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DIVERSIFICATION AND PERSISTENCE AT THE ARID–MONSOONAL INTERFACE: AUSTRALIA-WIDE BIOGEOGRAPHY OF THE BYNOE'S GECKO (*HETERONOTIA BINOEI*; GEKKONIDAE)

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Late Neogene aridification in the Southern Hemisphere caused contractions of mesic biota to refugia, similar to the patterns established by glaciation in the Northern Hemisphere, but these episodes also opened up new adaptive zones that spurred range expansion and diversification in arid-adapted lineages. To understand these dynamics, we present a multilocus (nine nuclear introns, one mitochondrial gene) phylogeographic analysis of the Bynoe's gecko (*Heteronotia binoei*), a widely distributed complex spanning the tropical monsoon, coastal woodland, and arid zone biomes in Australia. Bayesian phylogenetic analyses, estimates of divergence times, and demographic inferences revealed episodes of diversification in the Pliocene, especially in the tropical monsoon biome, and range expansions in the Pleistocene. Ancestral habitat reconstructions strongly support recent and independent invasions into the arid zone. Our study demonstrates the varied responses to aridification in Australia, including localized persistence of lineages in the tropical monsoonal biome, and repeated invasion of and expansion through newly available arid-zone habitats. These patterns are consistent with those found in other arid environments in the Southern Hemisphere, including the South African succulent karoo and the Chilean lowlands, and highlight the diverse modes of diversification and persistence of Earth's biota during the glacial cycles of the Pliocene and Pleistocene.

KEY WORDS: **Aridification, habitat reconstruction, phylogeography, Pleistocene, Pliocene, population expansion.**

Environmental change is a major driver of biotic diversification. Phylogeographic studies have demonstrated clearly the importance of past climate fluctuations in promoting diversification as lineages retreated to refugia, diverged in allopatry or across environmental gradients, and subsequently expanded their distributions during interglacials (Avise 2000). These patterns are well established in the Northern Hemisphere, where the glaciation cycles during the Pleistocene have left signals of high genetic diversity in refugia and of postglacial colonization via population expansion (Hewitt 2004). The Southern Hemisphere was less glaciated, but instead experienced aridification starting in the mid-Miocene that changed landscapes in South America (Ortiz-Jaureguizar and Cladera 2006), South Africa (Richardson et al. 2001; Cowling et al. 2009), and Australia (Martin 2006; Byrne et al. 2008). The effects of aridification on diversification both contrast with and mirror those of glaciation in the Northern

Hemisphere. One important difference is that aridification opened up a new adaptive zone, allowing the invasion and diversification of lineages whose ancestors originated in different biomes (Cogger and Heatwole 1981; Crisp et al. 2009). For example, the more recently arisen succulent karoo biome in the Cape Floristic Region of South Africa harbors younger plant lineages compared to the older, more mesic fynbos biome, suggesting that recent aridification in the late Miocene had an important role in producing this biodiversity hotspot (Richardson et al. 2001; Cowling et al. 2009; Verboom et al. 2009). In contrast, aridification during the Pliocene and Pleistocene in the Chilean lowlands resulted in the movement and diversification of mesic-adapted *Chaetanthera* (Asteraceae) in the wetter and newly formed highlands of the Andes (Hershkovitz et al. 2006). A macroevolutionary analysis of Southern Hemisphere plants revealed overall conservatism of biome-association, but also a predominance of within-continent shifts from sclerophyll woodland to arid biomes, mostly in the last 15 million years (Crisp et al. 2009). Here we examine the history of a widespread Australian reptile, highlighting and contrasting processes of diversification and persistence across the interface between semiarid and arid biomes.

Two of the largest biomes in Australia are the arid zone in the interior and the tropical monsoonal forests and savannas in the north (Fig. 1), both with a deep history, but their contemporary biotic compositions were shaped during the Pliocene and Pleistocene as a result of dramatic climatic changes (Byrne et al. 2008; Bowman et al. 2010). Major steps toward aridification in the Australian arid zone commenced in the mid-Miocene, after a period of relative climatic stability characterized by warm and wet environments (Martin 2006). The accumulation of Antarctic ice and the lowering of sea levels prompted aridification that drastically altered the landscape by the late Miocene; rainforests contracted to the north, east, and into small pockets in the south, as dry open woodland and chenopod scrubland replaced wet and swamp vegetation in central Australia (Martin 2006). A brief period of warm mesic conditions returned in the early Pliocene followed by intense aridification and climatic cycling during which the contemporary stony and sandy deserts formed. Dune systems

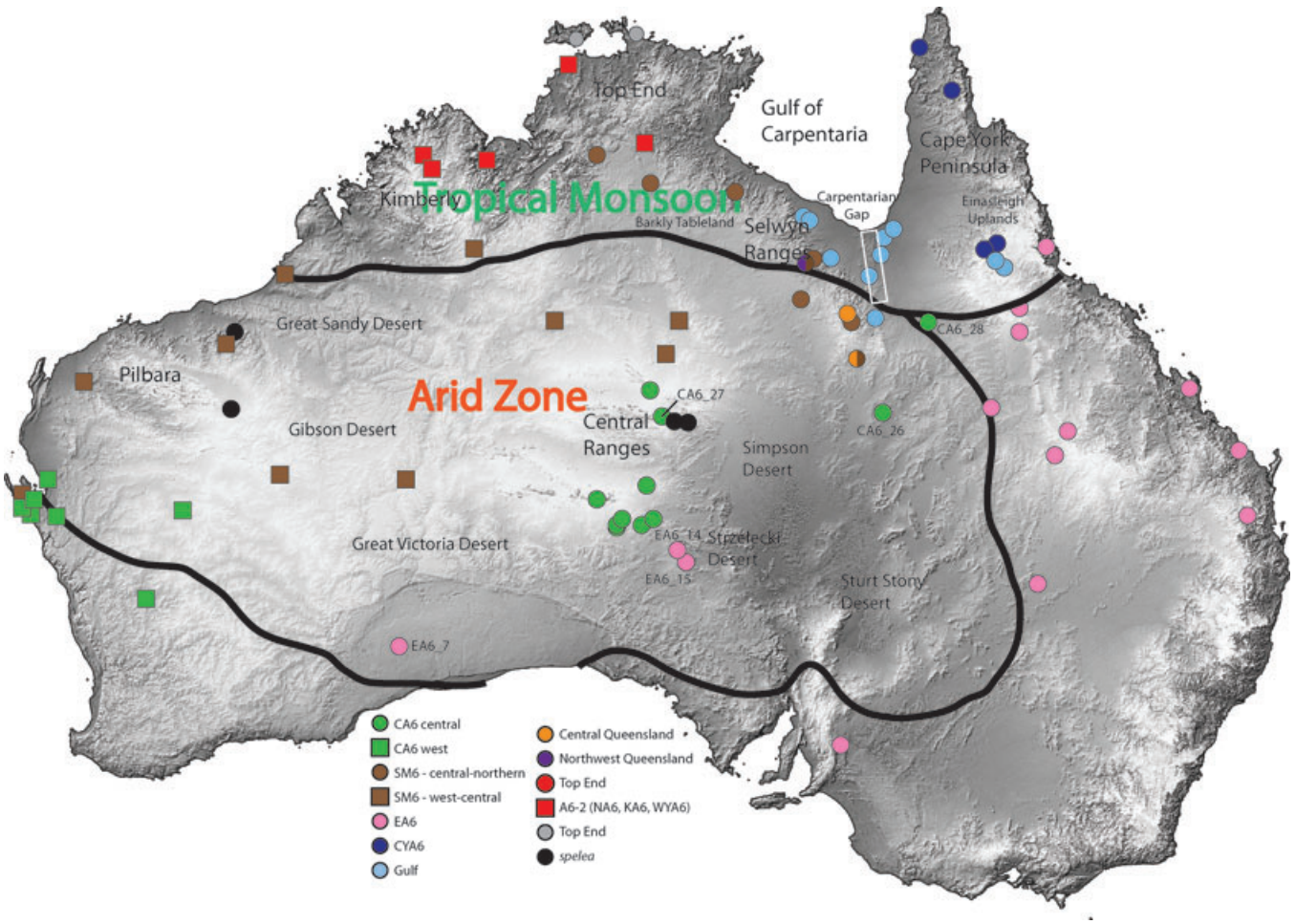

Figure 1. Sampling of *Heteronotia* used in this study. Major geological features and samples mentioned specifically in the text are labeled. The white box represents the Carpentarian Gap.

expanded during cold, hyperarid periods of the mid-late Pleistocene (Fujioka et al. 2005, 2009; Martin 2006; Byrne et al. 2008). The incursions of these dry climates influenced Australia in much the same way that glaciers influenced the Northern Hemisphere, by pushing the biota into isolated refugia (Byrne et al. 2008).

North of the arid zone is the tropical monsoon biome, marked by a dry winter and wet summer, with at least 85% of annual precipitation occurring during the summer cyclone season; Braby 2008; Bowman et al. 2010). The diversity of savanna or open woodland habitats in the tropical monsoon, and topographic complexity due to numerous dissected sandstone plateaus, gorges, and escarpments, supports more mesic environments that could have served as localized refugia during glacial periods. This contrasts with the large expanses of topographically uniform stony and sandy deserts of the arid zone (Woinarski et al. 2007). As part of the warm and wet tropical habitats that spanned Australia in the early-mid Miocene, the tropical habitat contracted northward as aridification intensified during the late Pliocene. The rise of the Tibetan Plateau between ~7.2 and 3.4 mya and the closing of the Isthmus of Panama established the contemporary ocean and wind currents conducive to monsoonal conditions (Haug and Tiedemann 1998; An 2000). In the Pleistocene, the monsoonal tropics experienced fluctuating environmental changes concomitant with global glacial-interglacial cycles; the tropics experienced weaker monsoons, cooler temperatures, and the development of more arid-adapted vegetation (e.g., *Eucalyptus* with grass understory) (Bowler 1982; Kershaw 1986; Bowler et al. 2001; Hesse et al. 2004; Marshall and Lynch 2006).

Relatively little attention has been paid to the historical biogeography of the monsoonal tropics or its interface with the arid zone (Bowman et al. 2010; Crisp et al. 2004). In their review of arid zone biogeography, Byrne et al. (2008) reiterated a long-held hypothesis that arid zone lineages diversified from mesic-adapted ancestors during the late Miocene/Pliocene, when aridification commenced, and that diversity is thought to have persisted in topographically complex refugial areas through the Pleistocene glacial cycles (Keast 1961; Cogger and Heatwole 1981; Schodde 1982). Late Miocene/Pliocene divergence has been documented in several Australian arid-zone taxa, including *Acacia* and chenopod shrubs (reviewed in Crisp et al. 2004; see also Crisp et al. 2009), dasyurid marsupials (Blacket et al. 2001), *Warramaba* grasshoppers (Kearney and Blacket 2008), *Ctenotus* and *Egernia* skinks (Chapple and Keogh 2004; Rabosky et al. 2007), *Diplodactylus* geckos (Pepper et al. 2006; Oliver et al. 2009), agamid lizards (Hugall et al. 2008), elapid snakes (Kuch et al. 2005; Sanders et al. 2008), and old endemic rodents (Rowe et al. 2008). Several studies have examined divergence and demographic history of taxa spanning the monsoonal woodlands of northern Australia, with particular reference to biogeographic barriers such as the Carpentaria Gap (e.g., Jennings and Edwards 2005; Lee and Edwards 2008). However, the monsoonal tropics are, in general, understudied (Crisp et al. 2004), and few studies address diversification and demographic history of taxa that span the arid and monsoonal biomes in Australia. Those that do typically have sparse sampling (Edwards 1993; Kuch et al. 2005; Joseph and Wilke 2007). In this context, we cannot yet discriminate between hypotheses that propose widespread late Pleistocene replacement of the monsoonal biome by arid systems, with monsoonal habitat persisting on the Sahul (Australia-New Guinea) shelf (Ford and Blair 2005), versus regional or localized persistence despite a weakened monsoon (Bowman et al. 2010).

