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Mitochondrial introgression via ancient hybridization, and systematics of the Australian endemic pygopodid gecko genus *Delma* ☆

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ABSTRACT

Of the more than 1500 species of geckos found across six continents, few remain as unfamiliar as the pygopodids – Family Pygopodidae (Gray, 1845). These gekkotans are limited to Australia (44 species) and New Guinea (2 species), but have diverged extensively into the most ecologically diverse limbless radiation save Serpentes. Current phylogenetic understanding of the family has relied almost exclusively on two works, which have produced and synthesized an immense amount of morphological, geographical, and molecular data. However, current interspecific relationships within the largest genus *Delma* Gray 1831 are based chiefly upon data from two mitochondrial loci (16s, ND2). Here, we reevaluate the interspecific relationships within the genus *Delma* using two mitochondrial and four nuclear loci (RAG1, MXRA5, MOS, DYNLL1), and identify points of strong conflict between nuclear and mitochondrial genomic data. We address mito-nuclear discordance, and remedy this conflict by recognizing several points of mitochondrial introgression as the result of ancient hybridization events. Owing to the legacy value and intraspecific informativeness, we suggest the continued use of ND2 as a phylogenetic marker. Results identify strong support for species groups, but relationships among these clades, and the placement of several enigmatic taxa remain uncertain. We suggest a more careful review of *Delma australis* and the 'northwest Australia' clade. Accurately assessing and addressing species richness and relationships within this endemic Australian Gekkotan genus is relevant for understanding patterns of squamate speciation across the region.

1. Introduction

1.1. The Pygopodidae Boulenger 1884

While subdigital lamellae have repeatedly developed and disappeared within Gekkota, the Pygopodidae, or flap-footed geckos, remain the only limb-reduced group within the infraorder (Gamble et al., 2012). Pygopodids are characterized by an absence of forelimbs, imbricate body scales, and extensive reduction of hindlimbs. Despite the obvious morphological schism between pygopodids and other gekkotans, evidence for the close relationship between these groups has been recognized and supported for a considerable period of time (Boulenger, 1885; Shute and Bellairs, 1953; McDowell and Bogert, 1954; Underwood, 1957; Miller, 1966; Wever, 1974; Greer, 1989). Recent molecular studies have supported the position of pygopodids within the Pygopodoidea as well as within the Gekkota, and molecular (Jennings et al., 2003; Oliver and Sanders, 2009), morphological (Kluge, 1974, 1976; Daza and Bauer, 2012), and karyotypic (Gorman and Gress, 1970; King and King, 1977) data unequivocally support the monophyly of the Family Pygopodidae and individual genera within the family, as well as a sister relationship between the Pygopodidae and Carphodactylidae.

Current taxonomy recognizes 45 species across seven genera; *Aprasia* Gray 1839 (13 spp.), *Delma* Gray 1831 (22 spp.), *Lialis* Gray 1835 (2 spp.), *Ophidiocephalus* Lucas & Frost 1897 (1 sp.), *Paradelma* Kinghorn 1926 (1 sp.), *Pletholax* Cope 1864 (1 sp.), and *Pygopus* Merrem 1820 (6 spp.). Substantial morphological divergence from the tetrapodal-squamate body plan, geographic dispersal, and ecological diversification has led to a unique radiation of limbless gekkotans. Natural history and ecology of genera and species vary greatly: fossorial myrmecophiles, *Aprasia*; terrestrial squamate-specialist ambush predators, *Lialis*; shrub-swimmers, *Delma concinna*, *Pletholax*; arthropod-generalists, *Pygopus*; and a species with nectarivorous habits, *Paradelma* (Tremul, 2000; Kutt et al., 2003; Wilson and Swan, 2013). Diurnality in the majority of pygopodid species occurs as a secondarily derived trait, and belies their

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gekkotan morphology, specifically: vertical pupils, the lack of a fovea (the sensitive retinal region found in many diurnal organisms) and absence of oil droplets in the visual cells of the eye (Greer, 1989; Röll, 2000, 2001). These characteristics suggest a nocturnal origin for the pygopodids, behavior shared with ancestral geckos. Of the pygopodids, *Delma* represents the most speciose genus, despite considerable phenotypic conservatism (Gamble et al., 2015).

