient evolutionary history associated with the Australo-Papuan landmass that far pre-dates the current proximity of the Asian continent (Frost et al., 2006; Read et al., 2001). Biota that have existed on the continent since Gondwanan times are primarily distributed in the eastern or southwestern mesic regions (Crisp and Cook, 2013; Slatyer et al., 2007). However, most diversity in *Uperoleia* exists in the northern monsoonal tropics region of Australia, with a significant number of species in both the arid zone and eastern mesic region. Thus, *Uperoleia* species are ideally suited to examine of the role of climate and landscape on diversification in Gondwanan biota.

We use dense taxon sampling for all available species of *Uperoleia* and a large multi-locus data set to generate a robust molecular phylogeny for the genus based on multiple independent loci. We then use this phylogeny, and the timing of divergence events, to evaluate patterns of diversification between the three biomes inhabited by *Uperoleia*. Our specific goals were to (1) establish the evolutionary relationships between *Uperoleia* species; (2) identify the biome of origin of the *Uperoleia* genus; (3) examine the influence of climatic fluctuations on the evolution of arid distributed *Uperoleia*; and (4) determine whether tolerance of aridity evolved once or multiple times. In addition, our fine-scale sampling allows us to quantify phylogeographic structure within 25 of the 28 recognized *Uperoleia* species and to address problematic issues relating to taxonomy and species distributions.

2. Materials and methods

2.1. Field collection of specimens and tissue samples

We addressed three issues when choosing our specimens for genetic analysis and targeting additional fieldwork: (1) maximum geographic spread for each species; (2) possible contact zones between parapatric species; and (3) geographical gaps in existing collections. In total, we used 589 tissue samples representing 25 of the 28 described *Uperoleia* species. The three species not represented were *U. marmorata* (not seen since 1841), *U. orientalis* (not seen since the early 1900s, (Tyler et al., 1981a)), and *U. arenicola* (not collected since 1978, and we were unsuccessful in recent field trips to find it). *Spicospina flammocaerulea* was used as the outgroup in all analyses as it represents the monotypic sister genus to *Uperoleia* (Frost et al., 2006; Read et al., 2001). Specimen data are provided in Appendix 1, and distribution maps of tissues are illustrated in Fig. 2. Data points in Fig. 2 represent the individuals in the mitochondrial phylogeny, except where the specimen carries a different nuclear DNA haplotype, as discussed below. We were unable to acquire or map explicit location details for museum tissues numbered Up0816-824 (*U. aspera* & *U. mjobergi*), or Up765-770 (*U. inundata*) but included the tissues in our genetic analyses to compensate for the low total number of available samples, especially for *U. aspera* and *U. mjobergi*.

2.2. DNA extraction, amplification, and sequencing

For all 589 samples, including the outgroup species *S. flammocaerulea*, we generated a 783 base pair (bp) *16S* data set to obtain an estimate of the number of species lineages in the genus. We selected 294 of the samples as representatives of every *16S* clade for additional sequencing for five nuclear exons (Table 1). Of the 589 samples included in this study, 342 individuals have not been included in any previous studies of the genus.

Genomic DNA was isolated using Proteinase K digestion and a modified sodium acetate extraction. Table 1 lists the oligonucleotide primer pairs used in PCR amplification and sequencing, as well as PCR amplification protocols and gene length. PCR products were purified using EXOSAP-IT (Affymetrix, Inc.). BIGDYE TERMINATOR 3.1 (Applied Biosystems) was used for cycle sequencing, and capillary electrophoresis was completed on an ABI 3130XL GENETIC ANALYZER

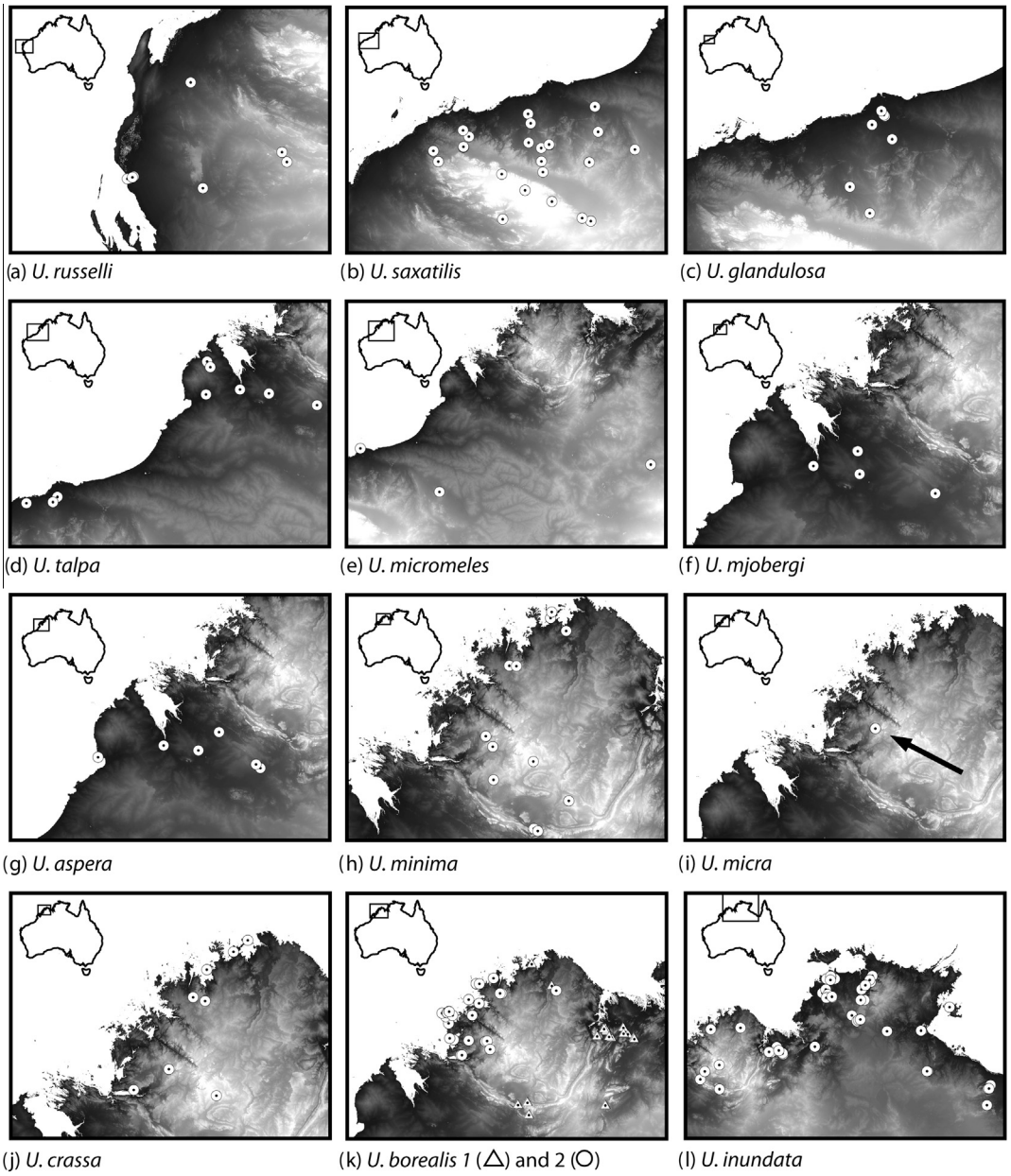

Fig. 2. Distribution map of the samples sequenced for 24 species of the *Uperoleia* genus. Samples are listed by mitochondrial clade placement, except in h, m, o, p, and s, where samples are assigned to species as determined in Catullo et al. (2013). If an individual was placed in separate mitochondrial and nuclear clades, it is shown as a member of the nuclear clade (see Appendix). Species are projected onto a digital elevation model, with low to high elevation gradients ranging from black to white.