We fill this void through a multilocus, continent-wide study to examine the history of the *Heteronotia binoei* complex, a widespread Australian endemic gecko previously found to include multiple chromosome races and parthenogenetic lineages (Moritz 1993). We use broad sampling from across the distribution of sexual *H. binoei*, including (and sometimes extending) the ranges of known races, and compile a dataset of nine nuclear intron markers and mitochondrial DNA for molecular phylogenetic analyses using both traditional and recently developed, coalescent methods for inferring species trees and the timing and extent of range fluctuations. We test the hypothesis that arid zone lineages are derived from mesic ancestors and provide an approximate time-scale for these events. We also test for Pleistocene-scale demographic expansions, which we expect were stronger in the relatively homogenous arid zone (with fewer and more separated refugia) relative to the more topographically complex monsoonal tropics.

Methods

STUDY SYSTEM AND TAXONOMIC SAMPLING

Heteronotia binoei is a widespread Australian vertebrate species complex spanning multiple biomes. There are three named species of *Heteronotia*: *H. spelea*, *H. planiceps*, and *H. binoei* (Bynoe's gecko), with a collective distribution that spans nearly the entire continent except for the cold southwest and southeast from which they are excluded physiologically (Kearney and Porter 2004). *Heteronotia binoei* is itself a complex assemblage of chromosomal races, each of which occupies distinct regions and are often parapatric (Moritz et al. 1990; Fig. 1). Comparative cytogenetic analyses (Moritz 1984; Moritz et al. 1990) delineated these *binoei* variants into cytotypes based on C-banding patterns and the morphology of chromosome 6. These are: SM6 (sub-metacentric chromosome 6, broadly distributed in northern Australia from western Queensland to the west coast), A6 (acrocentric chromosome 6, broadly distributed in eastern, central and southern Australia, and includes the CA6, CYA6, and EA6 cytotypes), and the A6–2 (distributed in the Kimberly and Top End, and includes the WYA6, KA6, and NA6 cytotypes) (Fig. 1). Additionally, there are multiple triploid parthenogenetic lineages in the central and

western deserts derived via hybridization between the CA6 and SM6 races (Moritz 1993; Strasburg et al. 2007). Previous studies using allozymes and karyotypes served to define the sexual chromosome races but did not resolve their relationships (Moritz et al. 1990). More recent mtDNA analyses addressed the phylogeography and historical demography of three widespread chromosomal races (CA6, SM6, EA6; Strasburg and Kearney 2005), but as yet there is no species-wide analysis with which to infer the biogeographic history of the entire complex.

Our sampling covers the range of sexually reproducing *H. binoei* with extended sampling in some areas, and includes previously karyotyped samples from all of the major chromosomal variants (Fig. 1; see Appendix for sample information, and Fig. S1 for current knowledge of the distribution of the lineages). A parallel analysis of the saxicolous species of *Heteronotia*, *H. spelea* and *H. planiceps*, is underway (M. Pepper, unpubl. data). As *Dixonius* is the sister group to *Heteronotia*, we used *D. vietnamensis* for the outgroup (Jackman et al. 2008).

MARKER DEVELOPMENT

We chose to use intron markers because they typically evolve faster than coding DNA, and because the opportunity to anchor primers in conserved exons alleviates polymerase chain reaction (PCR) amplification optimization across divergent taxa, including the outgroup. Most markers currently available in reptile systematics are exons (Townsend et al. 2008), whose phylogenetic signals are most useful for more divergent groups, necessitating the development of novel intron markers for our study.

To isolate genes for primer design, we constructed liver and testes SMART cDNA libraries from a male CA6 individual (Sambrook and Russell 2001). We screened the library by PCR (via standard blue-white selection), sequencing ~280 clones whose inserts were at least 400 bp. We used the gene cluster database Metazome (www.metazome.org), to identify the genes with conserved synteny across multiple genomes (implying orthology) and whose membership did not include multiple paralogous copies; in our case, we focused only on tetrapod genomes for gene identification and screening. For each of the genes identified in Metazome, we used Ensembl transcript information to determine the exon-intron structure in the chicken or mouse ortholog. Assuming the conservation of intron position between chicken (or mouse) and *Heteronotia*, we designed primers in exons to amplify the intervening introns using Primer3 (Rozen and Skaletsky 2000). We selected introns that amplified consistently across the diversity of *Heteronotia* as well as the outgroup (*Dixonius*; *erh* is the only intron that did not amplify for *Dixonius*). Our final collection of working markers included one mitochondrial gene (*nad2*) and nine nuclear introns; Table 1 documents the PCR conditions and annotations for all the markers that we used in this study.

AMPLIFICATION AND SEQUENCING

We used the same general PCR protocols for all of the markers. Each PCR occurred in a total volume of 25 μL that included 10 mM Tris, 5 mM KCl, 2 mM $MgCl_2$, 0.04 mM of each dNTP, 1 U *Taq* DNA Polymerase, 0.1 μM each primer, and 10–25 ng of DNA. The amplification protocol for all PCR reactions was: 94°C, 3 min; 37 cycles of 94°C 30 sec, annealing temperature (Table 1) 30 sec, 72°C 1 min; 72°C 10 min; final rest at 10°C. We visualized PCR products on 1% agarose gels stained with ethidium bromide, purified successful amplifications with ExoSAPIT (0.2 μL per 22 μL PCR product incubated at 37°C for 30 min followed by a 15-min denaturation step at 80°C; USB Corporation, Cleveland, OH), sequenced with BigDye version 3.1 chemistry (Applied Biosystems, Inc. [ABI], Foster City, CA), and collected the sequence data using an ABI3730 genetic analyzer (ABI). To avoid sequencing problems associated with homopolymer tracts, or with larger introns, we often used internal sequencing primers (in addition to the PCR primers) in the sequencing reactions.

We used CodonCode Aligner (version 2; CodonCode Co., Dedham, MA) to assemble contigs for each gene. Heterozygous indels often prevented initial contig assembly. We used a feature in CodonCode Aligner that subtracts out two competing sequences from a chromatogram when heterozygous indels are an issue; this produces two "pseudoalleles," which are not properly phased alleles based on population genetic theory. In instances when the program could not tease apart the pseudoalleles or when it was difficult to call a base (e.g., in homopolymer tracts), we coded those regions as missing data, later refining the base calls using the alignment of all samples as a guide.

For each gene we used MUSCLE version 3.6 (Edgar 2004) to construct multiple alignments, which we refined by eye with the sequence editor in Geneious version 4.5 (Biomatters, Auckland, New Zealand). We grouped the nuclear sequences into cytotypes, and in some instances (EA6 and SM6) into additional groups based on preliminary phylogenetic results. We used PHASE 2.1.1 to analytically phase the data (running the algorithm 10 times), using the resulting, highest-probability haplotypes for further analyses (Stephens and Donnelly 2003). Because of the limited length and low variation in the exons, we truncated each nuclear locus to include only intron sequence, and removed tRNA sequences from the *nad2* alignment. Perl scripts aided in developing concatenated, partitioned datasets and input files for the subsequent phylogenetic and demographic analyses (scripts available from MKF).

DIVERSITY AND DEMOGRAPHIC ANALYSES

We characterized the genetic variation in the markers and looked for evidence of population expansion by estimating basic population genetic measurements (nucleotide diversity, Tajima's *D*, Fu's *Fs*, population pairwise distances) of the phased nuclear

Table 1. Marker information used in this study.

Gene	Ensembl[1]	Intron	Size (bp)[2]	TA (°C)	Primers (5′-3′)[3]	GenBank accession numbers
snrpd3	ENSGALG00000006596	1	620	62	exon 1: ATATTGTGACTTGTGAGACCAATACG exon 2: CGAATGTACACTTGCTCTAACTGTG	GU388096– GU388199
erh	ENSGALG00000017384	3	1750	62	exon 3: TTGATGATTTGGCTGATCTTAGCTG exon 4: CCAGTCTTTATTGTAGGGCTGGTATG	GU387479– GU387581
rpl14	ENSGALG00000011523	1	900	62	exon 1: ACTGGTAGCAATTGTGGATGTTATCG exon 2: GAACTTGAGAACGAAGTCAGTCAGTTG	GU387888– GU387988
rpl35	ENSGALG00000001039	2	1500	72	exon 2: CAGAGTGCTGACAGTCATTAACCAGAC exon 3: GTCTTCAGACCCTCTTCGTGCTTG	GU387989– GU388095
bzw1	ENSGALG00000008220	2	700	70	exon 2: CTTCTGGAGCAAAGCTTGATTATCG exon 3: ATCGTTTCTAGGTCTTCCTGTGCTG	GU387289– GU387393
lztfl1	ENSGALG00000011810	1	450	65	exon 1: TGAAGTAATTAACTACATGCGATTTGCAC exon 2: TCCAGCATATCTGACACTTCATCTATTG	GU387688– GU387790
frih	ENSGALG00000007220	5	1250	70	exon 5: AAGAACATCAACCAAGCTCTCTTGGAC exon 6: TGCTTGATAGCCTGCACTTGATCC	GU387582– GU387687
dynll1	ENSGALG00000020999	1	1500	65	exon 1: TGATCAAGAATGCGGATATGTCTGAG exon 2: TCTTCCCACAATACAGTGCCAAGTAG	GU387394– GU387478
nmes1	ENSMUSG00000033213	3	1400	65	exon 3: ATAAACAAACGTGGTAATCCGGAACC exon 4: CTCTTCAATGCGTTTCCACTCCTG	GU387791– GU387887
nad2	-	-	1041	55	*tRNAI:* AAGGACTACTTTGATAGAGT[4] *tRNAA:* AAAGTGTTTGAGTTGCATTCAG[4]	GU388200– GU388302

[1]Accession number for the gene in chicken (ENSGALG) or mouse (ENSMUSG).
[2]Intron sizes are approximate as measured in sample CA6_06.
[3]Primers anneal in the indicated exon.
[4]From Strasburg and Kearney (2005).

genes and *nad2* as implemented in Arlequin v. 3.1.1 (Excoffier et al. 2005); these measurements were quantified in nine phylogenetically distinct groups based on the gene trees (see below). To explore differences among well-sampled lineages in demographic history we generated Extended Bayesian Skyline Plots using the phased nuclear data (Heled and Drummond 2009); these graphs plot effective population size through time, providing a temporal reference to demographic events such as bottlenecks and expansions. Extended Bayesian Skyline Plots explicitly incorporate multiple, independent loci, and estimate the number of coalescent intervals of population size changes, both advantages over Bayesian Skyline Plots (Heled and Drummond 2009). We calibrated the plots by setting the mutation rate priors for each of the nuclear markers to closely match the distributions estimated from the divergence dating exercise described below. Each marker was assigned the HKY+G+I model of nucleotide substitution. Because the molecular clock may be an adequate representation of molecular evolution at the intrapopulation level (Yang 2006), we enforced a strict molecular clock to simplify the coalescent model and thereby helping the analyses to converge. We ran four replicate analyses, each 40,000,000 generations long, for each well-sampled group (CA6, SM6, EA6, Gulf, A6–2), as implemented in BEAST version 1.5.3 (Drummond and Rambaut 2007). We assessed convergence primarily by comparing the resulting replicate plots, making sure each replicate produced the same demographic patterns.

