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Phylogenetic evidence of historic mitochondrial introgression and cryptic diversity in the genus *Pseudemoia* (Squamata: Scincidae)

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ABSTRACT

The Australian scincid genus *Pseudemoia* comprises six morphologically similar species restricted to temperate south-eastern Australia. Due to the high degree of morphological conservatism, phylogenetic relationships and taxonomic status within the *Pseudemoia entrecasteauxii* complex (comprising the nominal species *P. entrecasteauxii*, *P. cryodroma*, and *P. pagenstecheri*) remains unresolved. To further investigate the phylogenetic relationships and taxonomic status of *Pseudemoia* spp., and to test the hypothesis that *P. cryodroma* evolved from hybridization between *P. entrecasteauxii* and *P. pagenstecheri*, we sequenced one mitochondrial locus (ND4) and five nuclear loci (β-globin, LGMN, PRLR, Rhodopsin, RPS8). While we find strong support for the monophyly of the *P. entrecasteauxii* complex, there exists marked incongruence between the mitochondrial and nuclear markers, particularly in regards to the high altitude specialist, *P. cryodroma*. The most parsimonious explanation of this discordance is historic mitochondrial introgression, although a hybrid origin for *P. cryodroma* cannot be completely rejected. Within *P. pagenstecheri sensu lato*, we identified a strongly supported, highly divergent yet morphologically cryptic lineage restricted to northern New South Wales. Although more weakly supported by the nuDNA, we also identified a second geographically distinct lineage of *P. pagenstecheri s.l.*, which may warrant separate conservation management. Our study reveals a more complex evolutionary history of the genus *Pseudemoia* than previously appreciated and contributes to our understanding of the biogeography and evolution of Australian mesic zone fauna.

1. Introduction

Australia's reptile fauna is amongst the most diverse in the world. Members of the family Scincidae comprise approximately half of this diversity, with over 431 species in 40 genera (Wilson and Swan, 2013). In Australia, skinks are the dominant terrestrial vertebrate group in terms of species richness and distribution, which is in contrast to most regions of the world where skinks are a minor component of the reptile diversity (Wilson and Swan, 2013). Skinks inhabit every part of the Australian continent, and range from oviparous, leaf litter generalists to viviparous, rock-dwelling specialists. Despite high overall diversity, ecologically similar skink species are often morphologically conserved, with few reliable external characters distinguishing species. Even when present, these characters are often subtle, such as the presence or alignment of a single scale. Consequently, molecular phylogenetic studies frequently indicate the presence of cryptic species, which suggests that the true number of species is far greater than currently recognized (Couper et al., 2006; Horner and Adams, 2007; Hoskin and Couper, 2012; Kay and Keogh, 2012).

The Eugongylus group represents one of the five major lineages of the subfamily Lygosominae, three of which are found in Australia (Honda et al., 2000; Skinner et al., 2011). The ancestor of this lineage is thought to have colonized Australia from South-east Asia during the Oligocene (Honda et al., 2000; Skinner et al., 2011) and occupied the once-widespread mesic biome (Byrne et al., 2011). As the Australian continent began to aridify in the Miocene (Bowler, 1982; Hope, 1982; Nix, 1982), few Eugongylus taxa colonized the arid zone, with the majority of genera restricted to the retracting mesic zone of eastern Australia. Throughout the Plio-Pleistocene, these habitats experienced further contraction and fragmentation during glacial periods followed by limited expansion during inter-glacial periods (Bowler, 1982; Markgraf et al., 1995; Nix, 1982). By the end of the Pleistocene, the mesic zone, and therefore

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a large proportion of Eugongylus taxa, was constrained to the continent's eastern and southern coasts and the island of Tasmania (Bowler, 1982; Markgraf et al., 1995).

The impact of the decline and fragmentation of the mesic zone on the evolution of Eugongylus taxa is reflected in its current phylogenetic diversity. Major splits within this group first appear in the Miocene (Skinner et al., 2011), corresponding to the start of aridification (Bowler, 1982; Hope, 1982). The formation of dry habitat corridors, uplift of mountain ranges, and flooding of marine basins during the Plio-Pleistocene are thought to have further fragmented mesic habitats and created barriers to gene flow (Chapple et al., 2011c). This has been particularly well studied in north-eastern Australia where biogeographic breaks such as the Black Mountain Corridor, Burdekin Gap, and St. Lawrence Gap have been shown to correspond to interspecific and/or intraspecific divergence in the Eugongylus genera *Carlia* (Dolman and Moritz, 2006; Stuart-Fox et al., 2002), *Lampropholis* (Chapple et al., 2011c), and *Saproscincus* (Moussalli et al., 2005), as well as frogs (James and Moritz, 2000; Schäuble and Moritz, 2001) and birds (Joseph and Moritz, 1994). In contrast, little research has examined the historical biogeography of any taxa endemic to temperate south-eastern Australia, much less the Eugongylus group (but see Chapple et al., 2011b; Dubey and Shine, 2010).

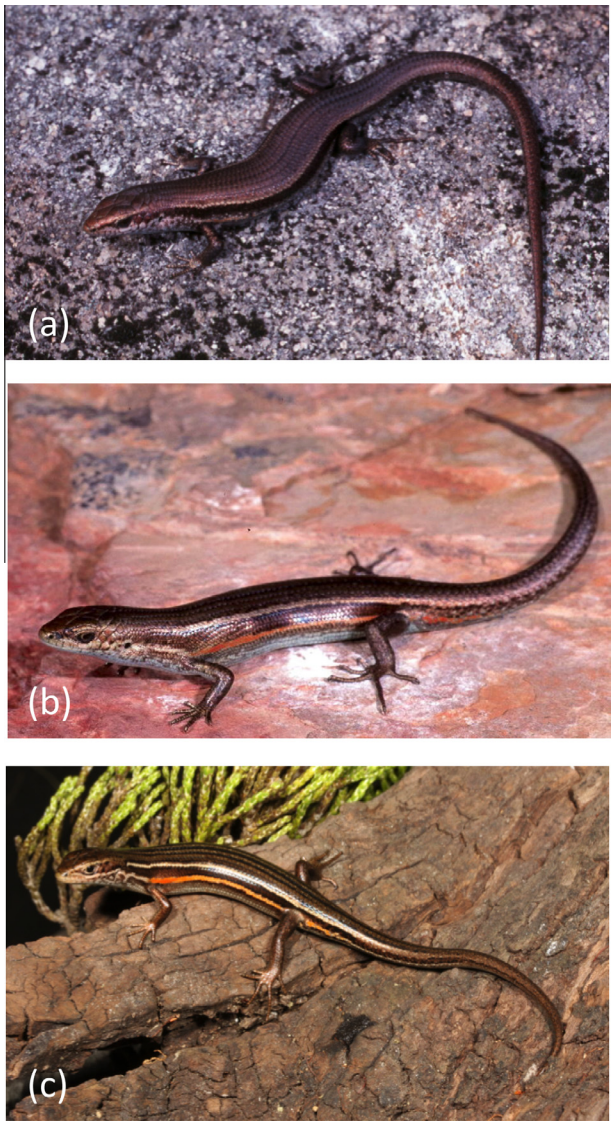

Fig. 1. Representative *Pseudemoia* males: (a) *P. entrecasteauxii*; (b) *P. cryodroma*; and (c) *P. pagenstecheri*.