(Applied Biosystems). Sequences were assembled in SEQUENCER 4.10.1 (Gene Codes Corporation), and all coding regions were checked for stop codons. Where individuals were found to carry mitochondrial haplotypes that did not match the species designation of their nuclear haplotype, laboratory processes were repeated to confirm the results.

2.3. Phylogenetic analyses of mitochondrial and nuclear data sets

Alignments were created using the MUSCLE algorithm (Edgar, 2004) in EBIOX (www.ebioinformatics.org) and then manually adjusted. Phylogenetic trees were estimated independently for the mitochondrial and concatenated nuclear datasets. Bayesian inference (BI) was performed using MRBAYES 3.0.4b (Ronquist and Huelsenbeck, 2003) on the freely available Bioportal server (www.bioportal.uio.no; (Kumar et al., 2009). Partitioning schemes and models for MRBAYES were implemented based on the program PARTITIONFINDER (Lanfear et al., 2012) and were selected using the lowest BIC score from the set of available models for each program. Our partitions and models were: *16S* by gene using GTR + I + Γ, the first codon position for *A2AB*, *BDNF*, *NTF3*, and *RAG1* using HKY + I + Γ, all second codon positions plus the first codon position for *BMP2* using HKY + I; the third codon position for *A2AB* using GTR + Γ; the third codon positions for *BDNF*, *BMP2*, and *NTF3* using K80 + I + Γ; and the third codon position for *RAG1* using HKY + I + Γ. We ran each dataset with four chains for 50,000,000 generations with a sample frequency of 1000 generations. The first 20% of the run was discarded as burnin. Convergence of parameter values was assessed using the program TRACER v1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Edges with posterior probabilities of 0.90 were considered significant.

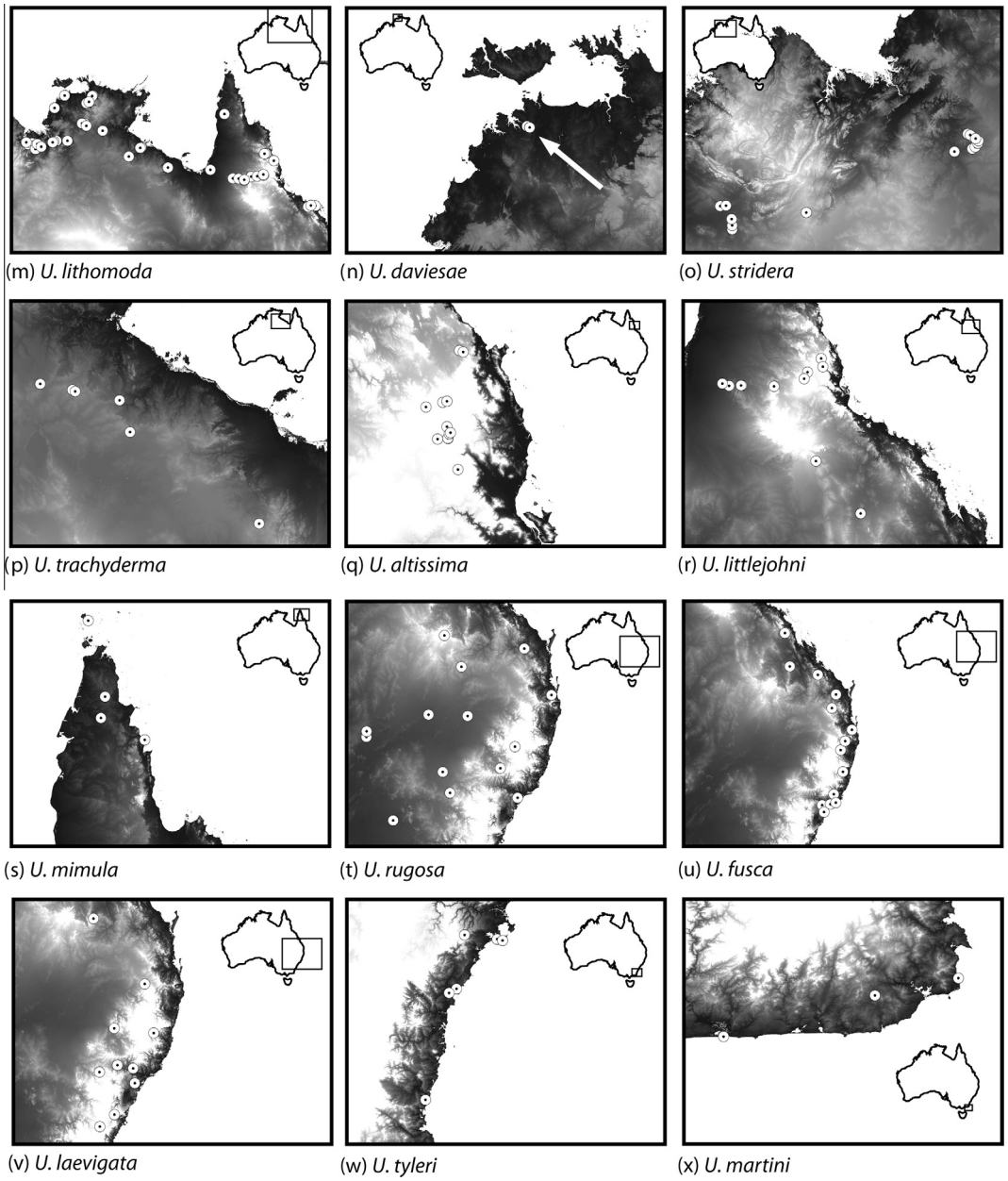

Fig. 2 (*continued*)

We inferred phylogenetic relationships under maximum likelihood (ML) using the thorough bootstrapping algorithm in RAXML-VI-HPC (Stamatakis, 2006), using the same partitioning schemes as the BI analyses and the GTRGAMMA substitution model. Default parameters were used, with a total of 1000 bootstrap replicates. Edges with bootstrap values of 70 were considered significant.