PHYLOGENETIC ANALYSES

Though the distinction between gene trees and species trees is now widely recognized, as are the potential pitfalls of concatenating independent nuclear genes in phylogenetic analysis (Degnan and Rosenberg 2009; Edwards 2009), there is, as yet, no coalescent-based method that can use multilocus data to simultaneously identify independently evolving lineages and infer the relationships among these. This problem is especially vexing at the interface between population (phylogeography) and species (phylogeny) scale analyses. Migration or within-lineage population subdivision violates key assumptions of the coalescent-based phylogenetic methods (Liu et al. 2009), yet among recently diverged populations heterogeneity of individual gene trees is inconsistent with the premise of a multilocus analysis using concatenation. Given these limitations, an emerging strategy is to use phylogenetic analysis of concatenated sequences (or some other clustering approach) to group individuals into consistently supported

groupings, and then use coalescent approaches to infer relationships among these (Leaché 2009).

We used MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) to infer gene trees for each of the mtDNA and the phased nuclear markers. Each gene tree analysis involved two runs, each with four chains each (three heated, one cold, default heating parameters), for 20,000,000 generations (6,000,000 burnin), using models selected under the AIC from those evaluated in MrModelTest version 2.3 (Nylander 2004); the mtDNA analyses were partitioned by codon. Our primary goal for these inferences was to obtain a general assessment of the extent of incomplete lineage sorting.

To cluster individuals into lineages and provide one estimate of relationship among these, we employed MrBayes version 3.1.2 to conduct a partitioned Bayesian analysis of the concatenated nuclear data. Because alleles from the same individuals were typically homogeneous, we used genotype data instead of the phased data; preliminary analyses demonstrated that trees using haplotypes and genotypes were broadly congruent, and did not significantly change our interpretation. Each intron represented an independent partition. The analysis consisted of two runs, each with four chains (three heated, one cold; default heating parameters), for 20,000,000 generations (6,000,000 burnin).

Our individual gene tree analyses revealed instances of incomplete lineage sorting among the nine nuclear genes and/or low support for relationships, curtailing any efforts to deduce species relationships from any single gene. We therefore employed the recently developed Bayesian Estimation of Species Trees (BEST version 2.2; Edwards et al. 2007; Liu and Pearl 2007; Liu 2008; Liu et al. 2008), a hierarchical method that estimates the joint posterior distribution of gene trees and species trees. This method requires a priori designation of species and assigning sequences to those species is highly sensitive to post-divergence introgression among lineages (Leaché 2009; Liu et al. 2009). We therefore divided our data based on the monophyletic groups that were completely consistent between the mitochondrial and concatenated nuclear analyses, resulting in 12 "species" for the BEST analysis. Because BEST and other species tree methods appear more sensitive to missing data than concatenation (Edwards 2009), we removed one gene (*dynll1*) so that all ingroup samples were represented by complete data. Most of our "species" included at least two individuals and introgressed individuals were excluded (Leaché 2009; Appendix). The BEST analysis used a partitioned dataset of eight introns (genotypes) and *nad2* with four runs (one chain), models inferred using MrModelTest version 2.3 (see above), for 200,000,000 generations, sampling every 20,000 (60,000,000 burnin). The BEST-specific parameters included an inverse gamma distribution for the population size prior ($\alpha = 3$; $\beta = 0.03$) and a uniform gene mutation prior (0.5, 1.5). As all runs produced the same topology with nearly the same posterior probabilities, we combined runs to generate a single consensus tree.

We assessed convergence of all the Bayesian MCMC phylogenetic analyses (MrBayes and BEST) by examining likelihood and parameter estimates over time using Tracer version 1.4.1 (Rambaut and Drummond 2007); in general, we discarded the first 30% of each Bayesian run as burnin before calculating the posterior probabilities using the remaining trees. Except for one MrBayes run (*nmes1*), all parameters had effective sample sizes (ESS) greater than 200, and most were greater than 300 upwards to over 1000; thus, most runs had at least several hundred independent samples from the MCMC chains, a good indication that the analyses adequately sampled the posterior distributions. We also used the Web-based tool Are We There Yet (AWTY) to verify the stabilization of posterior probabilities of nodes (which indicates that the tree topologies are sampled proportionally from the posterior distribution) (Nylander et al. 2008). Finally, we examined the independent runs of each of the analyses to make sure they reached the same maximum in tree space before combining them into single consensus trees.

To test the few observed conflicts between the mtDNA and nDNA gene trees, we evaluated alternative hypotheses using Shimodaira-Hasegawa (SH) tests (Shimodaira and Hasegawa 1999). We first used RAxML version 7.0.4 to construct maximum likelihood trees using partitioned mitochondrial and nuclear trees under the GTR-GAMMA model choice (Stamatakis 2006) of both unconstrained and constrained topologies. Using these alternative topologies, we employed both RAxML and PAUP v.4b10 (Swofford 2003; 1000 bootstraps with full optimization) to conduct the SH tests.

To determine whether arid zone lineages are recent derivatives from mesic-adapted ancestors, we reconstructed ancestral habitats. We first coded each individual as either "mesic" (tropical monsoon or eastern forest) or "arid zone," as depicted in Figure 1, although we included the southern extension of the Selwyn ranges as "mesic" because its complex topography harbors potential refugia. Given the results of the SH tests, we chose the concatenated nuclear tree to reconstruct the maximum likelihood (Markov k-state 1 parameter model; Lewis 2001) habitat preferences of internal nodes using Mesquite version 2.6 (Maddison and Maddison 2009; the maximum likelihood results were entirely consistent with a parsimony analysis; we repeated the maximum likelihood analysis using the BEST topology, and arrived at the same conclusions). We also used the Bayesian MCMC method in BayesTraits version 1.0 (Multistate; Pagel et al. 2004; available at www.evolution.rdg.ac.uk) to provide posterior probabilities of the ancestral distribution reconstruction at the six deepest nodes in the *binoei* clade. This method incorporates phylogenetic uncertainty in the estimation of trait rate parameters and ancestral states by integrating over a posterior distribution of phylogenetic

trees (Pagel et al. 2004). We selected 500 trees, sampled at even sampling intervals, from the post-burnin posterior distribution of the concatenated nuclear Bayesian phylogenetic analysis (first run), as a representative of the posterior tree distribution from which to estimate the trait rate parameters and ancestral states. As our interest chiefly lies in determining the ancestral habitat preference of *binoei*, we reconstructed the habitat preference of only the six deepest *binoei* nodes. To do this, we used the most recent common ancestor (MRCA) approach (Pagel et al. 2004), which involves the trait inference of the MRCA for selected taxa; we chose samples such that the MRCA of those samples were the six deepest *binoei* nodes in the concatenated nuclear tree. The analysis used a uniform (0, 100) prior for the rate parameters, and ran for 500,050,000 generations with a burnin of 50,000. We examined the output in Tracer version 1.4.1 to confirm stationarity of log-likelihoods and that acceptance rate of the MCMC chain was within 20–40%.

DIVERGENCE DATING

The lack of a fossil record for *Heteronotia* and related genera precludes a rigorous divergence dating analysis using current Bayesian phylogenetic approaches. However, the opportunity to infer important biogeographic scenarios in Australia's arid zone using the *binoei* system warrants an attempt to date the major nodes. We used a calibration point of 5.47 million years [Normal distribution$\sim$(5.47, 0.8)] for the primary *binoei* split, as inferred by Strasburg and Kearney (2005) and based on the Macey et al. (1998) calibration of 0.6–0.7%/Myr substitution rate of *nad2* in agamid lizards. We set also the root node age prior (*Dixonius-Heteronotia* split) to a Normal (25, 4) distribution (million years) that represents a conservative timing of the Australia-Asia contact that initiated faunal exchange between the two regions. Bayesian divergence dating was conducted using BEAST version 1.5.3 (Drummond and Rambaut 2007) by using the genotype sequences from only those samples with complete data (except for WYA6, Central Qld, and *Dixonius*, for which we included one sample each that lacked one gene in the data matrix), constraining the monophyly of the major groups inferred from the phylogenetic analyses described above, assigning the GTR + I + G model for each nuclear marker, and employing lognormal relaxed clocks. We ran four replicate analyses for 100,000,000 generations each, reviewed convergence criteria using Tracer version 1.5 (Rambaut and Drummond 2009), combined parameter and tree files using LogCombiner version 1.5.3 after confirming each run converged to the same posterior distribution and after removing the first 10,000,000 generations as burnin from each run, and summarized the results as a maximum clade credibility tree using TreeAnnotator version 1.5.3 (Drummond and Rambaut 2007).

Results

MARKER AND DATA CHARACTERISTICS

The nuclear introns that we developed ranged from small (*lztfl1*; 450 bp) to large (*erh*; 1750 bp) with respect to typical molecular markers (Table 1). Some markers would not amplify for particular individuals, but our dataset is nearly complete (92% complete by locus). The nuclear alignment was 7082 bp in length after removing exons and ambiguously aligned regions (such as homopolymer tracts). We tested the utility of these markers on other geckos, and found that many of them work in other gekkonines (e.g., *Gehyra*, *Lepidodactylus, Stenodactylus*, *Hemidactylus, Cyrtodactylus*) as well as diplodactylines (*Diplodactylus*, *Rhynchodeura*), and they may be applicable to other lineages in Gekkota (M. Fujita, unpubl. data).

Paralogy can confound phylogenetic inference if different samples contain alternative loci. If multiple loci encoding our markers exist, we expect our sequences to be heterozygous at multiple positions, depending on the age of the putative duplication event. However, we found that for those groups with the greatest sampling (e.g., CA6, EA6, SM6), there were several individuals homozygous for all of our markers. Additionally, the individual gene trees of phased haplotypes showed that alleles from a single individual largely grouped together and within the appropriate lineage, not in divergent groups as might be expected with genes that were duplicated early in the history of the group. Thus, we are confident our markers are single copy.