Within the Eugongylus group, the genus *Pseudemoia* provides an ideal model with which to investigate the complex evolutionary history of temperate taxa endemic to south-eastern Australia. *Pseudemoia* comprises six species with yet-to-be resolved phylogenetic relationships, all of which are restricted to temperate south-eastern Australia (Wilson and Swan, 2013). Congeners are morphologically conserved and have variable color patterning that overlaps between species, even in sympatry (Clemann, 2002; Fig. 1). Prior to allozyme and karyotype analyses by Hutchinson and Donnellan (1992), *P. entrecasteauxii* included what is now *P. cryodroma* and *P. pagenstecheri*, and to date there are still few external characters that consistently differentiate these species (Hutchinson and Donnellan, 1992). The endangered *Pseudemoia cryodroma* (DSE, 2013) is morphologically intermediate to and sympatric with the more widespread *P. entrecasteauxii* and *P. pagenstecheri*, and it has been hypothesized that *P. cryodroma* speciated as a result of hybridization between those two congeners (Hutchinson and Donnellan, 1992). Although subsequent genetic analyses combining mitochondrial and nuclear loci have been performed by Smith (2001); the sample size was insufficient to test for a hybrid origin. However, the conflicting relationships between *P. cryodroma*, *P. entrecasteauxii*, and *P. pagenstecheri* among the allozyme, karyotype, and combined mtDNA and nuDNA analyses (Hutchinson and Donnellan, 1992; Smith, 2001) do suggest that some form of introgression occurred during the evolution of these species.

Here we present a molecular phylogeny of *Pseudemoia*, with the aim of further resolving the evolutionary relationships within the *P. entrecasteauxii* complex. Using mitochondrial and nuclear loci, we investigate the monophyly of each species and assess evidence of past introgression between species. Of particular interest is the species origin and taxonomic status of the geographically restricted, high altitude species *P. cryodroma*. In addition, this study contributes to a growing body of work aimed at better understanding the evolutionary history and biogeography of the eastern Australian mesic biome.

2. Methods

2.1. Sampling

Our sampling regime included all *Pseudemoia* species and spanned the distributional range of each species, with 123 samples in total: *Pseudemoia baudini* ($n = 3$), *P. cryodroma* (24), *P. entrecasteauxii* (38), *P. pagenstecheri* (52), *P. rawlinsoni* (3), and *P. spenceri* (3) (Fig. 2; for sampling details see Table S1, Supporting Information). These samples were obtained from either the Australian Museum, South Australian Museum or collected in the field. Tissue samples for outgroup taxa within the Eugongylus group, namely *Acritoscincus duperryi* (NMVZ21485), *Cryptoblepharus plagiocephalus* (NMVD72651, NMVZ18962), *Morethia ruficauda* (NMVZ19114), and *Niveoscincus metallicus* (NMVD75207, NMVZ21551) were provided by Museum Victoria.

2.2. DNA extraction, amplification, and sequencing

Genomic DNA was extracted from samples using either a Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen, Hilden, Germany) or a GenCatch Blood and Tissue Genomic DNA Miniprep Kit (Epoch Life Sciences, Missouri City, Texas, USA). We amplified and sequenced the mitochondrial fragment NADH subunit 4 (ND4) and partial tRNAs for all samples ($n = 127$). We selected a subset of individuals ($n = 47$) representing each of the mtDNA clades, and sequenced these individuals for five nuclear loci: ß-globin and Rhodopsin (Dolman and

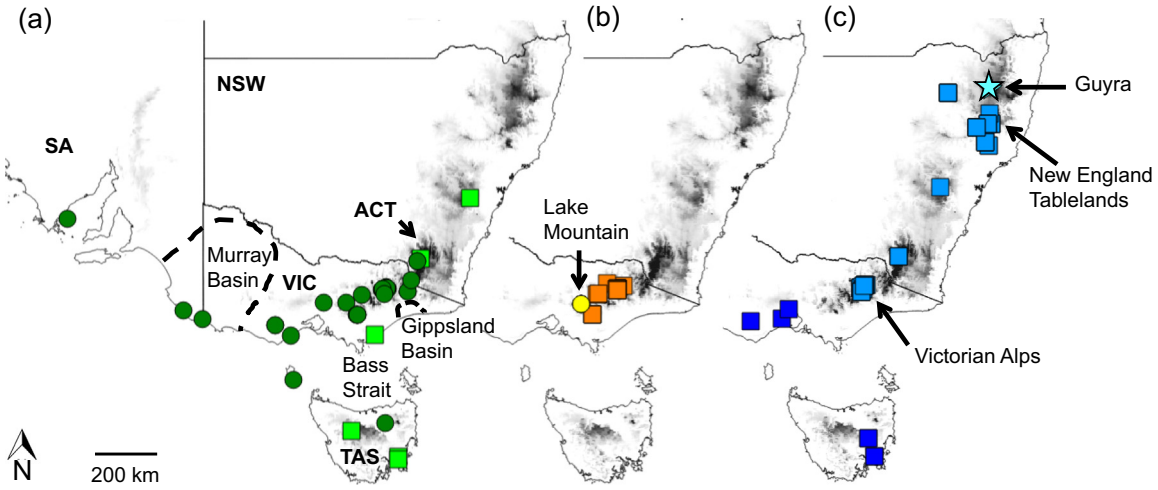

Fig. 2. Collection localities of *Pseudemoia* samples and relevant biogeographic regions. (a) *P. entrecasteauxii*; (b) *P. cryodroma*; and (c) *P. pagenstecheri*. Symbols represent mtDNA groupings designated in Fig. 3. State abbreviations are as follows: ACT = Australian Capital Territory; NSW = New South Wales; SA = South Australia; TAS = Tasmania; VIC = Victoria.