1.2. Delma Gray 1831

Delma are distinguished from all other pygopodids by the combination of several characters: head scales (including parietals) enlarged and symmetrical, anterior nasal scales nearly always in contact, nostril bordered by more than two scales (except in *D. impar*), external ear opening visible, usually fewer than 18 midbody scale rows, dorsal and ventral scales smooth, paired ventral scales, precloacal pores absent, tail 2–4 times snout-vent length (SVL). Plesiomorphic traits, including large hindlimb remnants – relative to other pygopodids – along with conspicuous ear openings, and generalist crushing dentition, support the basal position of *Delma* in the pygopodid radiation (Patchell and Shine, 1986).

Behaviorally however, little is known of this genus. Delmas often live in dense low vegetation, preferably tussock grasses and spinifex (*Triodia*), in which they are able to disperse quickly in a serpentine fashion (Wilson and Swan, 2013). We collected *Delma* both day and night, from a variety of cover types, including beneath spoil heaps, stones, logs, and dry grass clippings. Over open ground and in response to attempted predation, *Delma*, like other pygopodids (*Ophidiocephalus*; Ehmann, 1981) may jump, using the tail as a spring, adjusting the body into a sine wave configuration as it propels the body vertically or forward (Gans, 1974; Kluge, 1974; Bauer, 1986). Although other limb-reduced squamates appear to have converged on this behavior, e.g. trogonophid amphisbaenians (Gans, 1974), the viperid *Bitis caudalis* (Gans and Mendelssohn, 1972), and *Ophiosaurus* anguids (Cliburn, 1957), although the mechanics and outcome of the saltation event differ substantially (Bauer, 1986).

Delma appear to be active arthropod generalist hunters, with their diets almost exclusively insectivorous. *Delma butleri, D. fraseri*, *D. grayii*, *D. inornata*, *D. nasuta*, and *D. petersoni* – and presumably others – are both diurnal and nocturnal predators, dependent upon temperature (Patchell and Shine, 1986; Pianka, 2010). Although historical data (Jennings et al., 2003; Patchell and Shine, 1986) suggests a generalist diet for *Delma* species, recent preliminary data suggests the potential for prey specialization (Pianka, 2010). Patchell and Shine (1986) proposed a convergence in morphology and ecology between pygopodids and elapids, however *Delma* lack an equivalent in the Australian snake fauna in behavior and as insectivores. In other parts of the world, insectivorous snakes are not uncommon, e.g. *Chionactis*, *Opheodrys,* and *Tantilla* of North America; *Aparallactus* of Southern Africa; *Typhlopidae* found globally; *Oligodon* and *Eirenis* of Asia, and the absence of surface active insectivorous snakes in Australia may be attributable to the presence of *Delma* as serpentine arthropod generalists (Savitzky, 1983; Patchell and Shine, 1986). As current estimates of divergence dates suggest, perhaps the success and species richness of *Delma* is attributable to filling this available niche prior to the diversification of insectivorous snakes in Australia.

Hutchinson (1997) addressed mandibular and dentition variation in *Delma* in reference to the description of *Pygopus hortulanus* from a single mandible fossil. *Delma* retain the bicuspid crown condition of pygopodoid geckos, suggesting unspecialized dentition. This is indicative of a generalized diet of arthropods, and supports the position of *Delma* basally in the pygopodid tree. Dental synapomorphies of the genus include a slightly reduced splenial, with a moderately slender dentary, and posterior surangular foramina are narrowly separated. There is little divergence from this plan with the exception of *D. concinna*, which exhibits a substantially elongated mandible.

1.3. Delma

Type species: *Delma fraseri* Gray, 1831, by monotypy.

Content: *Delma australis* Kluge, 1974; *Delma borea* Kluge, 1974; *Delma butleri* Storr, 1987; *Delma concinna* Kluge, 1974; *Delma desmosa* Maryan, Aplin & Adams, 2007; *Delma elegans* Kluge, 1974; *Delma fraseri* Gray, 1831; *Delma grayii* Smith, 1849; *Delma haroldi* Storr, 1987; *Delma hebesa* Maryan, Brennan, Adams & Aplin, 2015; *Delma impar* Fischer, 1882; *Delma inornata* Kluge, 1974; *Delma labialis* Shea, 1987; *Delma mitella* Shea, 1987; *Delma molleri* Lütken, 1863; *Delma nasuta* Kluge, 1974; *Delma pax* Kluge, 1974; *Delma petersoni* Shea, 1991; *Delma plebeia* De Vis, 1888; *Delma tealei* Maryan, Aplin & Adams, 2007; *Delma tincta* De Vis, 1888; *Delma torquata* Kluge, 1974.