MITOCHONDRIAL RELATIONSHIPS AND PHYLOGEOGRAPHY

The *nad2* mtDNA tree recovered from the Bayesian analysis identifies multiple, strongly supported lineages emerging from a basal polytomy that includes *H. spelea* (Fig. 2A; M. Pepper, unpubl. data for a detailed analysis on the systematics and biogeography of the saxicolous *planiceps* and *spelea*). Several of the well-supported clades not only reflect the cytogenetic forms identified by Moritz et al. (1990), but also exhibit substantial variation and structure within each group (Fig. 2A). One divergent lineage comprises the monophyletic EA6 clade (excluding an introgressed CA6_28; see below), extending across mesic to semiarid woodlands of eastern Australia and to chenopod scrub of southern to western Australia, and its sister lineage, the CYA6 chromosome race of the monsoonal woodlands of Cape York. Another lineage corresponds to the CA6 group, which includes well-supported, sister clades of geographically disjunct western and central populations from Acacia-dominated arid habitats, and two divergent individuals from the Northern Territory (CA6_3) and western Queensland (CA6_26). A third lineage includes the SM6 race and various forms from the Kimberley and Top End monsoonal tropics. The SM6 form itself is paraphyletic in relation to the A6–2

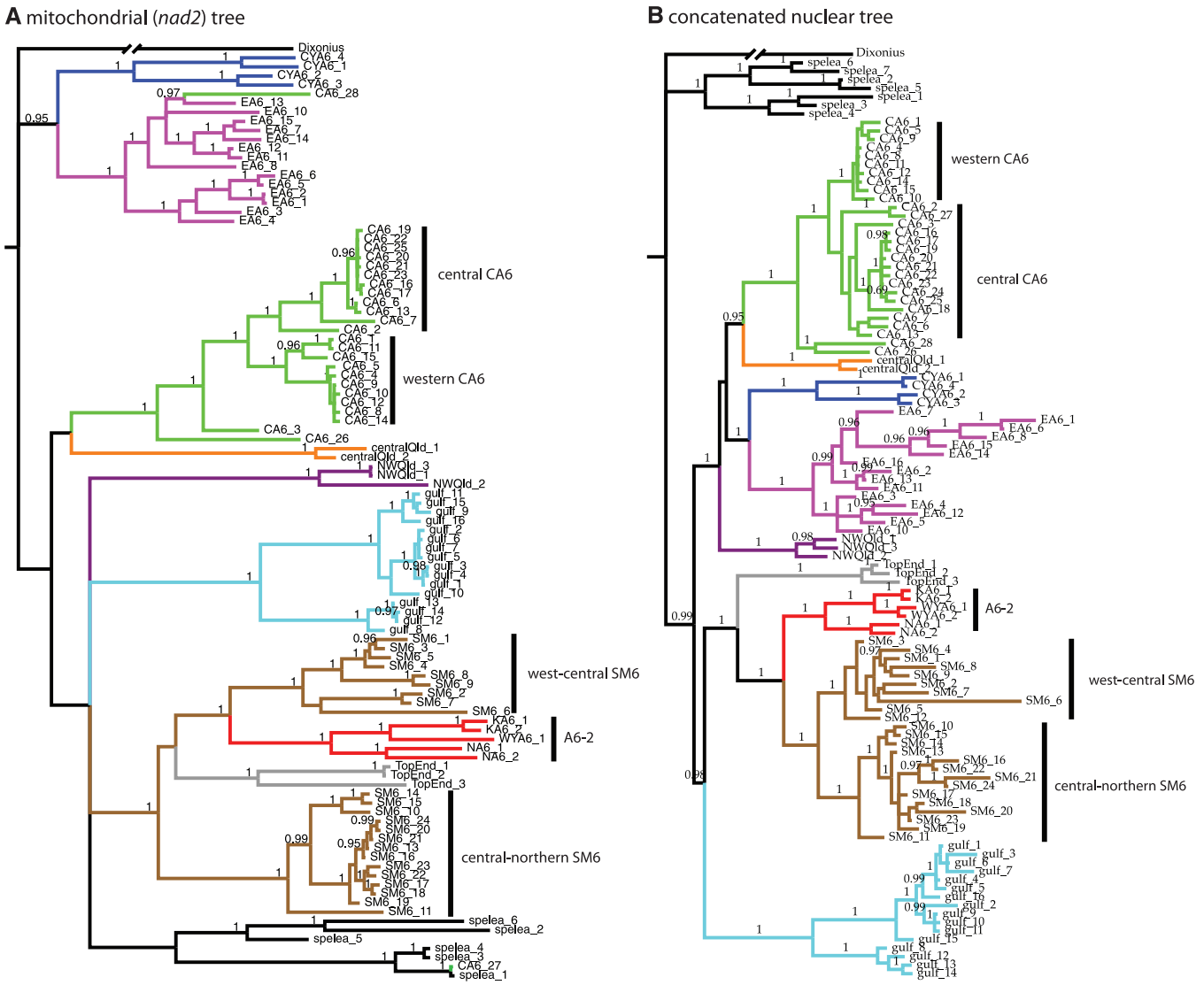

Figure 2. (A) Mitochondrial (*nad2*) tree, as inferred using MrBayes version 3.1.2 using codon-partitions. (B) Concatenated nuclear tree based on a partitioned Bayesian inference as implemented in MrBayes version 3.1.2. Color codes are as indicated in Figure 1. Posterior probabilities >0.95 are shown at well-supported nodes.

(KA6, WYA6, NA6 cytotypes) and a newly identified "Top End" lineage restricted to north-coastal populations (Fig. 1). The two SM6 clades are geographically distinct; a "west-central" group ranges from the west-central coast across the western deserts to north-central Australia occupying spinifex-dominated arid habitats, and a "central-northern" lineage is restricted to monsoonal woodlands and savanna of Northern Australia. In addition to the previously identified chromosomal groups, our sampling revealed novel, highly divergent mitochondrial clades (up to 12.08% sequence divergence; Table 2), which we call the "Central Queensland," "Gulf," and "Northwest Queensland" groups. Remarkably, all three of these newly identified lineages (and the divergent Top End lineage) occur in the monsoonal tropics. Two of these, the Central Queensland and Northwest Queensland lineages are restricted to the Selwyn Ranges. The Gulf lineage occupies monsoonal habitats from the Selwyn Ranges across the savanna-dominated Gulf plains (the "Carpentaria Gap") to the woodlands of the Einasleigh Uplands in the east.

LINEAGE RELATIONSHIPS INFERRED FROM NUCLEAR GENES

As expected, the nuclear genes exhibit far less variation than mtDNA, with inter-lineage divergences of 1.24–2.49% for nuclear genes, versus 4.64–12.08% for mtDNA (Table 2). Consequently, the gene trees typically have lower resolution than mtDNA and show instances of incomplete lineage sorting (Fig. S2). The gene trees often support monophyletic cytotypes, although the resolution varied extensively from gene to gene. In addition, it

Table 2. Population differences (Dxy).[1]

	CA6	Central Qld	CYA6	EA6	Gulf	NW Qld	Spelea	A6–2	SM6	Top End
CA6	-	0.0124	0.0162	0.0139	0.0184	0.0149	0.0183	0.0176	0.0158	0.0202
Central Qld	0.0994	-	0.0188	0.0176	0.0206	0.0176	0.0198	0.0194	0.0176	0.0218
CYA6	0.0723	0.1039	-	0.0147	0.0227	0.0196	0.0213	0.0213	0.0201	0.0249
EA6	0.0604	0.0865	0.0511	-	0.0198	0.0189	0.0205	0.0191	0.0177	0.0232
Gulf	0.1020	0.1207	0.1034	0.0898	-	0.0203	0.0234	0.0219	0.0202	0.0247
NW Qld	0.0912	0.1167	0.1136	0.0979	0.1167	-	0.0203	0.0169	0.0172	0.0205
Spelea	0.0578	0.0860	0.0672	0.0553	0.0810	0.0880	-	0.0226	0.0212	0.0253
A6–2	0.0869	0.1165	0.0866	0.0745	0.1012	0.1003	0.0761	-	0.0143	0.0185
SM6	0.0729	0.1059	0.0733	0.0564	0.0909	0.0890	0.0616	0.0464	-	0.020
Top End	0.0877	0.1178	0.0970	0.0742	0.1057	0.1029	0.0792	0.0755	0.0635	-

[1]**Based on Tamura-Nei corrected distances of combined nuclear (above diagonal, gray) and mitochondrial (*nad2*) data (below diagonal).**

was impossible to deduce relationships between the lineages (as defined in the mitochondrial gene tree) by comparing individual nuclear gene trees. The newly discovered lineages (Central Queensland, Gulf, Northwest Queensland, and Top End) were each monophyletic for half of more of the individual gene trees, a level of genealogical distinction on par with *Heteronotia spelea*.

In the concatenated Bayesian nuclear phylogeny each of the major lineages revealed by previous cytogenetic analysis and the current mtDNA phylogeny is monophyletic. This is despite the underlying heterogeneity of gene trees and supports use of these groupings as discrete lineages for further phylogenetic analysis. Further, the topology is largely concordant with the mitochondrial gene tree, and resolves many basal nodes for which mtDNA was uninformative (Fig. 2B). According to this analysis, the *binoei* complex is monophyletic relative to *H. spelea* (and also other taxa within *Heteronotia*; M. Pepper, unpubl. data) and consists of three major clades. The first clade includes the EA6, CYA6, CA6, and Central Queensland lineages and is broadly distributed, stretching from the east coast through the central and southern deserts to Western Australia. The second clade is the Gulf lineage, which is monophyletic and well differentiated, but whose placement is not entirely clear given inconsistencies between the mtDNA and BEST trees (see below). The third large clade, distributed across northern Australia excluding Cape York, includes the rest of the chromosomal groups (SM6, KA6, WYA6, and NA6), as well as the Top End lineage. Importantly, the newly discovered lineages (Gulf, Top End, Central Queensland and Northwest Queensland) are highly divergent for nuclear genes as well as mtDNA (Table 2) and their relationships are largely resolved. The Top End lineage is sister to other Kimberley and Top End forms (NA6, KA6, WYA6) and SM6, and the Gulf lineage is sister to all of these. The Central Queensland lineage is the sister group to CA6, and could represent a divergent, northwestern extension of that lineage. The Northwest Queensland lineage forms a trichotomy with eastern (EA6 + CYA6) and central (Central Queensland + CA6) clades.

There are few discrepancies between the mitochondrial gene tree and concatenated nuclear tree within the chromosomal lineages. In the nuclear tree, the SM6 race, though still separated into west-central and central-northern clades, is clearly monophyletic and sister to the A6–2 and Top End forms (KA6, WYA6, NA6, and Top End), rather than being paraphyletic with the latter. Within the CA6 group, the western isolate is monophyletic for nuclear as well as mitochondrial genes, but there is less support for monophyly of the central CA6. One individual, CA6_28 from Julia Creek, Queensland, clusters with EA6 in the mtDNA tree but forms a divergent CA6 clade along with another western Queensland sample (CA6_26) in the nuclear tree. The discordance between mtDNA and nuclear DNA for the Julia Creek sample most likely reflects localized introgression at the boundary between EA6 and CA6 lineages. Another example of localized introgression involves CA6 *binoei* and central Australian *H. spelea* where they are syntopic in the eastern MacDonnell Ranges (part of the Central Ranges); individual CA6_27 from Ross River, Northern Territory, groups with sympatric *spelea* mitochondrially but was consistently a CA6 for nuclear genes. Though our sampling was limited, the mitochondrial gene tree recovered a small western EA6 (EA6_7, EA6_14, EA6_15) clade that is not reflected in the nuclear concatenated tree. In general, however, the mitochondrial and nuclear trees are largely consistent and concordant with one another.