Phillips, 2004), RPS8 (Bell et al., 2010), LGMN (Singhal and Moritz, 2012), and PRLR (Townsend et al., 2008). To increase amplification success, internal primers were developed for ß-globin and LGMN using Primer3 v. 0.4.0 (Untergrasser et al., 2012). Polymerase chain reactions were performed in 20 μL reactions containing 0.5 μL of each primer (10 uM), 10 μL GoTaq Hot Start Master Mix (Promega, Madison, Wisconsin, USA), and 2 μL genomic DNA (diluted to 1:10). All primer sequences and PCR protocols are listed in Table S2 (Supporting Information).

PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) or gel purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany), and sequenced by Macrogen, Inc. (Seoul, South Korea). DNA sequences were edited in Geneious v. 6.1.2 (Biomatters, Auckland, New Zealand, available at: http://www.geneious.com/) and aligned using the default clustering algorithm. Heterozygous sites were coded using IUPAC ambiguity codes. All protein-coding regions were translated to amino acids using Geneious 6.1.2 (Biomatters), and no premature stop codons were observed. Sequences were deposited in GenBank (Table S1, Supporting Information).

2.3. Phylogenetic analyses

For the mtDNA gene region (full dataset) and the five nuclear loci (reduced 47 sample subset), we conducted Bayesian and Maximum Likelihood (ML) analyses using MrBayes 3.2 (Ronquist and Huelsenbeck, 2003) and RAxML 7.2.8 (Stamatakis et al., 2008), respectively. Prior to analysis, identical haplotypes for the mitochondrial gene region ND4 were omitted and a 17 bp segment from the middle of the ß-globin sequence was removed due to difficulties in alignment. We assessed partitioning schemes and models of best-fit based on Akaike Information Criterion (AIC) using MrModeltest 2.3 (Nylander, 2004) for each locus. Due to well-supported conflict in phylogenetic reconstruction between the mitochondrial and nuclear markers, we did not combine these two datasets into one analysis. For the nuclear dataset, we implemented a partitioned, mixed model in MrBayes, and a partitioned GTR + Γ model in RAxML. All Bayesian analyses consisted of two independent runs, each with four chains of Markov Chain Monte Carlo (MCMC). Analyses were run for five million generations, chains were sampled every 500 generations, and chain convergence was confirmed using an assessment of average standard deviation of split frequencies (<0.01). A maximum likelihood analysis was run for each locus with the suggested GTR + Γ model with 100 bootstraps using RAxML-HPC BlackBox (Stamatakis et al., 2008) on the CIPRES Science Gateway (Miller et al., 2010). Using only the full mtDNA dataset, we calculated uncorrected mean pairwise genetic distances between major clades using MEGA5 (Tamura et al., 2011).

2.4. Species trees and comparison of alternative topologies

We implemented a multi-species coalescent model (*BEAST) in BEAST 1.8 (Drummond et al., 2012) to generate species trees for the nuDNA only dataset, and the combined mtDNA and nuDNA dataset. For the *BEAST analyses, individuals were grouped *a priori* into 10 different "species" based on (1) current taxonomy and (2) mtDNA clades. Some loci could not be sequenced for all individuals; only 41 individuals were sequenced for ß-globin, 43 for LGMN, and 32 for RPS8. Missing data were randomly distributed among groups, and all samples were sequenced for at least five of the six loci with the exception of one outgroup (*A. duperryi*), which was missing data for two loci. Although missing data can be problematic (Lemmon et al., 2009), previous studies have demonstrated that including individuals with incomplete data is often better than excluding them (Roure et al., 2013; Wiens and Morrill, 2011). To assess the impact of missing data on our analyses, we first conducted a *BEAST analysis with all five nuclear loci and then reran the analysis omitting RPS8, which had the most missing data. For all analyses, we used an uncorrelated lognormal relaxed molecular clock and Yule speciation prior. Analyses were run twice for 50 million generations (sampling every 500 generations). Log and tree files for each pair of runs were combined using LogCombiner 1.8 (Drummond et al., 2012). Effective sample size (ESS) was examined in Tracer 1.5 (Rambaut and Drummond, 2007). Maximum credibility species trees were generated using TreeAnnotator 1.8 (Drummond et al., 2012), and the first 25% of trees were excluded as a burn-in. Trees were visualized in FigTree 1.4 (Rambaut, 2012).

We also calculated Bayes Factors to assess support for competing tree topologies in the concatenated nuDNA dataset. We implemented the stepping stone method (Xie et al., 2011) in MrBayes 3.2 to compare estimates of mean marginal likelihood for unconstrained, constrained, and negatively constrained topologies. Constrained topologies included only those topologies with the given groups constrained to be monophyletic. In contrast, negatively constrained topologies comprise topologies where the given groups are

Table 1
Fossil calibrations for estimation of *Pseudemoia* divergence times, including the settings implemented, for relaxed clock analyses in BEAST.

Fossil age	Description	Calibration	BEAST settings (lognormal distribution)	
			Zero offset	Std. dev.
Middle Jurassic (155 Mya)	Paramacelloidid scincomorph and potential anguimorph fossils (Evans, 1994, 1998; Evans and Chure, 1998)	Minimum divergence of Anguimorpha + Scincomorpha	155	1.4
Miocene (16–19 Mya)	Earliest record of *Oligosoma* in New Zealand (Lee et al., 2009)	Minimum age estimate of *Oligosoma*	16	0.8
Pliocene (3.6 Mya)	Scincid fossils from Riversleigh and Bluff Downs, Queensland, Australia (Hutchinson, 1992; Mackness et al., 2000; Shea and Hutchinson, 1992)	Common ancestor of *Bellatorias* + *Egernia* + *Tiliqua*	3.6	1.2

not monophyletic. The constraints tested reflected current taxonomy and major lineages well supported in the mtDNA analyses. We excluded *P. pagenstecheri* samples from Guyra, NSW (*P. pagenstecheri* A) from the *P. pagenstecheri* taxonomic constraints because this lineage forms a well-supported clade separate from the remaining *P. pagenstecheri* samples in both the mtDNA and nuDNA trees. Stepping stone estimates were run for 2 million generations. The strength of evidence in favor of a given constraint over the negative constraint was determined by calculating the Bayes Factor, the arithmetic difference between marginal likelihoods in log units, of the constrained topology compared to the negative constraint. Bayes Factors from 0 to 1 provide no evidence, 1 to 3 provide some evidence, 3 to 5 provide strong evidence, and greater than 5 provide very strong evidence in favor of the constrained topology whereas negative values show support in favor of topologies where the constraint is not present (Kass and Raftery, 1995).