Species were categorized as mesic, monsoonal, or arid on the basis of their mitochondrial clade distribution being within biome boundaries as defined in Fig. 1. If an individual carried nDNA of one species and a mtDNA haplotype of another species, it was considered a member of the nDNA species.

2.4. Species tree and divergence time estimates based on nuclear loci

We used the species assignments from our nuclear BI analyses as input data for species tree estimation. Due to the different tree topologies between the mitochondrial and nuclear data sets and extensive mitochondrial introgression (see results and also see Catullo et al., 2013), we excluded the mitochondrial data from our species tree assessment. We also removed any potential hybrid individuals (individuals that were assigned to one mitochondrial clade and different species' nuclear clade, see Figs. 3 and 4). For some species we had a large number of individuals, so to reduce computational time we trimmed clade representation to nine individuals per species in the well-sampled clades (see Appendix 1).

The program *BEAST v1.7 (Heled and Drummond, 2010) was used to estimate the species tree. As our species tree was based on slowly evolving nuclear exons, and additional partitioning can have substantial effects on the clock models (Simon Ho, per. comm.), we used a minimal partitioning approach. Partitioning was set by gene, with a single substitution model (*A2AB*: K80 + G; *BDNF*: K80 + I; *BMP2*: HKY + G; *NTF3*: HKY + I + G; *RAG1*: TrN + I + G) as selected under the BIC by MODELGENERATOR (Keane et al., 2006), and an individual strict clock. The divergence between *Uperoleia* and *S. flammocaerulea* was assigned a secondary calibration (28.82 Mya, normal distribution, standard deviation of 4.2653)

Table 1
Marker information used in this study.

Region	Primers	Sequence (5′–3′)	PCR profile	Primer source
Ribosomal large sub-unit (*16S*)	L16Sc H16Sd	GTRGGCCTAAAAGCAGCCAC CTCCGGTCTGAACTCAGATCAC	(94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min) × 30	Modified from Evans et al. (2003)
Alpha 2B adrenergic receptor (*A2AB*)	A2AB-F10 A2AB-R3	CATGCCMTTYTCYTTGGCMAACG CCCNGGYTTGATCTGRCTCAG	(95 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 54 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 53 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 52 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 51 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 50 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 49 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 48 °C for 15 s, 72 °C for 30 s) × 30	Modified from Funk et al. (2012)
Neurotrophin-3 (*NTF3*)	NTF3-F3 NTF3-R3	TCTTCCTTATCTTTGTGGCATCCACGCTA ACATTGRGAATTCCAGTGTTTGTCGTCA	(95 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 54 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 53 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 52 °C for 15 s, 72 °C for 30 s) × 30	Funk et al. (2012)
Bone morphogenetic protein 2 (*BMP2*)	BMP2-F7 BMP2-R2	AGACTATTGGACACCAGACTGGTACATCA CGRCACCCRCARCCCTCCACAACCA	(95 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 54 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 53 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 52 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 51 °C for 15 s, 72 °C for 30 s) × 2, (95 °C for 15 s, 50 °C for 15 s, 72 °C for 30 s) × 30	Modified from Townsend et al. (2008)
Brain-derived neurotrophic factor (*BNDF*)	BDNF-Fmb BDNF-Rmb	GACCATCCTTTTCCTKACTATGGTTATTTCATACTT CTATCTTCCCCTTTTAATGGTCAGTGTACAAAC		Modified from van der Meijden et al. (2007)
Recombination activating gene 1 (*RAG-1*)	AMP-Rag1F AMP-Rag1R1	AGCTGCAGYCARTACCAYAARATGTA AACTCAGCTGCATTKCCAATRTCACA		San Mauro (2010)

based on a multi-locus dated molecular phylogeny for Australian myobatrachid frogs with 12 calibration points (Keogh et al., unpublished data). The tmrca for *Uperoleia* was given a maximum constraint of 30 Mya. Three independent runs were executed in *BEAST, with each run consisting of 500,000,000 generations sampling every 5000 generations. The first 20% of trees were discarded as burnin. Convergence of parameter values was assessed using the program TRACER v1.5 (http://tree.bio.ed.ac.uk/software/tracer). Resulting tree and log files from each run were combined using the program LOGCOMBINER v1.7 (http://beast.bio.ed.ac.uk/Logcombiner).

3. Results

3.1. Sequencing

The nuclear alignment comprised 3093 characters, and coverage was nearly complete for all genes (*A2AB* – 98.6%; *BDNF* – 100%; *BMP2* – 95.6%; *NTF3* – 99.7%; *RAG1* – 100%). No individual was missing more than a single gene, and genes amplified for all species with the exception of *BMP2*, which would not amplify for *U. mjobergi* or the outgroup. All individuals sequenced for nuclear markers were one of the 589 individuals sequenced for *16S*.

3.2. Nuclear DNA

The concatenated nuclear MrBayes analysis recovered four well-supported species groups within the genus (Fig. 3). Nuclear Clade 1 (Fig. 3, Bayesian posterior probability (PP) group PP = 1) comprises *U. fusca*, *U. laevigata*, *U. tyleri* and *U. martini*. This species group is strongly supported (PP = 1). Crown group support for individual species was variable, with *U. fusca* strongly supported (PP = 0.93), *U. laevigata* weakly supported (PP = 0.85), and *U. tyleri* and *U. martini* forming a well-supported (PP = 1) clade but not being individually monophyletic.

Nuclear Clade 2 (Fig. 3, group PP = 1) comprises the species *U. micromeles* and *U. mjobergi*. *Uperoleia micromeles* forms a well-supported monophyletic group nested within *U. mjobergi* (PP = 0.99).

Nuclear Clade 3 was not well-supported (Fig. 3, group PP = 0.80), but comprises the "squelching" *Uperoleia*, which all have an elongated call with many pulses (R. Catullo, unpub. data). The species in this clade (with crown support) include *U. micra* (PP = 1), *U. daviesae* (PP = 1), *U. saxatilis* (PP = 1), *U. russelli* (PP = 0.99), *U. talpa* (PP = 1), two distinct clades of *U. borealis* (*U. borealis 1* [PP = 0.95], *U. borealis 2* [PP = 0.95]), and a clade comprising the species *U. inundata* and *U. crassa* (PP = 0.98). Except for the sister species relationship between *U. saxatilis* and *U. russelli*, interspecific relationships within this clade are not well resolved.