Our coalescent (BEST) analyses of lineage relationships included two individuals for several of the lineages, an approach that can improve the performance of the MCMC algorithm (Liu et al. 2008). Except for the basal location of the Gulf lineage, the tree itself is consistent with the combined nuclear tree (Fig. 3), yet is similar to the mitochondrial tree in that the deeper nodes had low posterior probabilities. The A6–2 lineage, which contains

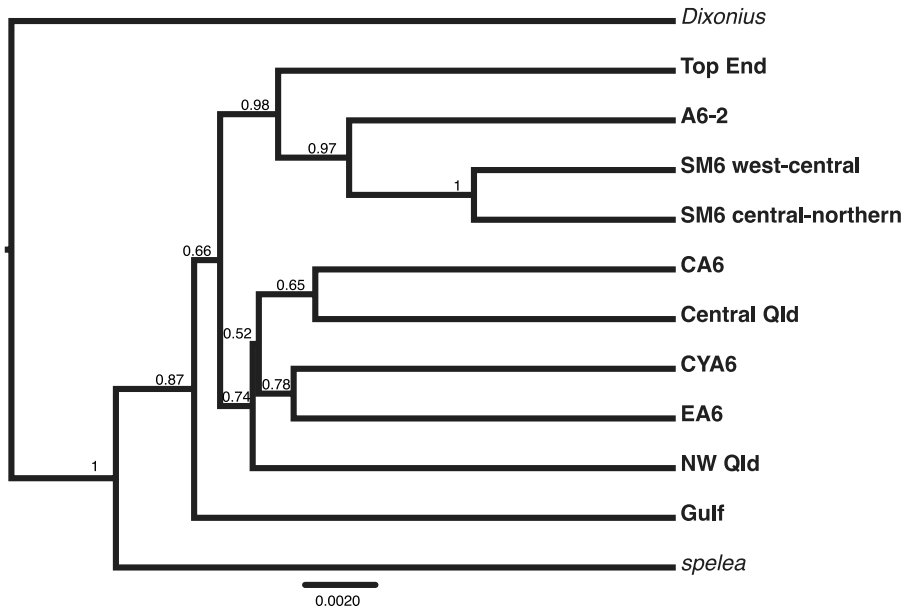

Figure 3. BEST species tree inferred using both the nuclear and mitochondrial (*nad2*) data.

KA6, WYA6, and NA6, is sister to a monophyletic SM6, in agreement with the concatenated nuclear analysis (Fig. 2B) but not the mtDNA tree (Fig. 2A). Because priors on BEST reflect the characteristics of the data (mutation rate, diversity), we also conducted a BEST analysis without the faster-evolving mtDNA (results not shown). The species trees from these two BEST analyses are consistent with each other, though the nuclear-only tree had increased support for *binoei* and for the basal placement of Gulf, with weaker support for relationships among the other lineages.

We applied SH tests to determine whether the few differences between mtDNA, concatenated-nuclear, and BEST trees were statistically significant. To do this, we used maximum-likelihood trees inferred using RAxML. The resulting trees, for both the partitioned nuclear and mitochondrial data, were entirely consistent with the corresponding MrBayes trees (results not shown). The first SH test examined the possible, but weakly supported paraphyly of *binoei* in the mitochondrial tree. The log-likelihood difference between the ML mitochondrial tree and the tree constrained with a monophyletic *binoei* was not statistically significant based on the SH test as implemented in RAxML and PAUP ($P = 0.441$ in PAUP). The second and third SH tests examined the monophyly of SM6, which is alternatively either strongly supported as paraphyletic in the mitochondrial tree, or is monophyletic in the nuclear tree. With the mitochondrial data, the SH test could not reject the monophyly of SM6 ($P = 0.093$). With the nuclear data, we constrained the west-central SM6 clade to have a sister relationship to A6–2, as inferred with the mitochondrial data; this tree was significantly different than the ML unconstrained tree ($P =< 0.001$). Because the mitochondrial data could not reject the monophyly of SM6, and because nuclear data strongly support monophyly of SM6 over paraphyly, we treat SM6 as monophyletic hereafter. The fourth test examined whether a sister relationship between the Gulf lineage and the rest of *binoei* (as inferred using BEST) was significantly different from its placement as sister to an SM6+A6–2 clade (as inferred from the concatenated nuclear data). The two topologies were not statistically significant ($P = 0.12$), indicating that the concatenated nuclear and BEST trees are not in strong conflict with one another.

ANCESTRAL HABITAT RECONSTRUCTION

Based on the concatenated nuclear gene tree, ancestral state reconstructions of habitat (arid vs. mesic) support the hypothesis that *H. binoei* had a mesic origin, with multiple relatively recent invasions of the arid zone (Fig. 4). The basal branches are dominated by lineages from the monsoonal tropics, and mesic habitats are strongly inferred as ancestral. Three of the major groups of *binoei* occur in the arid zone (EA6, SM6, and CA6) and each one is inferred to be a separate colonization of arid habitats. CA6 is the only chromosomal race whose entire distribution is almost completely within the arid zone. The west-central clade of SM6 occurs within the arid zone based on our sampling, while the central-northern clade largely occurs in the tropical monsoon region (albeit extending into the arid zone in the southern Selwyn Ranges). Superficially, it appears that EA6 invaded the arid zone multiple times, but it should be noted that our sampling of this widespread taxon is sparse. Samples of the north-central SM6 lineage identified as arid are from Camooweal, at the boundary between the monsoonal tropics and arid zone. We also

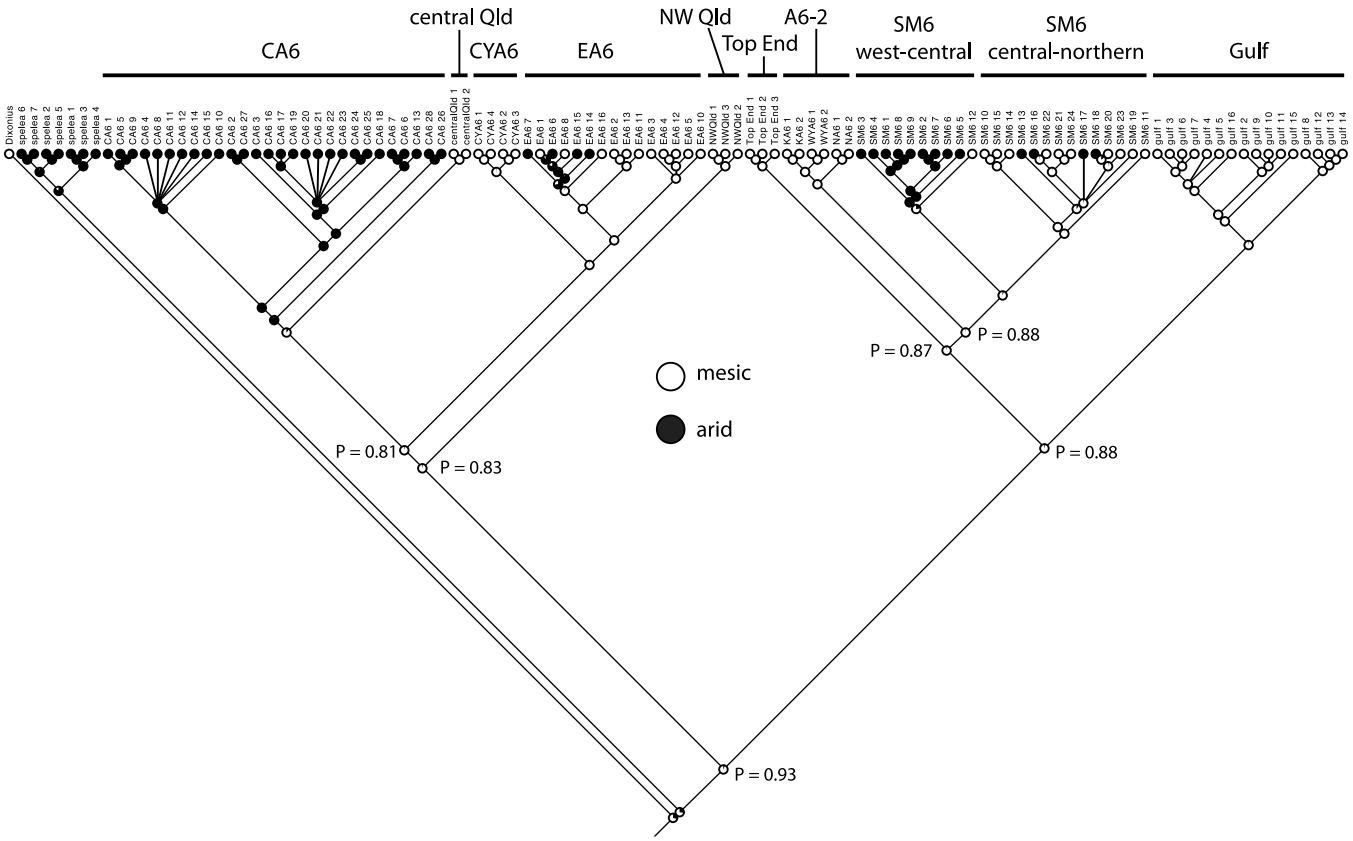

Figure 4. Reconstruction of ancestral habitat preferences for *Heteronotia binoei*. BayesTraits posterior probability values for "mesic" habitat are indicated at six of the *binoei* basal nodes.

repeated the maximum likelihood analysis with Gulf as the basal lineage, as suggested by the BEST tree, and arrived at the same conclusion.

DIVERGENCE DATING AND DEMOGRAPHIC HISTORY

Estimating divergence times using an indirect calibration—in this case the Strasburg and Kearney (2005) estimate of divergence of EA6, SM6, and CA6 at 5.47 Myr, itself based on the Macey et al. (1998) estimation of *nad2* substitution rates—is far from ideal (Graur and Martin 2004). Nonetheless, we proceeded in hope of providing at least a crude estimate of when particular splits occurred, and the geological and climatic events that might have played a role in the diversification of *Heteronotia* (Fig. 5). At the base of the chronogram are divergences that include several major splits within *Heteronotia*, including between (1) *spelea* and *binoei*, (2) the three main *binoei* groups, and (3) the major lineages (such as CYA6 + EA6 and CA6). These dates fall within the Pliocene, and at this time the ancestral lineages inhabited mesic environments (Figs. 4 and 5). A second round of diversification occurred more recently (Pleistocene), resulting in within-cytotype splits (e.g., divergences within the "Gulf" lineage, CYA6, A6–2, and within each of the SM6 clades). It was during this period that just three cytotypes—CA6, EA6, and SM6—populated the arid zone (Fig. 4). Subsequently, the split between the western and central arid zone population of CA6 occurred in the mid Pleistocene (0.72 ± 0.42 mya).

To infer demographic fluctuations in response to the intense climate cycles of the late Pleistocene, we examined nucleotide diversity and tested for population size change using summary statistics (Table 3; only those groups with the largest sample sizes are shown) and Extended Bayesian Skyline Plots (Fig. 6). Nucleotide diversity, although generally low (0.3–1.9%), was highest in those lineages with some or all of their ranges in mesic or monsoonal habitats (CYA6, Gulf, SM6, A6–2 and EA6) than in the widespread, arid zone CA6 lineage. Tajima's *D* measures were significant in several of the nuclear markers for CA6, EA6, and SM6, and for a few markers in "Gulf," but were not significant for any group with *nad2*. Similarly, Fu's *Fs* was significant (and large) for every nuclear gene in CA6, all but one locus in EA6 and SM6, but with more mixed signals for the monsoonal Gulf and A6–2 lineages. For mtDNA Fu's *Fs* was significant only for CA6. While signatures of population expansion are expected for the wide-ranging arid zone lineages, the indication of expansion in the Gulf lineage indicates that even those lineages in the tropical monsoon biome were affected by climatic oscillations of the Pliocene and Pleistocene.