2.5. Estimates of divergence time

Divergence times for the major clades within *Pseudemoia* were estimated using a relaxed clock method and Yule speciation prior in the program BEAST 1.8 (Drummond et al., 2012). We only included a subset of in-group taxa from the mtDNA dataset in order to have a more balanced tree. To accurately place calibration points, additional sequences were downloaded from GenBank and included in the analysis (GenBank accession numbers are provided in Fig. S3, Supporting Information). Since fossils are a minimum estimate of age, we used lognormal distributions for three fossil calibration points (Table 1). Each in-group for the fossil calibration points was constrained to be monophyletic, and a random starting tree was employed. The analysis was run for 50 million generations (sampling every 500 generations) using an uncorrelated lognormal relaxed molecular clock and a GTR + I + Γ model of evolution. The output was examined in Tracer 1.5 (Rambaut and Drummond, 2007) to check that stationarity had been reached, ensure sufficient estimated sample size (ESS), and assess autocorrelation of rates from ancestral to descendant lineages. The program Tracer was also used to determine mean substitution rates. Trees were summarized using TreeAnnotator 1.8 (Drummond et al., 2012), and the first 25% of trees were excluded as a burn-in. Trees were visualized in FigTree 1.4 (Rambaut, 2012).

3. Results

3.1. Mitochondrial genealogy

The ND4 locus included the ND4 protein-coding gene and two tRNAs for a total of 798 bp, 329 variable sites, and 282 parsimony informative sites (Table 2). Phylogenetic analysis of the mtDNA supports the monophyly of the *P. entrecasteauxii* species complex, comprising the three nominal species *P. pagenstecheri*, *P. entrecasteauxii* and *P. cryodroma*, and a sister relationship between *P. baudini* and *P. rawlinsoni* (Fig. 3). The relationship between these two clades and *P. spenceri* remains unresolved. Within the *P. entrecasteauxii* species complex, there are three well-supported lineages: (1) *P. entrecasteauxii* A which comprises the majority of *P. entrecasteauxii* samples from New South Wales (NSW), South Australia, Victoria and Tasmania, and *P. cryodroma* A which represents a single southern population from Lake Mountain, Victoria; (2) *P. pagenstecheri* A, a highly divergent lineage from Guyra, NSW at the northern-most part of its range, which forms a well-supported sister relationship with the first lineage; and (3) *P. pagenstecheri* B from high elevation areas in NSW and the Victorian Alps, *P. pagenstecheri* C from lowland regions of western Victoria and Tasmania; *P. cryodroma* B, comprising the majority of *P. cryodroma* from the Victoria Alps; and *P. entrecasteauxii* B, which includes samples from across a broad geographic range, spanning from NSW to Tasmania. Uncorrected net mean pairwise genetic distance between the three lineages was high, ranging from 6.9% to 9.8% (Table 3).

Table 2
Fragment size, number of variable and parsimony informative sites for in-group taxa, and model of evolution applied to each locus.

Locus	Fragment size (bp)	Variable sites	Parsimony informative sites	Model
ND4	798	329	282	GTR + I + Γ
β-globin	735	83	52	GTR + I + Γ
RPS8	594	48	29	HKY + Γ
LGMN	288	27	18	HKY + I
PRLR	544	33	20	GTR + Γ
Rhodopsin	365	12	10	HKY

Phylogenetic diversity varies within each of the three major lineages of the *P. entrecasteauxii* species complex. For the first lineage comprising *P. entrecasteauxii* A and *P. cryodroma* A, phylogenetic diversity is low, with no genetic differentiation between the two nominal species. There is shallow phylogeographic substructure within *P. entrecasteauxii* A, with a genetic break between eastern and western Victorian populations, as well as between populations on either side of the Murray Basin (see Fig. 2). In contrast, there is high phylogenetic diversity and strong phylogeographic structure within the third lineage, with a maximum net sequence divergence of 6.5% between *P. pagenstecheri* B and *P. pagenstecheri* C. With the exception of two clades, namely *P. pagenstecheri* C and *P. entrecasteauxii* B, this third lineage predominantly represents high altitude (>900 m) populations ranging from the New England Tablelands in north-east New South Wales to the central Highlands of Victoria (see Fig. 2). In contrast, *P. pagenstecheri* C is restricted to the lowlands (<300 m) of western Victoria and Tasmania.

3.2. Multi-locus nuclear DNA analyses

The nuclear dataset included 43 individuals of *Pseudemoia* spp. sequenced for up to five loci, comprising 2524 bp of which 210