Nuclear Clade 4 (Fig. 3, group PP = 1) comprises the "clicking" *Uperoleia*, which all have a short, sharp call (R. Catullo, unpub. data). Within this clade there is an additional well-supported divergence, with all the species present in Cape York forming a clade (Clade 4 east, PP = 0.99) and all the western species forming another (Clade 4 west, PP = 1). Clade 4 east comprises the species *U. altissima* (PP = 1), *U. littlejohni* (PP = 1), *U. lithomoda* and *U. mimula*. *Uperoleia mimula* forms a well-supported clade (PP = 1) nested within *U. lithomoda* (PP = 1) but is divergent in call (Catullo et al., 2013). Clade 4 west comprises the species (with crown support) *U. aspera* (PP = 1), *U. glandulosa* (PP = 1), *U. minima* (PP = 0.99), *U. rugosa* (PP = 1), *U. stridera* and *U. trachyderma*. *Uperoleia trachyderma* forms a well-supported clade (PP = 1) within *U. stridera* (PP = 0.94). The extensive mitochondrial introgression and topological differences, unless discussed below, do not influence our primary conclusions.

3.3. Mitochondrial gene tree

The mitochondrial Bayesian analysis generally recovered the same four well-supported species groups within the genus, with the exception of the placement of *U. micra*, and clarified the number of lineages within species and their distributions (Fig. 4). However, we recovered different clade relationships between groups, with clade 2 being the sister group to clade 3 in the mtDNA phylogeny (PP = 1) instead of as an outgroup to both clades 3 and 4, as in the nDNA phylogeny.

Mitochondrial Clade 1 (Fig. 4) comprises *U. fusca*, *U. laevigata*, *U. tyleri* and *U martini*, similar to the nDNA phylogeny. However, the

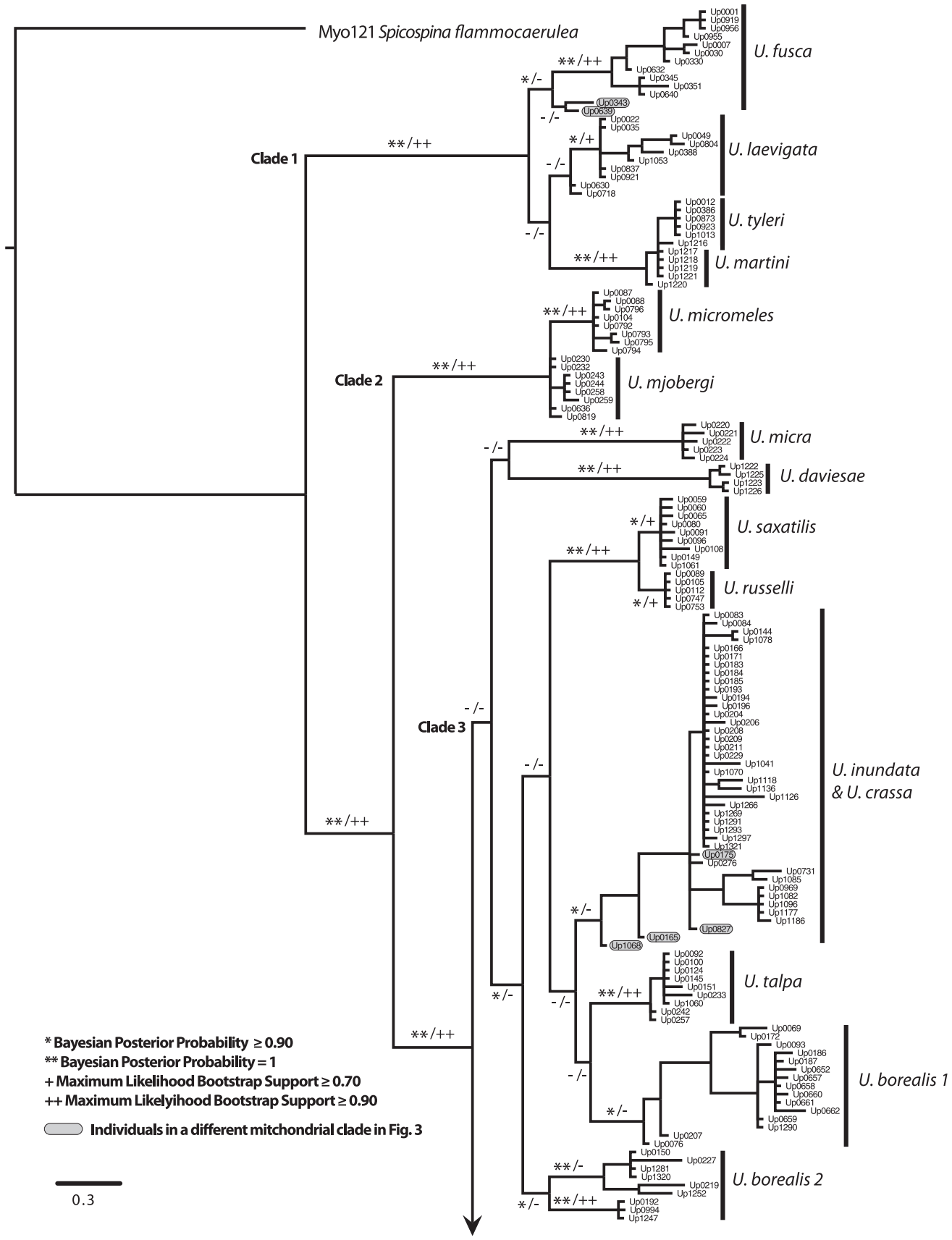

Fig. 3. Concatenated nuclear phylogeny, as inferred by Mr.Bayes 3.0.4.b. Bayesian posterior probabilities and maximum likelihood bootstrap supports are shown on branches. Individuals highlighted in grey were assigned to a different mitochondrial clade in Fig. 3.