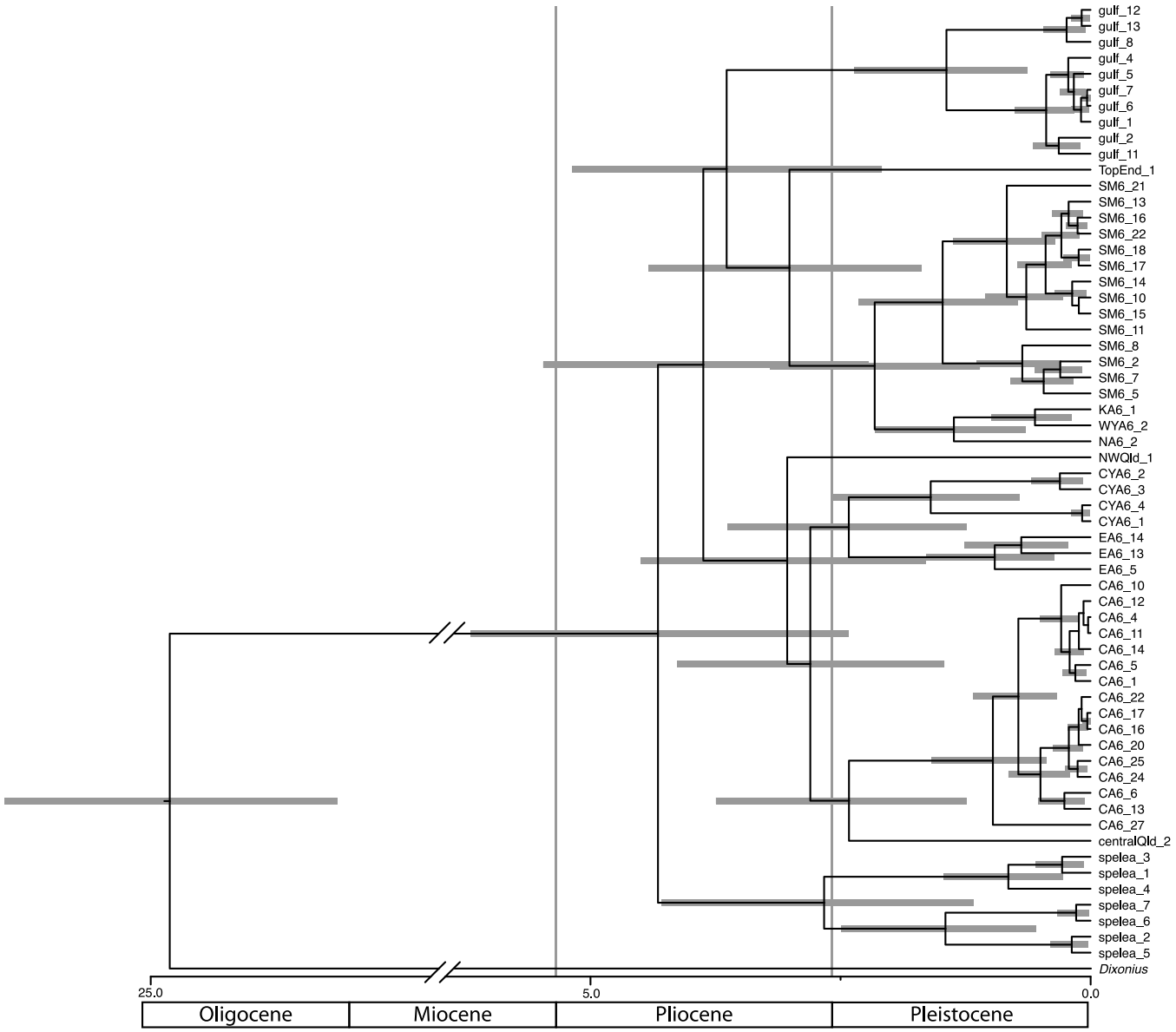

Figure 5. Chronogram inferred using relaxed molecular clocks implemented in BEAST version 1.5.3, using a subset of the nuclear data, with time axis indicated on the bottom. Nodal bars are 95% confidence intervals. Gray vertical lines demark boundaries of the Pliocene.

The Extended Bayesian Skyline Plots show that all the arid zone lineages that we examined (CA6, EA6, SM6) experienced population expansions in the Pleistocene (Fig. 6). Expansion of EA6 and SM6 commenced ~1 mya, perhaps in response to the accruing habitat as the arid zone expanded during Pleistocene. In contrast, CA6 experienced a noticeable population expansion starting at ~0.5 mya; this roughly corresponds to the west-central divergence and may represent a westward expansion from the more diverse central populations. For comparison with monsoonal tropics lineages, we constructed Extended Bayesian Skyline Plots for the Gulf and A6–2 lineages. Despite their more restricted distributions in the tropical monsoon biome, they also experienced population expansions during the Pleistocene (Fig. 6); A6–2 experienced only a slight increase starting at ~2 mya, while the Gulf lineage appears to have had a steady population size up until the late Pleistocene.

Discussion

Multilocus phylogeographic and phylogenetic methods provide powerful approaches for elucidating the biogeographic processes that drove diversification of recently diverged groups in the Australian arid and monsoonal zones, where much of the paleoecology remains uncertain. Several recent studies have highlighted the dangers of relying too heavily on inferences drawn from single markers (Maddison 1997; Degnan and Salter 2005), primarily

Table 3. Diversity and Demographic Summary Statistics.

Gene[1]	CA6 n=28			EA6 n=16			SM6 n=24			gulf n=16			A6–2 n=6		
	D[2]	Fs	ND	D	Fs	ND	D	Fs	ND	D	Fs	ND	D	Fs	ND
snrpd3	**−1.996**	**−24.156**	0.004	−1.423	**−25.811**	0.006	**−1.579**	**−24.565**	0.006	**−1.597**	**−10.623**	0.008	−0.255	**−7.739**	0.008
erh	−1.032	**−7.373**	0.005	**−1.935**	**−3.868**	0.005	**−1.679**	−3.213	0.004	−0.423	−3.225	0.004	−0.303	1.056	0.006
rpl14	**−2.266**	**−10.995**	0.006	−0.475	**−4.296**	0.012	−0.929	**−12.636**	0.008	0.772	**−6.247**	0.010	−0.529	0.140	0.008
rpl35	**−2.454**	**−24.146**	0.003	−0.478	**−9.298**	0.012	**−1.742**	**−17.294**	0.006	**−1.569**	**−26.939**	0.003	0.124	−1.631	0.013
bzw1	**−2.145**	**−25.683**	0.004	**−1.584**	**−23.816**	0.009	−0.913	**−24.108**	0.015	0.806	**−25.092**	0.011	0.448	**−6.505**	0.010
lztfl1	**−1.906**	**−24.190**	0.006	**−1.773**	**−21.313**	0.005	−0.987	**−24.202**	0.013	1.517	**−26.911**	0.006	−1.032	**−9.831**	0.012
frih	**−1.772**	**−10.702**	0.005	−1.232	**−11.419**	0.007	−0.232	**−9.720**	0.008	−1.169	**−4.804**	0.003	0.146	**−5.834**	0.005
dynll1	**−1.657**	**−5.980**	0.006	−0.780	**−4.399**	0.006	−1.314	**−12.763**	0.008	−0.761	−1.765	0.004	0.895	0.094	0.004
nmes1	**−1.751**	**−10.130**	0.004	−0.213	−1.701	0.019	**−1.826**	**−4.301**	0.010	0.093	−2.761	0.016	0.140	0.028	0.004
	average:		0.005			0.009			0.009			0.007			0.008
nad2	−0.805	**−4.614**	0.066	−0.251	1.721	0.074	0.475	−2.838	0.086	0.602	−2.812	0.048	0.400	2.007	0.081

[1]Nuclear gene calculations are based on phased data.

[2]D, Tajima's D; Fs, Fu's Fs; ND, nucleotide diversity; bold indicates significance.

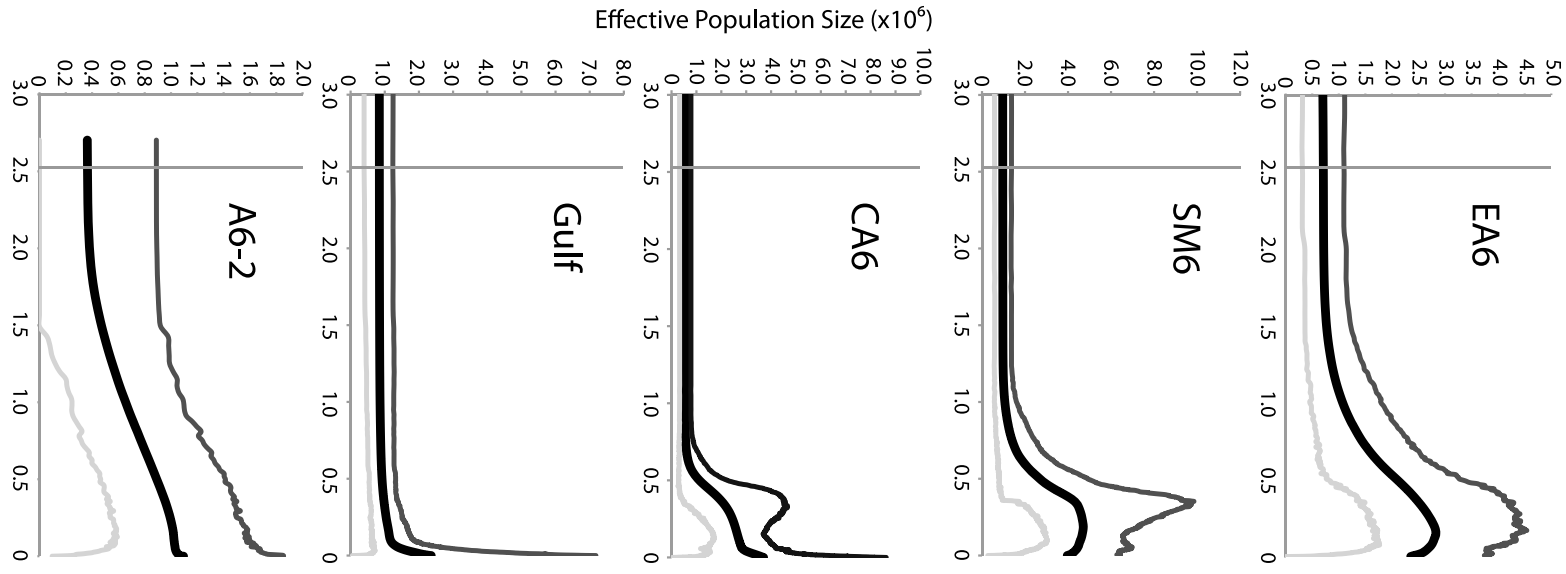

Figure 6. Extended Bayesian Skyline Plots showing trends in population size through time of CA6, SM6, EA6, Gulf, and A6–2 lineages, showing the mean (black lines) and the 95% confidence intervals (light and dark lines). Gray vertical lines indicate the onset of the Pleistocene at 2.59 mya.

focusing on incomplete lineage sorting as a common source of gene tree-species tree discordance (Maddison 1997; Edwards 2009). By incorporating multiple unlinked markers, it is possible to capitalize on the signal in that discordance and estimate the underlying pattern of lineage, as opposed to gene, divergence. The philosophical position that the species tree, rather than a collection of gene trees or a concatenated data tree, is the target of phylogenetic analysis has motivated the development and evolution of new inference methods (Edwards et al. 2007; Liu 2008; Kubatko et al. 2009). One strength of the present study is that we exploit multilocus analyses to explore historical processes of diversification and persistence in a low-dispersal species complex spanning mesic, arid, and semiarid biomes of Australia, mirroring recent studies of diversification history of avian species with disjunct distributions across the monsoonal tropics (Jennings and Edwards 2005; Lee and Edwards 2008). Our analyses confirm the cohesion and distinctiveness of previously defined chromosomal races of *H. binoei*, but also reveal entirely new lineages in northern Australia. Given a robust phylogeny of the lineages, we were able to test hypotheses about the pattern and process of diversification relative to aridification of Australia. Further, for the widespread and better-sampled lineages, we were able to test for demographic fluctuations associated with the relatively severe climatic fluctuations of the late (<400 kya) Pleistocene.