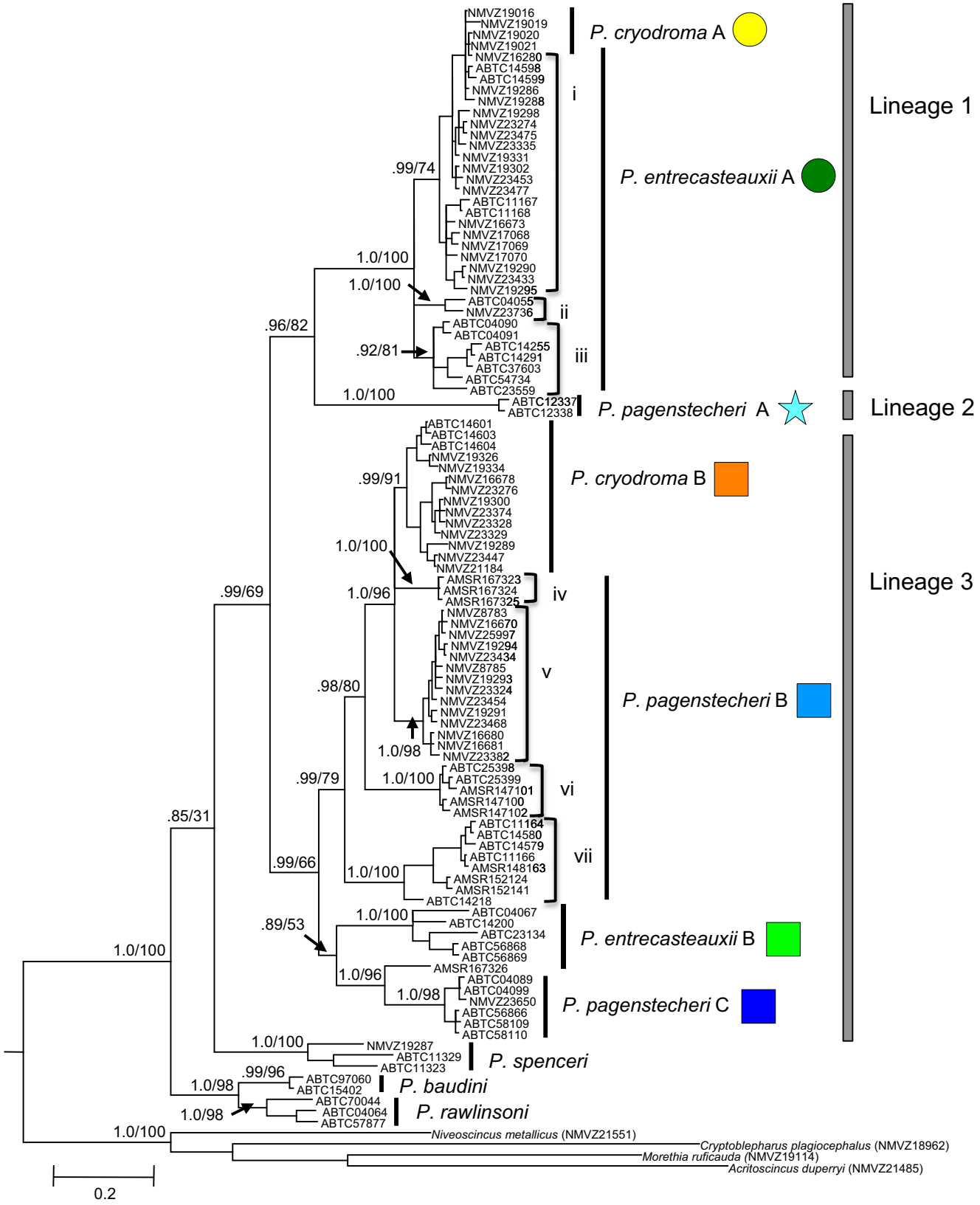

Fig. 3. Bayesian tree based on ND4 haplotypes. Bayesian posterior probabilities ⩾0.95 (left) and ML bootstraps ⩾70 (right) are presented next to internal nodes. Symbols correspond to those shown in Fig. 2. Roman numerals indicate geographic subgroups: i = New South Wales and northeast Victoria; ii = central Victoria; iii = western Victoria, South Australia, and Tasmania; iv = Snowy Mountains; v = Victorian Alps; vi = Barrington Tops; vii = New England Tablelands.

Table 3
Percent uncorrected net mean pairwise genetic distances (mean ± standard error) using the full mtDNA dataset between mtDNA clades designated in Fig. 3.

	Clade	1	2	3	4	5
1	*P. baudini*					
2	*P. entrecasteauxii* complex Lineage 1 (*P.e.*A, *P.c.*A)	12.0 ± 0.52				
3	*P. entrecasteauxii* complex Lineage 2 (*P.p.*A)	13.4 ± 0.25	9.4 ± 0.51			
4	*P. entrecasteauxii* complex Lineage 3 (*P.c.*B, *P.p.*B, *P.e.*B, *P.p.*C)	8.7 ± 0.62	6.9 ± 0.74	9.8 ± 0.51		
5	*P. rawlinsoni*	4.5 ± 0.47	10.4 ± 0.48	12.1 ± 0.49	7.5 ± 0.63	
6	*P. spenceri*	8.2 ± 0.28	8.3 ± 0.64	10.5 ± 0.15	6.5 ± 0.52	6.7 ± 0.70