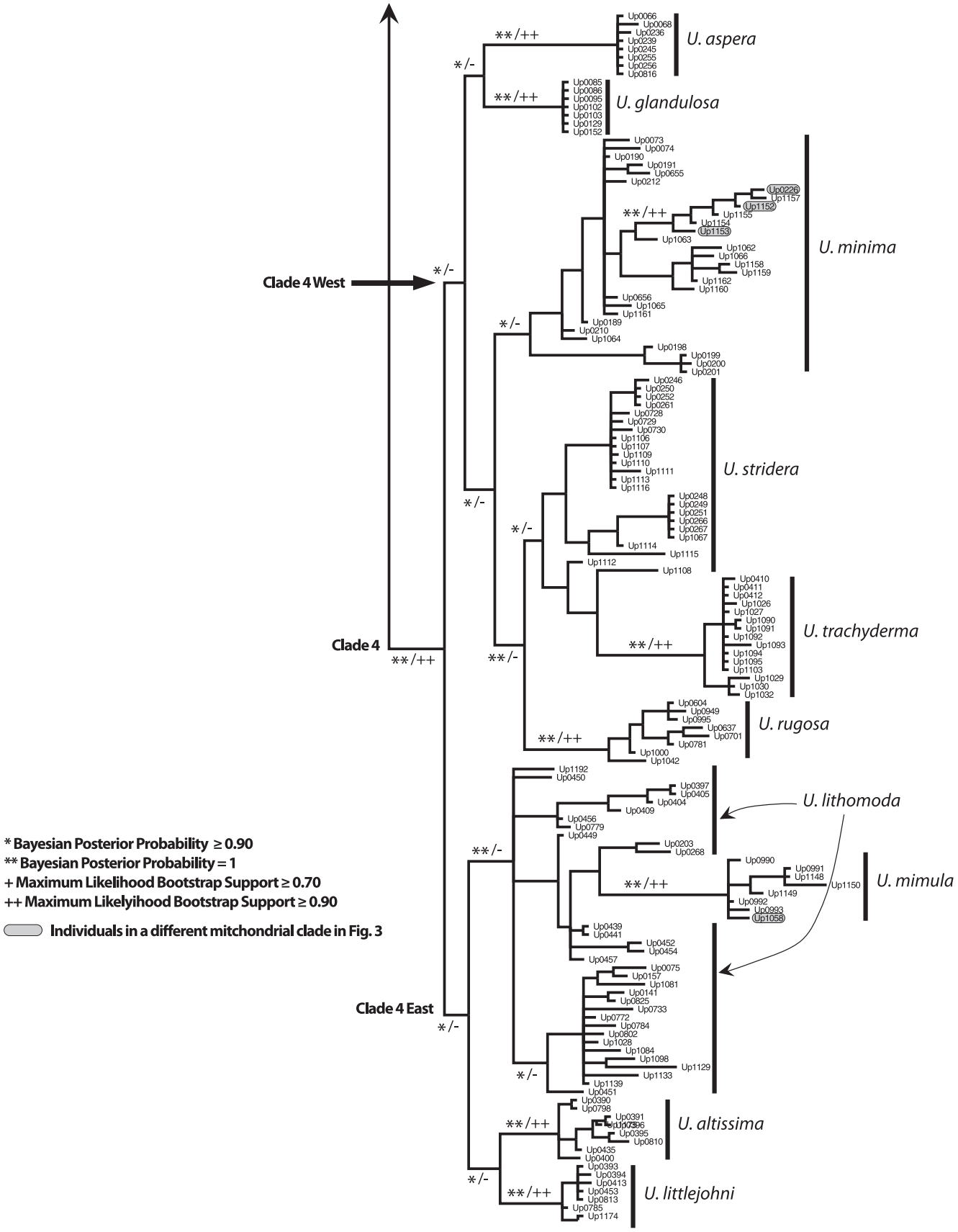

Fig. 3 (*continued*)

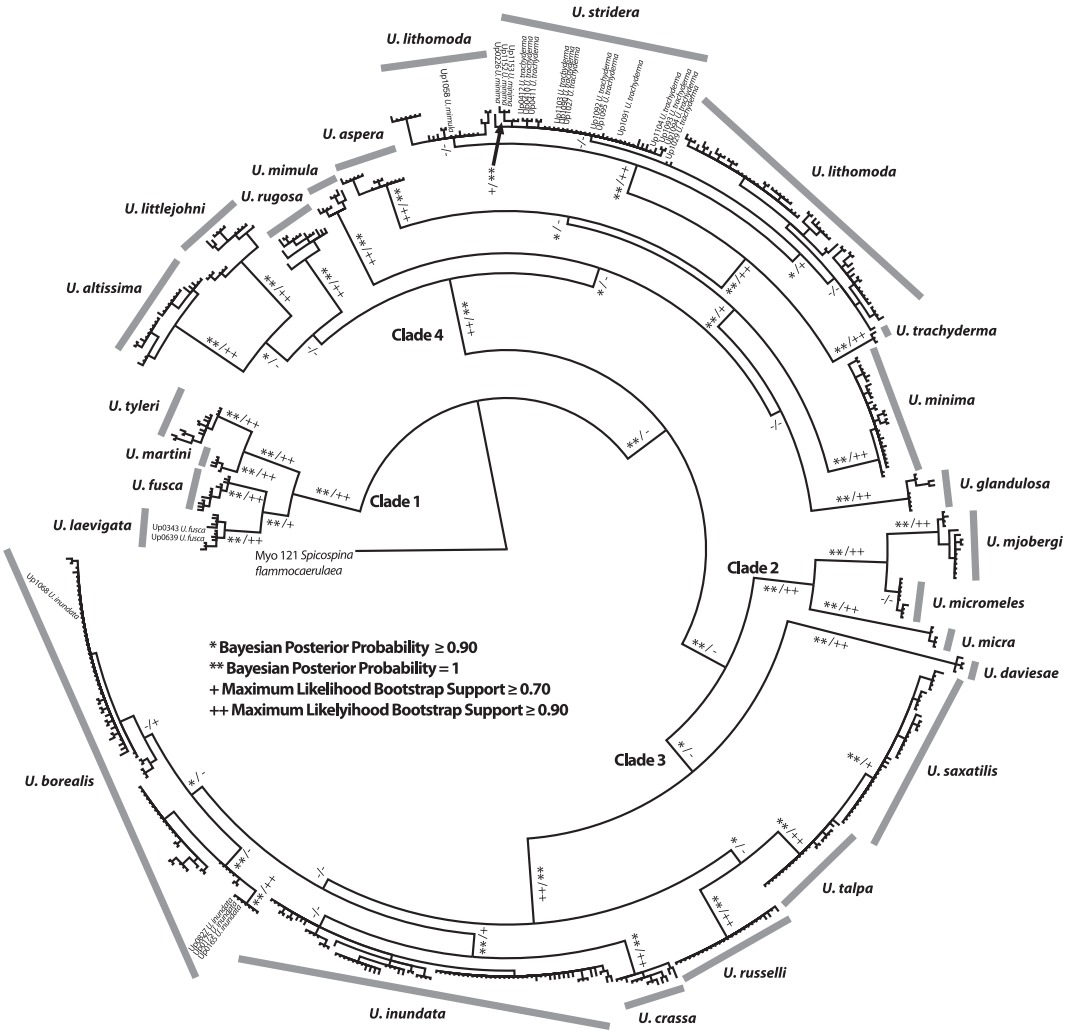

Fig. 4. Mitochondrial (*16S*) tree, as inferred by Mr.Bayes 3.0.4.b. Bayesian posterior probabilities and maximum likelihood bootstrap supports are shown on branches. Individuals with names are assigned to a different species in the nuclear phylogeny, and names indicate the final nuclear clade of that sample.

mtDNA phylogeny strongly supports (PP = 0.99) *U. laevigata* as the sister group to *U. fusca,* whereas it is poorly supported as the sister group to the *U. tyleri/U. martini* clade in the nDNA phylogeny. The species relationships within the group and crown support for each species were all strongly supported (PP > 0.99). Two nDNA *U. fusca* (Up0343 and Up0639) are strongly supported as having a *U. laevigata* mtDNA haplotype (PP = 1).