IDENTIFICATION AND RELATIONSHIPS OF *H. BINOEI* LINEAGES

Given data from both mtDNA and nine independent nuclear genes, we were able to confidently identify genealogically distinct lineages and their relationships, using a combination of methods. Though coalescent methods for inferring lineage relationships from component genes trees appear to be an improvement over existing concatenation or consensus type approaches (Degnan and Rosenberg 2009; Edwards 2009), such methods are still very sensitive to deviations from assumptions, especially post-divergence introgression (Leaché 2009; Liu et al. 2009). Accordingly, we used both approaches for nuclear genes, and compared the results to the mtDNA gene tree; the results across all three approaches were essentially concordant.

The multilocus molecular results presented here confirm and extend previous efforts, based on allozymes, chromosomes, and mtDNA, to identify historical lineages within this widespread species complex. The chromosome races and cytotypes identified previously (Moritz et al. 1990; Moritz 1984) were relatively homogeneous across 24 polymorphic allozyme loci (mean within $D_{NEI} = 0.07$) and distinct from each other (mean between $D_{NEI} = 0.21$). Further, the SM6 and CA6 lineages also differ substantially in thermal physiology and development rates (Kearney and Shine 2004a,b), in keeping with their distinct environmental niches (Kearney et al. 2003). Though there is substantial phylogeographic structure within each widespread chromosome form (SM6, CA6, EA6, A6–2, CYA6), our molecular data confirm the monophyly of each of these. The one possible exception, the paraphyly of SM6 relative to A6–2 forms in the mtDNA gene tree, was rejected in favor of the strong support for monophyly in the multilocus nuclear analysis.

In parallel with other recent studies of widespread lineages in the Australian arid and semiarid zones is our discovery of several new lineages (Pepper et al. 2008; Oliver et al. 2009), in the present case all from the monsoonal tropics of northern Australia. These include the Gulf lineage from the tropical savannas south of the Gulf of Carpentaria, the Top End lineage from north coastal Top End, and two geographically restricted lineages (Central Queensland and Northwest Queensland) from the Selwyn Ranges of northwest Queensland. Additionally, the deep divergences between these lineages are as great as those between the previously identified cytotypes. Interestingly, most of these lineages are distantly related, yet at least four overlap in the Selwyn Ranges in northwest Queensland (Central Queensland, Northwest Queensland, Gulf, and SM6), identifying this area as a hotspot of phylogenetic diversity and endemism (Rosauer et al. 2009). Several of these lineages are sufficiently distinct genealogically that they could be considered as distinct species. Our extended sampling has also revealed other instances of close parapatry (<100 km) between other combinations (Fig. S1): e.g., CA6 and SM6 in coastal WA; EA6 and CA6 in interior South and Western Australia and in western Queensland; and EA6, Gulf and CYA6 on the Einsleigh Uplands of northeast Queensland. We are continuing our investigations of species boundaries by examining these contact zones to quantify the extent of local introgression among the lineages and will present a formal taxonomic revision of the complex once those studies are completed.

DIVERSIFICATION AND PERSISTENCE OF *HETERONOTIA*

A long-held view is that the long-term aridification of Australia, together with concomitant intensification of fires, has both led to extinction and retraction of mesic biomes to coastal refugia and enabled diversification of arid-adapted lineages (reviewed in Crisp et al. 2004; Byrne et al. 2008). Our analyses of the *H. binoei* complex add further evidence by demonstrating the repeated evolution of arid-adapted lineages from mesic, mostly northern ancestors during the Pliocene, with subsequent within-lineage structuring through the Pleistocene cycles.

The major splits in *Heteronotia* occurred during the Pliocene (Fig. 5), after a Miocene colonization of Australia from a common ancestor with *Dixonius*. During this time period, Australia experienced its first strong aridification with expansion of *Acacia* and eucalypt-dominated woodlands, and perhaps isolation of semiarid biomes during the still poorly understood late Miocene

arid period (Martin 2006; Byrne et al. 2008). The brief return of warm, mesic conditions in the early Pliocene would have allowed incipient species to expand before again experiencing the onset of intense aridification in the mid-late Pliocene (Martin 2006; Byrne et al. 2008). Most of the *binoei* lineages originated during this period, most likely in ancestrally favored mesic habitats such those that now occur in the monsoonal tropics. Reconstruction of mesic versus arid environments on the phylogeny of *H. binoei* lineages provides strong evidence for a mesic ancestry (Fig. 4), with three independent origins of arid zone lineages tentatively dated to the Pleistocene (Figs. 4 and 5).

During the mid-late Pleistocene, especially from 400 kya, the climatic cycles were more pronounced (Kershaw et al. 2003; Hocknull et al. 2007; Byrne et al. 2008), which is expected to have greatly modified species ranges in arid and monsoonal regions. This had led to suggestions that species retracted their ranges to topographic refugia during cold and hyperarid glacial-periods, especially during the last glacial maximum, with the most recent reexpansion during the Holocene (Keast 1961; Cogger and Heatwole 1981; Schodde 1982). Two complementary approaches to testing this hypothesis are (1) to identify refugia as areas with enhanced species endemism, and (2) in widespread species, to test for genetic signatures of isolation between refugial areas and post-glacial expansion from them. Suggested refugia in the arid and monsoonal areas of Australia include the dissected areas of the Kimberley, Top End, and Selwyn Ranges in the monsoonal tropics, the Central Ranges in the core arid zone, and the Pilbara-Hamersley Ranges toward the central west coast (Morton et al. 1995; Byrne et al. 2008). Several studies have identified the Kimberley and Top End regions as pronounced centers of endemism in the north, but this is less obvious for the Central Ranges (Laffan and Crisp 2003; Slatyer et al. 2007; Rosauer et al. 2009). Recent studies of the Pilbara-Hamersley Ranges have revealed substantial microendemism of lineages (Pepper et al. 2008), and the same is true of rock-restricted species of *Heteronotia* for the Pilbara, Kimberley, and Central Ranges (M. Pepper, unpubl. data). Until now, evidence for narrow endemism in the Selwyn Ranges has been sparse, largely because of lack of knowledge (Morton et al. 1995); however, there are species of reptiles (*Gehyra robusta*; King 1983; *Demansia flagellatio*; Wells and Wellington 1985) and one rock wallaby (*Petrogale purpureicollis*; Eldridge et al. 2001) locally endemic to these rocky ranges, and several more species have population isolates there (i.e., *Ctenotus alacer*, *Varanus mitchelli*; see Wilson and Swan 2003).

The multilocus analyses of the *H. binoei* complex reinforces the notion that the monsoonal tropics include multiple refugial areas, especially in regions with relatively complex topography. This evidence for localized persistence is contrary to the hypothesis that this biome shifted entirely northwards, onto the exposed Sahul shelf, during the LGM (Ford and Blair 2005). For *H. binoei*, the Kimberly and Top End host genetically distinct populations of the A6–2 lineage (KA6 and WYA6 in the Kimberly, and NA6 in the Top End), and another divergent lineage ("Top End") is restricted to the north coast of the Top End. Of particular note is the discovery of two divergent lineages (Northwest Queensland, Central Queensland) restricted to the Selwyn Ranges. Northwestern Queensland is a particularly diverse region for *Heteronotia*, as several divergent lineages (SM6, Gulf, Central Queensland, Northwest Queensland) overlap where the savanna plains of the Gulf and Barkly Tablelands intersect with the rocky hills and gorges of the Selwyn Ranges (Fig. 1). These lineages represent deep divergences, having arisen during the short period when the other major groups of *Heteronotia* originated in the Pliocene. Thus, the evidence from *Heteronotia* provides strong support for the hypothesis that the Selwyn Ranges, along with dissected areas of the Top End and Kimberley, acted as a discrete refugium during the Pleistocene climate cycles. Strong signatures of population expansion for the Gulf lineage suggest that this lineage could have expanded from the Selwyn Ranges, or an as yet unidentified refugium, as savanna habitats expanded across the Gulf plains during the late Pleistocene/Holocene.

Phylogeographic patterns within widespread lineages of *H. binoei* are also informative about persistence and expansion of arid-zone populations in response to mid-late Pleistocene cycles. Previous analyses using Nested Clade Analysis of mtDNA suggested fragmentation between central and western populations of CA6 and expansion of SM6 from central to western Australia, both in the early Pleistocene; and late Pleistocene expansions of the EA6 lineage from eastern to the southern and western arid zone (Strasburg and Kearney 2005). Range fluctuations driven by late Pleistocene climate change also set the stage for hybridization between western populations of CA6 and SM6 lineages, giving rise to multiple triploid parthenogenetic lineages that subsequently expanded rapidly through the western, central, and southern deserts (Moritz 1993; Kearney et al. 2006). Our multilocus analyses support and extend these inferences. The CA6 lineage is spatially structured, with a lower diversity in the western isolate than in central Australia, and an inferred divergence time in the mid-Pleistocene. The disjunct distribution of CA6 may have arisen from an expansion of sandy, spinifex-dominant deserts that split the *Acacia* shrub habitat preferred by CA6 (Kearney et al. 2003), although an *Acacia* corridor extending across the sandy deserts from central into western Australia could have provided a means of migration during this period (Chapple et al. 2004; Shoo et al. 2008). Similarly, the SM6 lineage includes two divergent sublineages of the same age that diverged $\sim$1.5 ($\pm$0.79) mya; one spread through the central and western deserts (combined nuclear nucleotide diversity = 0.00342), and the other in the monsoonal woodlands and savanna in northern Australia (combined nuclear nucleotide diversity = 0.00589). Both summary statistics and

coalescent (Extended Bayesian Skyline Plots) analyses support strong population growth in the arid zone CA6 and SM6 lineages, especially as the climate cycles intensified from the mid Pleistocene onwards.

Our results for *Heteronotia* are consistent with a growing number of studies that have revealed substantial and unrecognized diversity in the arid zone and monsoonal regions of Australia. This pattern is also seen in other reptile species complexes, including *Diplodactylus* and *Heteronotia spelea* geckos in the Pilbara (Pepper et al. 2006; M. Pepper, unpubl. data), the king brown snake (*Pseudechis australia*; Kuch et al. 2005), earless dragons (*Tympanocryptis*; Shoo et al. 2008), *Gehyra* geckos (Sistrom et al. 2009), and diplodactyline geckos (Oliver et al. 2009). This diversity originated in the Pliocene or earlier, during a period that fluctuated between increasing aridity and a brief return of mesic conditions, that may have fractured populations among multiple, localized refugia. In addition, a few lineages of *Heteronotia* (CA6, EA6, SM6) experienced range expansions into and throughout the arid zone accompanying mid-late Pleistocene climatic cycling. Examples in other systems, including several birds (Driskell et al. 2002; Joseph et al. 2002; Joseph and Wilke 2007; Kearns et al. 2009), sexual and asexual *Warramaba* grasshoppers (Kearney and Blacket 2008), crayfish (Nguyen et al. 2004), and parthenogenetic lineages of *Heteronotia* (Strasburg et al. 2007) comprise a growing body of evidence supporting range expansion as general biotic response to Pleistocene climate fluctuations in the Australian arid zone. These results reinforce emerging paleoecological evidence for large-scale habitat fluctuations, yet also localized persistence within refugia. More of such analyses are needed to document the species and phylogenetic endemism within these and other potential refugia, with obvious relevance to conservation (Moritz 2002; Morton et al. 1995; Carnaval et al. 2009; Rosauer et al. 2009).