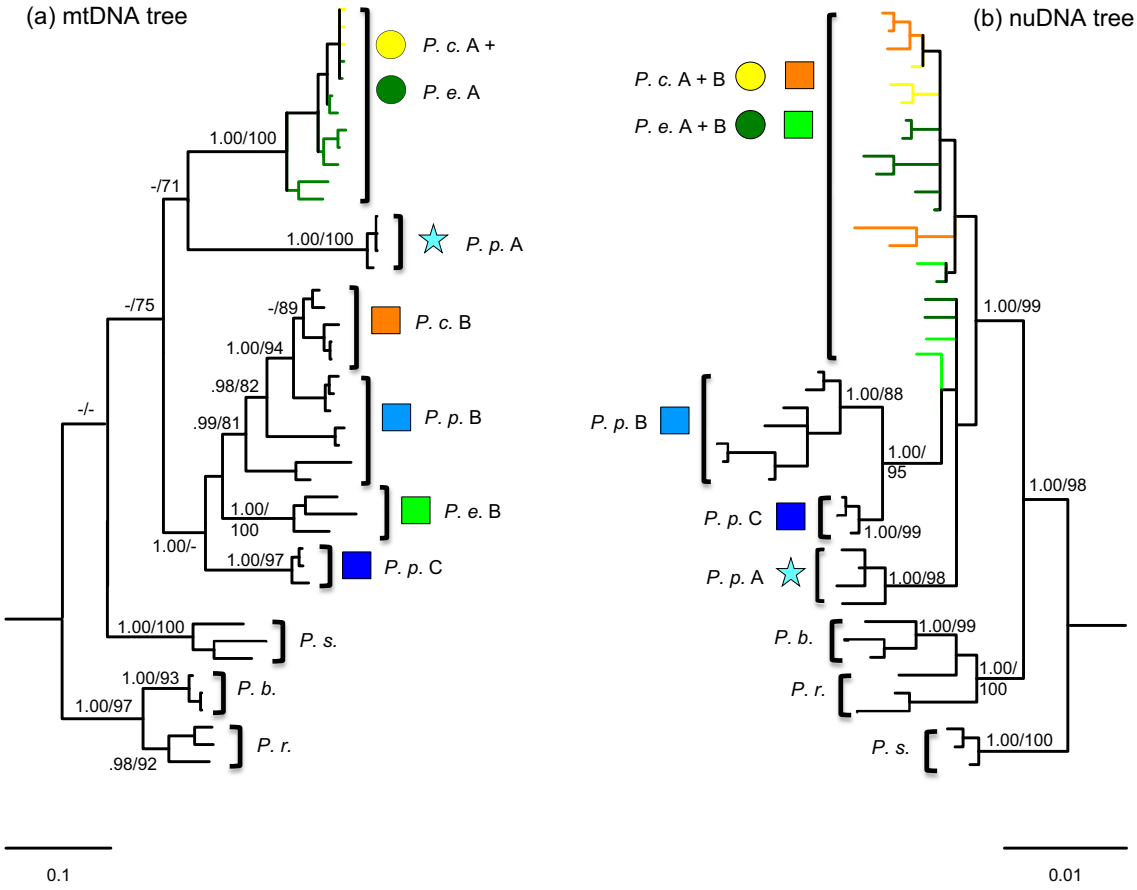

Fig. 4. Bayesian trees based on the reduced mtDNA dataset (left) and the concatenated nuDNA dataset (right). Bayesian posterior probabilities ⩾0.95 (left) and ML bootstraps ⩾70 (right) are presented next to internal nodes. Species abbreviations are as follows: *P. b.* = *P. baudini*; *P. c.* = *P. cryodroma*; *P. e.* = *P. entrecasteauxii*; *P. p.* = *P. pagenstecheri*; *P. r.* = *P. rawlinsoni*; *P. s.* = *P. spenceri*.

were variable and 128 were parsimony informative (Table 2) across *Pseudemoia*. Within the *P. entrecasteauxii complex*, 132 bp were variable and 78 were phylogenetically informative. Best-fit models determined using MrModeltest 2.3 are given in Table 2. Bayesian trees based on the five individual nuclear loci provided minimal phylogenetic resolution, particularly in regards to the lineages within the *P. entrecasteauxii complex* (Fig. S1, Supporting Information). Concatenating all nuclear genes and applying a partitioned, mixed model confirmed the monophyly of *P. spenceri*, the sister relationship between *P. baudini* and *P. rawlinsoni* as inferred from the mtDNA genealogy, and the monophyly of the *P. entrecasteauxii* complex. Within the latter, there was notable divergence of *P. pagenstecheri* A, a well-supported clade comprising *P. pagenstecheri* B and C (Fig. 4) and, although not well supported, a cluster comprising *P. cryodroma* A and B. In the *BEAST nuDNA only analysis, *P. pagenstecheri* B and C formed a monophyletic group to the exclusion *P. cryodroma* B (Fig. 5). This was also observed when the nuDNA and mtDNA were combined (Fig. S2, Supplementary Information). The relationships among *P. cryodroma* A and B, and *P. entrecasteauxii* A and B were unresolved.

We constrained and compared alternative tree topologies using the concatenated nuDNA dataset by assessing Bayes Factors. Constraining groups based on taxonomy consistently resulted in positive Bayes Factors, indicating that they were favored over the negative constraints that taxonomic groups were not monophyletic (Table 4). Despite the lack of support in the nuDNA tree, we observed the highest positive Bayes Factor for constraining *P. entrecasteauxii* over the negative constraint that this species was not monophyletic. In contrast, constraining groups based on mtDNA lineages always produced negative Bayes Factors, providing evidence in favor of the negative constraint. In particular, the negative constraint that *P. cryodroma* B and *P. pagenstecheri* B are not monophyletic was strongly favored (Bayes Factor > 3) over the monophyly of these two groups. This provides key evidence that there is significant conflict in the placement of *P. cryodroma* B between the mtDNA and nuDNA.

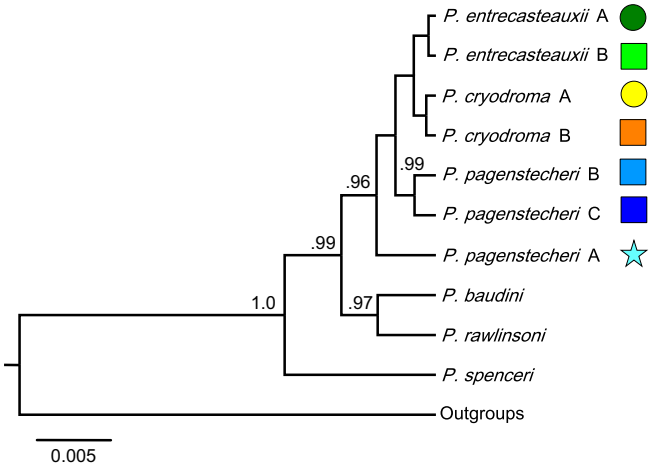

Fig. 5. Species tree generated using the multi-species coalescent analysis in *BEAST based on the nuDNA dataset. Bayesian posterior probabilities ⩾0.95 are presented next to internal nodes.

Table 4
Marginal likelihood for unconstrained, constrained, and negatively constrained nuDNA tree topologies. Bayes Factors (BF) were calculated using the arithmetic difference between marginal likelihood in log units for positive (+) and negative (−) constraints.

Constraint	Marginal likelihood (ln)		BF
None	−7702.90		NA
P.c. A + B	(+) −7711.38	(−) −7712.74	1.36
P.e. A + B	(+) −7709.80	(−) −7718.16	8.30
P.p. B + C	(+) −7702.93	(−) −7707.53	4.60
P.c. A + *P.e.* A (lineage 1)	(+) −7710.76	(−) −7709.83	−0.93
P.c. B + *P.p.* B + *P.e.* B + *P.p.* C (lineage 3)	(+) −7726.54	(−) −7713.79	−12.75
P.c. B + *P.p.* B	(+) −7709.08	(−) −7705.79	−3.29
Taxonomy (*P.c.* A + B, *P.e.* A + B, *P.p.* B + C)	(+) −7707.98	(−) −7710.99	3.01
mtDNA phylogeny (lineages 1 + 3)	(+) −7724.90	(−) −7710.21	−14.69

3.3. Estimates of divergence times

The relaxed lognormal clock analysis of the mtDNA dataset produced the same in-group topology as the Bayesian and ML analyses (Fig. S3, Supporting Information). The coefficient of variance was estimated to be 0.629 (95% highest posterior density: 0.443–0.838), indicating that the data is not strictly clock-like. While there was a weak trend toward a positive correlation in the rate of parent to child branches (covariance = 0.45), the wide 95% highest posterior density (−0.1643 to 0.257) suggests that the uncorrelated lognormal relaxed clock model was appropriate for this dataset. The most recent common ancestor for the *P. entrecasteauxii* complex is estimated to have occurred in the late Miocene – 8.1 Ma (95% CI: 4.0–14.0 Ma). The split between the *P. cryodroma* A and *P. entrecasteauxii* A samples, as well as the divergence between the *P. cryodroma* B and *P. pagenstecheri* B samples dates to the Plio-Pleistocene – 2.2 Ma (95% CI: 0.9–5.5 Ma) and 2.6 Ma (95% CI: 0.6–4.9 Ma), respectively. Substitution rates (% million years^{-1}) were 2.0 ± 0.01 (95% CI: 1.41–2.53), which is consistent with other squamate studies (Feldman and Spicer, 2006; Pang et al., 2003).