Mitochondrial Clade 2 comprises *U. micromeles*, *U. mjobergi*, and *U. micra* (Fig. 4). All species relationships within this group and crown support for each species were strongly supported (PP = 1). However, *U. micra* was strongly placed (PP = 1) within combined Clades 3 and 4 in the nDNA phylogeny.

Mitochondrial Clade 3 (Fig. 4, group PP = 0.90) comprises *U. daviesae* (PP = 1), *U. saxatilis* (PP = 1), *U. talpa* (which is paraphyletic to *U. saxatilis*, PP = 1), *U. russelli* (PP = 1), a well supported clade (PP = 0.94) with three distinct clades of *U. borealis* (PP = 1 for *U. borealis* 1, PP = 0.97 for *U. borealis* 2, and PP = 1 for *U. borealis* 3), *U. crassa* (PP = 1), and *U. inundata* (PP = 1). Unlike the nDNA phylogeny, *U. talpa* is strongly supported (PP = 1) as the sister species to *U. saxatilis*.

Four *U. inundata* nDNA individuals carry *U. borealis* mtDNA haplotypes (Up0165, Up0175, Up0827, and Up1068). The two nDNA clades of *U. borealis* form a well-supported clade in the mtDNA phylogeny (PP = 0.9419). This clade was not found the nDNA phylogeny.

Mitochondrial Clade 4 (Fig. 4, group PP = 1) comprises *U. mimula* (PP = 1), *U. glandulosa* (PP = 1), *U. aspera* (PP = 1), *U. rugosa* (PP = 1), *U. altissima* (PP = 1), and *U. littlejohni* (PP = 1). Also included in this group are *U. mimula*, *U. lithomoda*, *U. trachyderma*, and *U. stridera*, a species complex covered in detail with morphological and acoustic data in Catullo et al. (2013). Within this group, *U. lithomoda* comprises two clades (PP = 0.67, PP = 0.84) and is here paraphyletic with respect to the *U. stridera* mitochondrial haplotypes (PP = 0.92). However, *U. lithomoda* and *U. stridera* form well-supported sister clades when the mtDNA gene ND2 is included (Catullo et al., 2013). *Uperoleia stridera* haplotypes are present in most individuals of *U. trachyderma* (Catullo et al., 2013) and three individuals of *U. minima*. The remaining *U. trachyderma* and *U. minima* mitochondrial haplotypes form strongly supported clades (PP = 1). One nDNA *U. mimula* (Up1058) carries a *U. lithomoda* mtDNA haplotype (PP = 1).

3.4. Biome assignment of species

All species, with the exception of *U. talpa*, were clearly distributed within a single biome as defined in Fig. 1. Four species were distributed solely within the arid zone (Fig. 2a–c and e). *Uperoleia talpa* (Fig. 2d) is a primarily monsoonal species, but is also present along the coastline on the edge of the arid zone. Due to this coastal

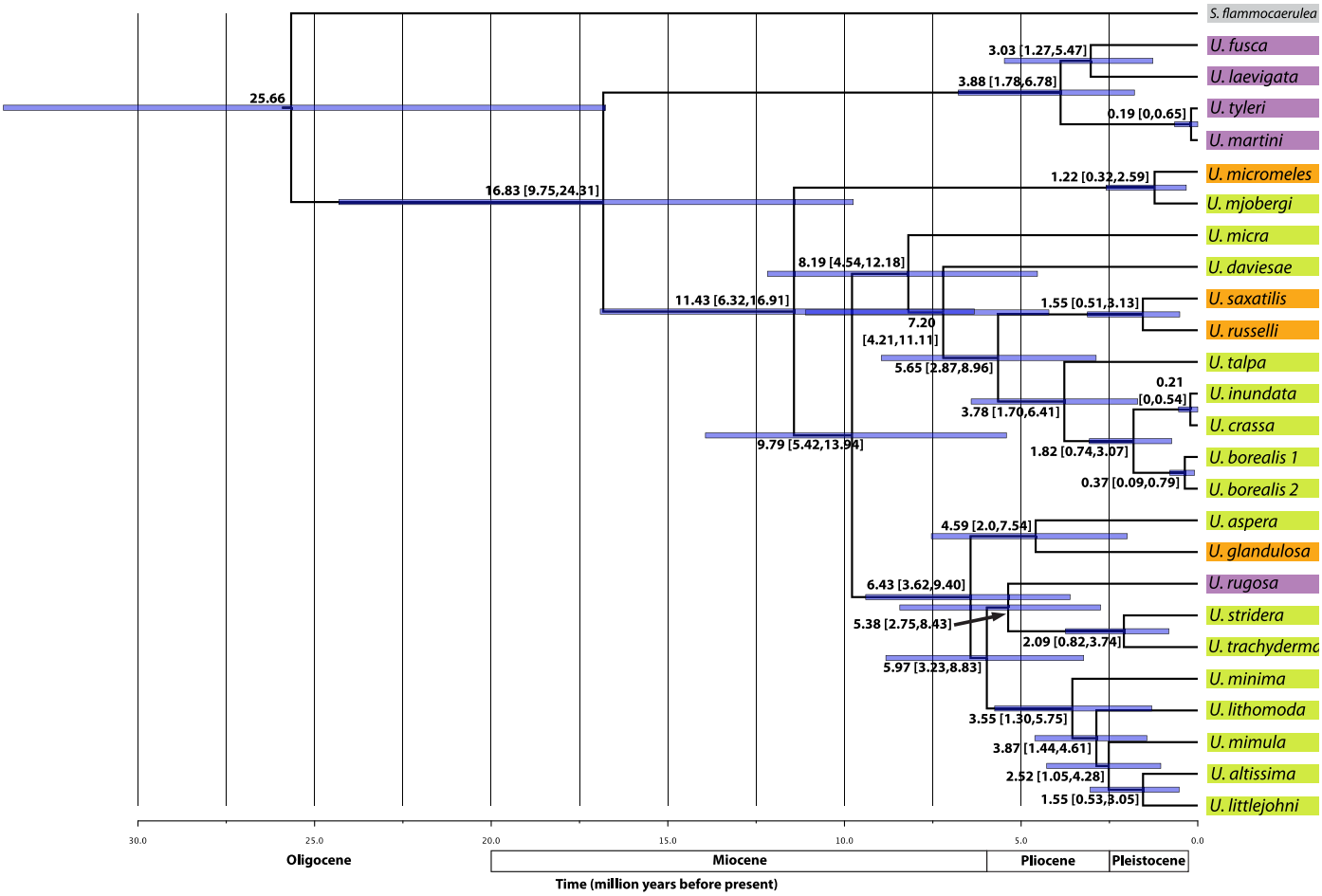

Fig. 5. Chronogram inferred using strict molecular clock in *BEAST version 1.7, using five nuclear exons. Numbers indicates age of node (mean[95%CI]). Nodal bars and brackets are 95% confidence intervals. Color of boxes indicates biome of species (gray = southwestern mesic, purple = eastern mesic, orange = arid zone, green = monsoonal tropics).

restriction within the arid zone, we consider this species to be primarily monsoonal. Fourteen species are found entirely within the monsoonal tropics biome (Fig. 2f–s). Five species are found in the eastern mesic region (Fig. 2s–x).