Transitions between biomes are uncommon (Crisp et al. 2009), but recent aridification (<15 mya) in the southern hemisphere has opened new habitats that allowed invasion from older (already inhabited) mesic environments into younger arid lands (Rabosky et al. 2007). These lineages are younger than their mesic-inhabiting ancestors, suggesting that adaptation to arid conditions is recent. Arid zones on three continents experienced very similar climatic changes and concomitant biotic responses to those changes (Richardson et al. 2001; Ortiz-Jaureguizar and Cladera 2006; Byrne et al. 2008; Cowling et al. 2009). These responses include recent diversification and population expansions into the new arid-adaptive zone as well as range fluctuations into and out of refugia. The patterns seen in *Heteronotia* are consistent with those seen in these other Southern Hemisphere systems—diversification and persistence in the monsoonal tropics, and range expansions in the arid zone. Continued work on identifying and characterizing refugia (such as the Selwyn Ranges described in this study) and greater emphasis on the history of diversification and demographic fluctuations across the interface of arid and mesic biomes of the Southern Hemisphere will contribute to a global view of biotic responses to climate change during the late Neogene.

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Appendix. Samples used in this study and their associated locality data.

ID[1]	Acc.[2]	Locality	State	Latitude	Longitude	karyotype?[3]
Arnhem_1	28209	Murgenella	NT	−11.5528	132.925	No
Arnhem_2	28215	Murgenella	NT	−11.5528	132.925	No
Arnhem_3	29977	Taracumbie Falls, Melville Island	NT	−11.6667	131	No
CA6_01	11662	Ninghan	WA	−29.4167	117.3	No
CA6_02	31229	Wigley Waterhole	NT	−23.633	133.883	Yes
CA6_03	31348	Native Gap	NT	−22.806	133.418	Yes
CA6_04	31355	Wooramel	WA	−25.7167	114.2833	Yes
CA6_05	31357	Wooramel	WA	−25.7167	114.2833	Yes
CA6_06	MKF99	Granite Downs	SA	−26.937	133.495	No
CA6_07	31651	Kulgera	NT	−25.8333	133.3	Yes
CA6_08	32331	Nanga	WA	−26.25	113.8167	Yes
CA6_09	32338	Nanga	WA	−26.25	113.8167	Yes
CA6_10	32344	Carrarang	WA	−26.4167	113.5167	Yes
CA6_11	32346	Ninghan	WA	−29.4167	117.3	Yes
CA6_12	32375	Tamala	WA	−26.7	113.75	Yes
CA6_13	32437	Chandler	SA	−27	133.317	Yes
CA6_14	32459	Billabong	WA	−26.817	114.617	Yes
CA6_15	32920	Yoothapinna	WA	−26.533	118.517	Yes
CA6_16	33880	Mimili	SA	−27	132.5	No
CA6_17	33881	Mimili	SA	−27	132.5	No
CA6_18	41618	Mt Woodroffe	SA	−26.3333	131.75	No
CA6_19	52485	Victory Well	SA	−27.054	132.506	No
CA6_20	52489	Victory Betty Well	SA	−27.023	132.424	No
CA6_21	52491	between Victory and Betty Well	SA	−27.023	132.424	No
CA6_22	52493	between Victory and Betty Well	SA	−27.023	132.424	No

Continued.

Appendix. Continued.

ID[1]	Acc.[2]	Locality	State	Latitude	Longitude	karyotype?[3]
CA6_23	52496	Everard Ranges	SA	−27.0833	132.4667	No
CA6_24	52497	Everard Ranges	SA	−27.0833	132.4667	No
CA6_25	52498	between Victory and Betty Well	SA	−27.023	132.424	No
CA6_26	9067	30 KM SE OF SPRINGVALE STN	QLD	−23.55	140.7	No
CA6_27	31441	Ross River	NT	−23.8	134.5667	No
CA6_28	72890	47k E Julia Ck	QLD	−20.712	142.18699	No
centralQld_1	82433	Phosphate Hill, Acacia Site	QLD	−21.8248	139.96485	No
centralQld_2	8942	37 KM NNE OF MOUNT ISA	QLD	−20.434	139.63105	No
CYA6_01	31241	Mapoon Mission	QLD	−11.9667	141.9	No
CYA6_02	77094	31k N Einasleigh on Mt Surprise Rd	QLD	−18.2945	144.0766	Yes
CYA6_03	77126	35k E Mt Surprise on Gulf Developmental Rd	QLD	−18.1477	144.3161	No
CYA6_04	SEW7634	Wolverton	QLD	−13.3171	142.9511	No
EA6_01	31925	Waterloo Station	QLD	−24.723	152.00803	No
EA6_02	31926	Waterloo Station	QLD	−24.723	152.00803	No
EA6_03	33018	Shoalwater Bay	QLD	−22.6868	150.44049	No
EA6_04	6995	Kennedy	QLD	−18.2053	145.95673	No
EA6_05	72898	22k S Torrens Ck	QLD	−20.9853	145.01258	No
EA6_06	72963	14k NW Longreach on Landsborough H/way	QLD	−23.3566	144.16654	No
EA6_07	31375	Rawlinna	WA	−31.0167	125.3333	Yes
EA6_08	3886	Cooyar Ck. Crssng	QLD	−26.7351	152.24962	No
EA6_10	72911	3k N Lolworth HS	QLD	−20.1833	145.0167	No
EA6_11	72967	Tambo dump	QLD	−24.8845	146.25321	No
EA6_12	77207	69k S Alpha on Alpha-Tambo Rd	QLD	−24.192	146.55742	No
EA6_13	79485	9k N NSW/QLD border on Mitchell Highway	QLD	−28.9235	145.73483	No
EA6_14	MKF118	Copper Hills	SA	−27.95	134.313	No
EA6_15	MKF129	Evelyn Downs	SA	−28.2047	134.48833	No
EA6_16	MKF35	∼9.8 km north of Bower, South Australia	SA	−34	139.35	No
gulf_01	32873	Westmoreland	QLD	−17.3333	138.2333	Yes
gulf_02	32874	Hells Gate	QLD	−17.4536	138.3575	No
gulf_03	32941	Hells Gate	QLD	−17.4536	138.3575	Yes
gulf_04	32944	Westmoreland	QLD	−17.3333	138.2333	Yes
gulf_05	32945	Westmoreland	QLD	−17.3333	138.2333	Yes
gulf_06	32950	Westmoreland	QLD	−17.3333	138.2333	No
gulf_07	32955	Westmoreland	QLD	−17.3333	138.2333	Yes
gulf_08	70786	Karumba Rd 42 Km S Karumba	QLD	−17.8736	140.83528	No
gulf_09	70807	Flinders River; Normanton	QLD	−17.67	141.07918	No
gulf_10	72768	Bang Bang Jumpup, On Burke Dev Rd, Wandoola T/off	QLD	−18.5333	140.6667	No
gulf_11	72874	Burke & Wills RH Dump	QLD	−19.2275	140.34733	No

Continued.

Appendix. Continued.

ID[1]	Acc.[2]	Locality	State	Latitude	Longitude	karyotype?[3]
gulf_12	77110	35k WNW The Lynd Junction on Einsasleigh Rd (Carpentaria Downs Station)	QLD	−18.7218	144.3244	No
gulf_13	77115	The Lynd Roadhouse	QLD	−18.8808	144.5471	No
gulf_14	77117	The Lynd Roadhouse	QLD	−18.8808	144.5471	No
gulf_15	MKF563	Lanark Station	QLD	−20.529	140.41405	No
gulf_16	MKF577	Yeldham Station	QLD	−18.6285	139.02338	No
KA6_01	31253	Drysdale	WA	−15.7	126.36667	Yes
KA6_02	31377	Doongan	WA	−15.3667	126.3	Yes
NA6_01	31408	Mataranka	NT	−14.9333	133.15	Yes
NA6_02	31424	Berrimah	NT	−12.4667	130.8333	Yes
NWQld_01	32946	Lawn Hill Station	QLD	−18.77	138.387	Yes
NWQld_02	32989	Lawn Hill Station	QLD	−18.77	138.387	Yes
NWQld_03	Q348	Lawn Hill Station	QLD	−18.77	138.387	Yes
SM6_01	31398	Nita Downs	WA	−19.086	121.677	Yes
SM6_02	31407	Warachope	NT	−20.6	134.25	Yes
SM6_03	31254	Notabilis Hill	WA	−25.657	125.556	Yes
SM6_04	31255	The Granites	NT	−20.5833	130.35	Yes
SM6_05	32406	Earahedy	WA	−25.4167	121.58333	Yes
SM6_06	32783	Mt Edgar	WA	−21.3333	120.05	Yes
SM6_07	32953	Barrows Creeks	NT	−21.55	133.8833	Yes
SM6_08	MKF302	Useless Loop	WA	−26.1391	113.40334	No
SM6_09	Q262	Nanutarra	WA	−22.55	115.5	Yes
SM6_10	31391	Daly Waters	NT	−16.25	133.3667	Yes
SM6_11	31410	Willeroo	NT	−15.2833	131.5833	Yes
SM6_12	31416	Halls Creek	WA	−18.2667	127.7667	Yes
SM6_13	31724	Camooweal	QLD	−19.9167	138.1167	Yes
SM6_14	32865	McArthur R Stations	QLD	−16.45	136.1167	Yes
SM6_15	32882	McArthur R Stations	QLD	−16.45	136.1167	Yes
SM6_16	32898	Camooweal	QLD	−19.9167	138.1167	Yes
SM6_17	32900	Camooweal	QLD	−19.9167	138.1167	Yes
SM6_18	32902	Camooweal	QLD	−19.9167	138.1167	Yes
SM6_19	32943	Lawn Hill NP	QLD	−18.7019	138.48455	Yes
SM6_20	32956	Lawn Hill Station	QLD	−18.77	138.387	Yes
SM6_21	32987	Lawn Hill Station	QLD	−18.77	138.387	Yes
SM6_22	82420	Phosphate Hill, Snappy Site	QLD	−21.8248	139.96485	No
SM6_23	MKF547	West Leichhardt	QLD	−20.5974	139.70423	No
SM6_24	MKF605	Adels Grove	QLD	−18.6946	138.53177	No
spelea_01	31199	Undoolya	NT	−23.6833	134.0333	Yes
spelea_02	31256	Bamboo Creek	WA	−20.9333	120.2167	Yes
spelea_03	31432	Ross River	NT	−23.8	134.5667	Yes
spelea_04	31636	Ross River	NT	−23.8	134.5667	Yes
spelea_05	32833	Bamboo Creek	WA	−20.9333	120.2167	Yes
spelea_06	11740	Newman	WA	−23.321	120.1	No
spelea_07	32937	Newman	WA	−23.321	120.1	No
WYA6_01	31381	Wyndham	WA	−15.4667	128.1	Yes
WYA6_02	31382	Wyndham	WA	−15.4667	128.1	Yes

[1]Australian Biological Tissue Collection Numbers, unless otherwise noted.

[2]Bold samples were used in the BEST analysis.

[3]Availability of karyotypes from C. Moritz (yes or no).

Supporting Information

The following supporting information is available for this article:

Figure S1. Sampling of all genetic samples (mtDNA sequenced and karyotyped individuals) to show the current knowledge of the distribution of the chromosomal races (Moritz et al. 1990; Strasburg and Kearney 2005; this study).
Figure S2. (A)–(I) Nuclear gene trees; only erh is unrooted; all others are rooted with Dixonius vietnamensis.

Supporting Information may be found in the online version of this article.

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