1.4. Prior phylogenetic assessment of Pygopodidae and Delma

Phylogenetic relationships within the Pygopodidae have been assessed in-depth on two occasions (Kluge, 1976; Jennings et al., 2003). Following a revision of the family in 1974, Kluge's (1976) phylogenetic assessment focused on morphological characters. Adding to this existing dataset, Jennings et al. (2003) used mtDNA (16S, ND2) and nDNA (MOS) markers in concert with morphology to propose the currently accepted phylogenetic understanding of pygopodids. Due to remarkably complete intergeneric and interspecific sampling, Jennings et al.'s phylogeny of the Pygopodidae has been used extensively (Lee et al., 2009; Oliver, 2009; Oliver et al., 2010b; Wiens et al., 2012; Garcia-Porta and Ord, 2013; Maryan et al., 2013; Pyron et al., 2013). However, morphological (Kluge, 1976) and nDNA (Jennings et al., 2003) assessments of interspecific relationships within *Delma* have been insufficient to provide well-supported phylogenetic reconstructions. Based on our contemporary understanding of the importance of strong mitochondrial *and* nuclear datasets, a 372 bp fragment of MOS is inadequate to properly reconstruct the nuclear evolutionary history of this genus, and is further discussed below (1.4).

Here, we expand upon Jennings et al.'s dataset, adding several newly described taxa and previously un-genotyped species of *Delma*, and include three additional nuclear markers (RAG1-1071 bp, MXRA5-793 bp, DYNLL1-1056 bp). We assess interspecific relationships *Delma*, within the largest genus, with the intention of contributing to our understanding of Australian squamate biogeography. Despite ambiguities in intergeneric relationships within the Pygopodidae, there is high support for placing *Delma* as the sister group to all remaining pygopodids. *Delma* represents a perplexing radiation within the Pygopodidae; despite a continent-wide distribution, moderate species richness (22 spp.), several deep molecular divergences, and adaptation to a variety of habitat types, the genus remains remarkably conservative in morphology, microhabitat, and diet (Jennings et al., 2003; Kluge, 1974, 1976; Oliver, 2009). Morphological conservatism and broadly overlapping ranges of closely related species highlight the potential for interesting evolutionary scenarios with *Delma*.

1.5. Historical gene flow and the persistence of species

Delimiting species and establishing accurate phylogenetic relationships is tantamount to our understanding of species units, ecology, and conservation. Incongruence in phylogenetic reconstruction, caused by convoluted evolutionary histories obscure our ability to infer micro- and macroevolutionary patterns.

Identification of discordance between mitochondrial and nuclear genealogies has increased dramatically in recent years (see Toews and Brelsford, 2012 for review). Many of these instances involve shared mitochondrial haplotypes (Leaché and McGuire, 2006; McGuire et al., 2007; Veith et al., 2012) that aid in isolating recent hybridization and mitochondrial introgression as the cause of such discordance. More difficult to tease apart are instances of ancient hybridization not currently visible in phenotype or contemporary genotype. While the development of new species from hybridization does occur (Seehausen, 2004), reticulations between closely related or sympatric lineages, in which both species maintain their independence are more common (Mayr, 1996; McCormack and Venkatraman, 2013). As more multilocus datasets are generated, we are likely to encounter many more instances in which individual species lineages persist beyond periods of introgression and gene flow.

Due to high frequency of sympatry, particularly between hypothesized sister taxa, and low morphological diversity, *Delma* appears to be a strong candidate for investigating hybridization and ancestral introgression. One of the more surprising findings of Jennings et al.'s (2003) phylogeny of the Pygopodidae is the paraphyly of *Delma fraseri* subspecies with regards to *D. grayii*. In light of this result, Jennings et al. (2003) elevated *Delma f. petersoni* to full species status, acknowledged the relationship is built upon mtDNA data, recognized the possibility of incomplete lineage sorting, emphasizing the need for more thorough genetic sampling. Because of the syntopy of phenotypically distinct *D. fraseri* and *D. grayii* along coastal Western Australia, and the geographically disjunct nature of morphologically similar *D. fraseri* and *D. petersoni*, we chose to investigate this system further through exhaustive sampling across the range of *D. fraseri* and *D. grayii*. Identifying conflict between morphological and molecular signals and addressing their discordance is invaluable to understanding species richness and evolution within this strongly divergent gekkotan lineage.