3.5. Species tree analysis and estimates of divergence times

The phylogenetic placement of species within the *BEAST analysis (Fig. 5) was largely congruent with the concatenated nuclear MRBAYES phylogeny, although posterior probabilities were generally lower than in the concatenated nuclear analyses. While the nuclear species tree and mitochondrial gene tree topologies are not identical, few of the changes influence the interpretation of biome-level divergences between species, as introgression generally appears to have happened within clades distributed in a single biome. The primary exception is *U. talpa,* which we interpret as having recently captured the *U. saxatilis* mtDNA haplotype and therefore consider the nDNA species tree to represent the accurate evolutionary relationship.

The estimated age of divergence between mesic Clade 1 and all other species is the mid-Miocene: 16.83 Mya (95% CI 9.75–24.31), while the age of divergence among the mesic species in Clade 1 is estimated in the mid-Pliocene (3.88 Mya [95% CI 1.78–6.78]). The age of divergence between Clade 2 and Clades 3 & 4 is estimated to have occurred during the mid-Miocene (11.43 Mya [95% CI 6.32–16.91]), while the divergence between Clades 3 & 4 occurred later in the Miocene (9.79 Mya [95% CI 5.42–13.94]).

The *U. glandulosa* (arid) and *U. aspera* (monsoonal) divergence is estimated at 4.59 Mya [95% CI 2.0–7.54], while the divergence between *U. rugosa* (mesic) and *U. trachyderma/U. stridera* (monsoonal) is estimated at 5.38 Mya [95% CI 2.75–8.43]. The divergence between *U. russelli/U. saxatilis* (arid) and U. *borealis 1 & 2/ U. crassa/U. inundata/U. crassa* (monsoonal) is estimated at 5.65 Mya [95% CI 2.87–8.96]. The age of divergence between *U. micromeles* (arid) and *U. mjobergi* (monsoonal) is estimated at 1.22 Mya [95% CI 0.32–2.59], and the *U. russelli* (arid) and *U. saxatilis* (arid) divergence is estimated at 1.55 Mya [95% CI 0.51–3.13].

4. Discussion

Our comprehensive and multi-locus nuclear species tree phylogeny for the genus *Uperoleia* provides a robust hypothesis of relationships among the species and a framework for evaluating biogeographic structure and the evolution of biome shifts in the group. Strong within-biome biogeographic structure was apparent in our nuclear and mitochondrial phylogenies. Despite the distribution of individual species within a single biome, we did not find that all the species within a biome formed a monophyletic group, illustrating the need for wide-scale sampling even when reconstructing evolutionary history within a single region. Various hypotheses regarding the origin and distribution of mesic, monsoonal, and arid zone taxa have been proposed previously, and below we review these hypotheses against our results.

4.1. Multi-locus phylogeny of Uperoleia

Our comprehensive sampling across the distributional range of 25 species has provided a means to identify species (which are otherwise cryptic) and their current distributions (Fig. 2). We supplemented the mitochondrial data with five independent nuclear genes for representatives of all major mitochondrial clades to produce a multi-locus species tree phylogeny for the genus. The genus comprises two major well-supported clades: eastern mesic (Figs. 3 and 4, Clade 1) *versus* all other *Uperoleia* species (Fig. 4, Clades 2, 3 & 4). Species in the second group are primarily distributed in the monsoonal tropics, but a single mesic species and a number of arid species also are present in the group (Figs. 2 and 5).

Our results indicate that, despite extensive morphological conservatism within the genus, the current morphology-based taxonomy and our molecular phylogeny are remarkably congruent (see Davies et al., 1986; Tyler et al., 1981a,b). Of the 25 sequenced taxa, most were recovered as well-supported clades (PP < 0.95) in our mitochondrial phylogeny, with a few exceptions. The *U. micromeles* group was poorly supported in the single-locus 16S data set, but this morphologically and ecologically distinct species forms a well-supported clade when the mitochondrial gene ND2 is included (Catullo et al., 2011). The only sequenced species for which we found no genetic support was *U. capitulata*. All specimens identified as *U. capitulata* grouped with *U. rugosa*, a species from which *U. capitulata* was split on the basis of gland enlargement and a single skull characteristic (Davies et al., 1986). We here synonymise *U. capitulata* with *U. rugosa*. All samples previously identified as *U. capitulata* are shown as *U. rugosa* in Fig. 2t.

The clade incorporating the species *U. borealis*, *U. crassa* and *U. inundata* appears to have diverged recently, and support for inter-species relationships is low. A number of *U. inundata* individuals appear to carry *U. borealis* mitochondrial haplotypes. *Uperoleia borealis* forms a clade in the mitochondrial phylogeny and species tree analyses (*U. borealis* 1 & *U. borealis* 2), but not sister clades in the concatenated nuclear phylogeny (Fig. 3). However, given the poor support for interspecific relationships in Clade 3 of the concatenated nDNA phylogeny, additional molecular data are needed to test the monophyly of *U. borealis*. *Uperoleia crassa* forms a monophyletic group in the mitochondrial phylogeny, although it is not divergent from *U. inundata* in either of the nuclear phylogenies. While they are sympatric, we suggest that further investigation with additional genetic markers and acoustic data is required to resolve their taxonomic status.

Repeated mitochondrial capture of the *U. stridera* haplotype is suggested by our data. In the concatenated nuclear phylogeny, *U. lithomoda* is well supported as a member of Clade 4 east, which comprises all species present on the Cape York peninsula (Fig. 3). In the mitochondrial phylogeny, *U. lithomoda* is paraphyletic with respect to *U. stridera* and is a member of Clade 4 west (Fig. 4; Catullo et al., 2013). In addition, most individuals of *U. trachyderma* carry a *U. stridera* mitochondrial haplotype. The *U. stridera* mitochondrial haplotype also occurs in three individuals of *U. minima*. These species have been analyzed morphologically and acoustically (Catullo et al., 2013), and recent taxonomic revisions show them to be well-supported species congruent with their nuclear haplotypes (Catullo et al., 2013, 2014). These data suggest that the *U. stridera* mitochondria have been captured by *U. lithomoda*, that a probable mitochondrial capture of the *U. stridera* mitochondria is in progress by *U. trachyderma*, and this has likely failed within *U. minima* (Catullo et al., 2013).