4. Discussion

Our molecular analyses reveal a more complex evolutionary and biogeographic history of the genus *Pseudemoia* than previously recognized. First, there exists well-supported incongruence between the mitochondrial and nuclear datasets in regards to the evolutionary relationships within the *P. entrecasteauxii* complex (*P. cryodroma*, *P. entrecasteauxii* and *P. pagenstecheri*). We discuss possible explanations for this incongruence, including historic mitochondrial introgression, and revisit the hybrid-origin hypothesis proposed for *P. cryodroma* (Hutchinson and Donnellan, 1992). Second, we identify two divergent lineages of *P. pagenstecheri*, one geographically restricted to northern NSW, and a second ecologically differentiated lineage in the montane regions of NSW and eastern Victoria. We then discuss and reappraise the current taxonomy of *Pseudemoia*, with particular emphasis on potential cryptic species. Lastly, we discuss the effects of biogeography on the structure observed within *P. entrecasteauxii* and *P. pagenstecheri*.

4.1. Discordance between mitochondrial and nuclear loci

Although the nuclear markers provide low overall resolution of relationships within *Pseudemoia*, there exists well-supported incongruence, particularly in the placement of *P. cryodroma*. Based on the mitochondrial dataset, *P. cryodroma* is found within two of the three major lineages within the *P. entrecasteauxii* complex, one represented predominantly by *P. entrecasteauxii* and the other predominantly by *P. pagenstecheri* (hereafter referred to as *P. entrecasteauxii sensu stricto* and *P. pagenstecheri sensu stricto*, respectively). Specifically, *P. cryodroma* from the Lake Mountain region are undifferentiated from *P. entrecasteauxii* A, whereas *P. cryodroma* from the Victorian Alps form a clade closely related to sympatric *P. pagenstecheri* and are nested within the *P. pagenstecheri* B clade. The results from the fossil calibrated tree and substantial mtDNA divergence (6.9%) between these two major mitochondrial lineages (*P. pagenstecheri s.s.* and *P. entrecasteauxii s.s.*) indicate that they diverged during the late Miocene or Pliocene. The nuclear genealogy, however, fails to differentiate between representatives of *P. cryodroma* from the two mitochondrial lineages. Furthermore, the nuDNA dataset strongly supports the monophyly of the clade encompassing *P. pagenstecheri* clades B and C to the exclusion of *P. cryodroma* from the Victorian Alps. This pattern of incongruence is repeated with *P. entrecasteauxii* B being nested with *P. pagenstecheri s.s.* in the mitochondrial genealogy, yet clearly showing close affinity to *P. entrecasteauxii s.s.* based on the nuDNA dataset.

The incongruence observed between the nuDNA and mtDNA genealogies is likely due to multiple instances of hybridization. Specifically, we suggest that mitochondrial introgression is the most parsimonious explanation for these patterns in the data. Mitochondrial introgression occurs when two species hybridize and the hybrid offspring repeatedly backcross with the paternal species while retaining the mtDNA of the maternal species. Over time, the nuDNA and morphology of introgressed individuals can become indistinguishable from the original paternal species (Funk and Omland, 2003). Evidence for mitochondrial introgression has been well-documented in several other lizard taxa (Jezkova et al., 2013; Leaché, 2009; McGuire et al., 2007; Olave et al., 2011; Rabosky et al., 2009). In this study, the incongruence between markers for the placement *P. cryodroma* B may be a consequence of interbreeding between male *P. cryodroma* and female *P. pagenstecheri*. Similarly, the incongruence between markers for *P. entrecasteauxii* B most likely reflects past interbreeding between male *P. entrecasteauxii* and female *P. pagenstecheri*. Given our estimated substitution rate of 2.0% per million years, and the genetic distinctiveness of the *P. cryodroma* B and *P. entrecasteauxii* B mtDNA clades, the data suggest that the introgression is an historic event that occurred during the Pliocene. It is currently unclear why *P. cryodroma* A does not form a distinct clade; however, one possibility is recent hybridization between *P. cryodroma* and *P. entrecasteauxii*. Since the current dataset does not provide enough

information to test this hypothesis, we highlight the need for additional research using more rapidly evolving genetic markers.

Though much less common in animals, hybrid speciation may also explain the conflicting phylogenetic placement of *P. cryodroma* in the mtDNA and nuDNA datasets. First, the placement of *P. cryodroma* within both *P. entrecasteauxii s.s.* and *P. pagenstecheri s.s.* lineages is consistent with a hybrid origin resulting from bidirectional hybridization. However, the mtDNA genealogy indicates that *P. cryodroma* A has recently diverged from *P. entrecasteauxii* A, yet the split between *P. cryodroma* B and its sister clade dates to the Pliocene. Furthermore, this pattern is not mirrored in the nuDNA phylogeny or previous allozyme data (Hutchinson and Donnellan, 1992). Second, karyotyping conducted by Hutchinson and Donnellan (1992) provides additional support for the hybrid origin hypothesis, showing that both *P. cryodroma* B and *P. pagenstecheri* B are metacentric at chromosome 9, while *P. entrecasteauxii* A is almost exclusively acrocentric for the same chromosome. Karyotype data is not available for *P. cryodroma* A, preventing a full assessment of the chromosome variation across the genetic lineages. Third, *P. cryodroma* is morphologically intermediate to the two potential parental species (Hutchinson and Donnellan, 1992). This is a common trait among hybrid species and exemplified by features such as the wing patterns of the butterfly *Heliconius heurippa* (Mavarez et al., 2006) and Italian sparrow plumage coloration (Hermansen et al., 2011). Microsatellite analyses using syntopic individuals from all three *Pseudemoia* species may provide information to distinguish these two hypotheses and enable the examination of recent gene flow.

The historical biogeography of *P. cryodroma*, *P. entrecasteauxii*, and *P. pagenstecheri* can also aid in interpreting the conflicting gene trees. Glaciation during the Plio-Pleistocene may have brought recently diverged species into contact, facilitating introgressive hybridization. Specifically, expansion of subalpine habitats during glacial periods may have increased overlap between the high elevation species *P. cryodroma* and both *P. entrecasteauxii* and *P. pagenstecheri*. Consistent with this hypothesis, the mtDNA phylogeny indicates that *P. cryodroma* populations A and B are most closely related to sympatric populations of *P. entrecasteauxii* and *P. pagenstecheri*, respectively. Subsequent retraction of subalpine habitats during interglacial periods may have allowed introgressed populations to continue to evolve in isolation.