Our data also suggest mitochondrial capture of the *U. saxatilis* mitochondrial haplotype by *U. talpa*. *Uperoleia talpa* forms a well-supported monophyletic group on a relatively long branch within Clade 3 of both nuclear phylogenies (Figs. 3 and 5). The sister species relationship of *U. saxatilis* and *U. russelli* is also well-supported in both nuclear phylogenies. However, in the mitochondrial phylogeny, *U. talpa* is paraphyletic with respect to *U. saxatilis* while *U. russelli* remains the well-supported sister group to this clade. Taxonomic revision of these species shows clear morphological and acoustic differences (Catullo et al., 2011).

The relative causes of mitochondrial capture within the group are not yet understood, and require further investigation. Incomplete lineage sorting provides a possible hypothesis; however, the relatively high phylogenetic distance between species and their close geographic proximity suggest that hypotheses involving the post-divergence transfer of mitochondria are more likely, with stochastic factors following hybridization a possible cause. Mitochondrial haplotypes have also been shown to have energetic effects in migratory birds (Toews et al., 2014). Similar benefits may be associated with acoustic breeding displays of anurans, although this hypothesis is yet to be tested.

4.2. What is the origin of the monsoonal tropics Uperoleia?

Two hypotheses were proposed by Bowman et al. (2010) regarding the origin of monsoonal tropics taxa: first, that they represent recent arrivals from Asia that crossed Wallace's line, or alternatively, that they represent ancient Gondwanan relicts that persisted in monsoonal refugia. Byrne et al. (2011) proposed a third hypothesis, that ancestors of Australian biota in general were adapted to mesic environments, and that climatic fluctuations in the Quaternary subsequently influenced their distributions and diversification. As Myobatrachid frogs are most closely related to South American taxa (Pyron and Wiens, 2011), and thus are an old Gondwanaland group, we can eliminate interactions with Asia as playing a major role in diversification. Thus we seek to understand whether *Uperoleia* persisted and diversified within the monsoonal tropics or diverged from mesic ancestors.

A long history on the southern mainland for the Myobatrachidae has been previously hypothesized, as an uneven proportion of species occur in the eastern and south-western mesic biome *versus* the monsoonal or arid biomes (Slatyer et al., 2007). The age of divergence between the two major monophyletic groups of *Uperoleia* (Clade 1 *versus* Clades 2, 3 & 4, Figs. 3 and 4) is associated with the peak moisture levels of the Miocene, a time of widespread forests and substantial rainfall in the center of the continent (reviewed in Bowman et al., 2010; Byrne et al., 2008). Given the primarily mesic distribution of myobatrachid frogs (Slatyer et al., 2007) and the distribution of all Clade 1 species in the eastern mesic region, our data suggest a mesic origin of *Uperoleia*. These clades share a common ancestor just prior to the gradual increases in aridity starting 12–10 Mya in the late Miocene (Byrne et al., 2008).

Our data do not support either of the Bowman et al. (2010) hypotheses, and instead support the Byrne et al. (2011) hypothesis: a single monsoonal origin in the late Miocene, as has been found in skinks and agamid lizards (Chapple and Keogh, 2004; Melville et al., 2011). The age of diversification between species in Clade 1 (3.88 Mya, Fig. 5) suggests that species-level divergence was associated with contraction into coastal refugia associated with the gradual drying of the Pliocene (reviewed in Byrne et al., 2011, 2008). This pattern of early Pliocene divergence has also been documented in the eastern mesic skink *Lampropholis* (Chapple et al., 2011). Mid- to late-Miocene aridification of central Australia has been implicated in the diversification of plants, marsupials, and various reptile groups (see Table 1 in Byrne et al., 2008; Melville et al., 2011).

4.3. Did climatic fluctuations dictate the evolution of arid-distributed Uperoleia, and did tolerance to aridity evolve once or multiple times?

The arid zone is a relatively new biome (<2.5 My, Byrne et al., 2008), and arid distributed taxa generally show one of two biogeographic patterns: a single arid origin followed by extensive diversification, or multiple independent derivations from mesic or monsoonal ancestors (reviewed in Byrne et al., 2008; Fujita et al., 2010). Our results on arid zone *Uperoleia* support the multiple independent derivations hypothesis, as we see several divergences between monsoonal and arid taxa within Clades 2, 3 and 4, as well a divergence between monsoonal taxa and *U. rugosa*, an eastern mesic species (Fig. 5). Despite evidence that the current dunefield deserts are young (<1 My, Fujioka et al., 2009), a number of studies have shown that the divergence time of arid taxa from mesic or monsoonal regions pre-dated the emergence of the dunefield deserts (Byrne et al., 2008). The diversification of *U. glandulosa*, the *U. russelli*/*U. saxatilis* clade (both arid), and *U. rugosa* (mesic) from monsoonal sister species all support this pattern, with the age of divergence in the late Miocene (approximately 6 Mya). This suggests that monsoonal *Uperoleia* spread into the inland regions during the wet, mild beginning of the Pliocene (Byrne et al., 2008), and independently adapted to changing conditions as aridification increased.

The arid species *U. micromeles* shows a much more recent divergence from its monsoonal sister species, as does the divergence between the sister arid species *U. saxatilis* and *U. russelli*. The age of these divergences coincides with the beginning of the Pleistocene and the generation of the extraordinarily xeric dunefield deserts (Fujioka et al., 2009). *Uperoleia micromeles* is distributed in the Great Sandy Desert, one of the most extreme arid habitats in Australia. Similarly, *U. russelli* is distributed in the sandy Gascoyne region, in stark contrast to the rocky Pilbara distribution of *U. saxatilis*. A number of studies have shown that the recent development of major sand dune systems in xeric regions has shaped diversification patterns (Melville et al., 2011; Pepper et al., 2011a,b; Scott et al., 2004; Sole et al., 2005). It is likely that *U. micromeles* and *U. russelli* adapted to the extensive aridity and unique geology of the Pleistocene dunefield deserts as they developed.

5. Conclusions

We provide the most complete molecular systematic study of the *Uperoleia* genus. Most described species were strongly supported by our molecular phylogenetic data, except for *U. capitulata,* which we synonymize with *U. rugosa*. Additional data are needed to address species boundaries within the *U. inundata* species group, and further work is required to investigate causes of extensive mitochondrial introgression and capture between species.

Our data suggest that monsoonal taxa diverged from mesic taxa during the aridification of the center of the Australian continent at the end of the Miocene, and subsequently diversified within the region. From these monsoonal ancestors, independent speciation events gave rise to arid adapted species. These events likely followed recolonization of central Australia during moist periods in the Pliocene and Pleistocene, with divergence from monsoonal relatives driven by the following arid periods. The lack of biome-level monophyly for any of our three target biomes illustrates the need for continent-wide representative sampling, even in biome-level studies of biogeographic history. Considerable phylogeographic structuring within the monsoonal tropics indicates a need for further investigations into the role refugia and past climate fluctuations played in the generation of diversity within the region.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.06.012.

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