4.2. Cryptic diversity and biogeographic structure

Our multi-locus data indicate that species richness in *Pseudemoia* is greater than currently appreciated. Specifically, there is strong support from both the mitochondrial and nuclear dataset for the hypothesis that the most northern lineage of *P. pagenstecheri sensu lato* (*P. pagenstecheri* A) represents a separate species. Uncorrected net mitochondrial sequence divergence between *P. pagenstecheri* A and its sister lineage *P. entrecasteauxii* A, is 9.4%, which is comparable to interspecific divergence observed within *Pseudemoia* and other skink genera (Chapple et al., 2011a; Chapple and Keogh, 2004; Dolman and Moritz, 2006; Dubey and Shine, 2010). This potential new species appears geographically restricted to Guyra in northern NSW; however, further work is needed to determine the species' geographic extent. Guyra is part of the Central volcanic province, which experienced volcanic activity during the Miocene (Coenraads, 1990) and the resulting lava fields could have divided previously continuous mesic habitats (Rix and Harvey, 2012). This region is also home to the endemic spider *Austrarchaea monteithi* (Rix and Harvey, 2012), one of two populations of the vulnerable turtle *Wollumbinia bellii* (Georges and Adams, 1996; Wilson and Swan, 2013), and a newly rediscovered disjunct population of the critically endangered frog *Litoria castanea* (Hero et al., 2004; Thompson et al., 1996). Cursory examination of voucher specimens does not show any major morphological differences between the Guyra lineage and other individuals identified as *P. pagenstecheri*; therefore, a detailed morphological analysis is required as its taxonomic status is formally assessed.

We observed greater genetic substructure within *P. pagenstecheri sensu stricto* than the main *P. entrecasteauxii* group (*P. entrecasteauxii* A), presumably due to differences in ecology. In alpine and sub-alpine areas *Pseudemoia pagenstecheri* is typically restricted to grasslands on disjunct plateaux (Wilson and Swan, 2013), which may have caused it to experience greater habitat fragmentation and therefore greater barriers to gene flow (Koumoundouros et al., 2009). In contrast, *P. entrecasteauxii* is a more generalist species occurring in a wider range of habitats (Wilson and Swan, 2013), allowing gene flow to be more continuous between occupied areas. This would explain the well-supported genetic substructuring within the mtDNA clade *P. pagenstecheri* B along the Great Dividing Range, a mountain range spanning eastern Australia, but lack of structure in sympatric *P. entrecasteauxii*.

Pseudemoia pagenstecheri s.s. showed deep mtDNA genetic divergence (6.5%) between individuals from high elevations in the Victorian Alps and NSW, and those in lowland regions of western Victoria. Although more shallow (2.9%), this split is mirrored in *P. entrecasteauxii*, and comparable to patterns observed in the lizards *Lampropholis guichenoti* (Chapple et al., 2011b) and *Rankinia diemensis* (Ng et al., 2014), and the frogs *Limnodynastes peronii* and *L. tasmaniensis* (Schäuble and Moritz, 2001). Since *Pseudemoia* are restricted to areas of high precipitation (mean annual rainfall above 600 mm; Hutchinson and Donnellan, 1992), the formation of dry habitat corridors during glacial cycles in the Plio-Pleistocene (Byrne et al., 2011) would likely have fragmented habitats, impeding gene flow. The genetic split between *P. pagenstecheri* B and C is also present in the concatenated nuDNA datasets, suggesting that these lineages have separate evolutionary trajectories and potentially represent two distinct species. Although individuals from both clades display variable stripe patterning, those from *P. pagenstecheri* B tends to have a thick, black vertebral stripe and two black paravertebral stripes while *P. pagenstecheri* C typically possesses a single vertebral stripe (M. Haines, pers. obs.). Therefore, we stress the need for thorough morphological analyses to determine whether there is sufficient support for further splitting *P. pagenstecheri s.s.* to reflect these two lineages. Such analyses may have conservation implications because populations of *P. pagenstecheri* C occurring in lowland grasslands have experienced extensive habitat loss in Tasmania and appear to have undergone recent precipitous declines in the volcanic plains of Victoria (N. Clemann pers. obs.), which has resulted in this species being listed as Vulnerable in both states (DPIPWE, 1995; DSE, 2013).

5. Conclusion

Our research demonstrates the importance of using both mtDNA and multiple nuDNA loci in a biogeographic context to investigate the evolutionary relationships between species. Based on the nuDNA, we suggest that the paraphyly of *P. cryodroma*, *P. entrecasteauxii*, and *P. pagenstecheri* in the mtDNA dataset is a result of multiple instances of historic mitochondrial introgression; however, hybrid speciation of *P. cryodroma* cannot be completely dismissed. Within the *P. entrecasteauxii* complex, we identified three genetically and geographically distinct lineages. The first lineage, *P. pagenstecheri* A from Guyra, NSW, is distinct from *P. pagenstecheri s.s.* in both the nuDNA and mtDNA datasets and sister to the widely distributed *P. entrecasteauxii* A clade in the latter. Within *P. pagenstecheri s.s.*, *P. pagenstecheri* B from high elevations

in the Victorian Alps and NSW forms a distinct clade within the nuDNA and is also separated from *P. pagenstecheri* C from lowland regions of western Victoria in mtDNA genealogies. Since multiple lines of evidence are needed to describe new taxa (de Queiroz, 2007), the isolated and restricted geographic distribution of *P. pagenstecheri* A and the ecological differentiation of *P. pagenstecheri* B and C provides critical support for describing these genetic lineages as distinct species. We recommend a detailed morphological appraisal to determine whether these lineages are truly cryptic. This study also provides evidence for geographic substructure within species, most prominently across the Great Dividing Range and between eastern and western Victoria. Because the relative impact of the latter barrier differed among *Pseudemoia* spp. and has only been noted in a handful of studies, future work on other ground-dwelling vertebrates found across this biogeographic divide would provide important phylogeographic comparisons. Thus, our study elucidates the evolutionary complexity within the genus *Pseudemoia* and provides a stepping-stone to future studies investigating recent gene flow between *Pseudemoia* spp. and contributes to our understanding of the evolution of Australian mesic zone fauna.

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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.2014.09.006.

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