By reconstructing a multilocus molecular phylogeny, we aim to address questions regarding the phylogenetic affinities and patterns of diversification within *Delma*, and challenge mitochondrially and morphologically biased hypotheses proposed by Jennings et al. (2003) and Kluge (1974, 1976). Analyses of novel molecular data includes testing for ancient and contemporary introgression events, to accurately unravel the phylogenetic history of *Delma*.

2. Materials and methods

2.1. Taxon sampling

All samples, along with locality data, voucher information, and GenBank accession numbers can be found in Table 1. Molecular sampling comprises 42 individuals (all adults). With the inclusion of *D. elegans*, *D. plebeia,* and several species described since the last molecular analysis of the group (*D. desmosa, D. hebesa, D. tealei*), our sampling covers all 22 currently recognized *Delma* species. Additionally, we have included members of all outgroup pygopodid genera. For intraspecific investigation of the sympatric species *D. fraseri* and *D. grayii*, we supplemented our sampling with an additional 75 individuals, representing all tissues available for these species in the Western Australian Museum collections.

2.2. Molecular methods

Genomic DNA was isolated via Qiagen DNeasy Tissue kits (Qiagen) from liver, heart, or tail tissue preserved in 95–100% ethanol. To take advantage of increased resolution as a result of multi-locus mito-nuclear datasets, we employed both mitochondrial (ND2) and nuclear markers (DYNLL1, RAG1, MXRA5, MOS) (Fisher-Reid and Wiens, 2011). Mitochondrial (mtDNA) and nuclear (nDNA) loci were amplified by polymerase chain reaction (PCR). Primers used for PCR amplification and sequencing are listed in Table 2. Standard 25 μL PCR reactions utilized; dH_2O, 5x Taqmaster PCR enhancer, 10x PCR Buffer, dNTPs, forward and reverse primers, Taq polymerase, and genomic DNA, and were carried out on an Eppendorf Nexus gradient thermocycler. Thermocycler amplification programs followed a standard protocol with varying annealing temperatures, relative to the loci and primers; initial denaturation period (95 °C, 2 min) followed by 34 cycles at 95 °C (30 s), 48 °C (35 s) annealing, and 72 °C (150 s) extension. Amplified PCR products were visualized using 1.5% agarose electrophoresis, purified via Agencourt AMPure magnetic bead system (Agencourt Bioscience), and stored in a refrigerator at 4 °C until sequenced. We performed cycle sequencing via BigDye Terminator v3.1 Cycle Sequencing kit using purified PCR product as a template, and sequencing product was purified using Agencourt CleanSeq magnetic bead system (Agencourt Bioscience). Amplified product was sequenced in both forward and reverse directions using an ABI 3730 XL sequencer, to allow for identification of heterozygous sites.

All sequences were assembled and edited in Geneious v.7, aligned by eye, and protein-coding loci were translated to amino acid sequence to maintain proper reading frame and avoid premature stop codons. tRNA secondary structure was addressed and aligned by eye for consistency. Mitochondrial genes were analyzed together because of shared evolutionary history as the result of physical linkage. Nuclear loci were analyzed individually to recognize individual locus discordance, and were also concatenated into a single nDNA dataset. Final aligned mitochondrial and nuclear sequences were 1480 bp (ND2) and 3019 bp (DYNLL1-783, MXRA5-787, RAG1-1071, MOS-378) respectively. Although the name C-mos has been used consistently in many squamate studies, the correct name for the gene is currently MOS (v-mos Moloney murine sarcoma viral oncogene homolog) (Alföldi et al., 2011).

2.3. Phylogenetic analyses

We used maximum likelihood (ML) and Bayesian inference (BI) methods to test for conflict between topologies and support values between analytical programs. The Akaike Information Criterion in PartitionFinder (Lanfear et al., 2012) was used to identify the most accurate models of evolution for each gene and codon position (Supplementary Table 1). For both ML and BI analyses, we analyzed nuclear loci individually, and as concatenated datasets in all possible combinations.

For ML analyses, we used RAxML 8.0 (Stamatakis, 2014), and divided the mitochondrial dataset into two partitions; ND2 and tRNAs; the nuclear dataset into four partitions; DYNLL1, MXRA5, RAG1, MOS; and employed the GTR + I + Γ model of evolution. When analyzed independently, individual loci were not partitioned by codon position because of RAxML's limits on evolutionary models, and were instead analyzed under GTR + I + Γ. Topology estimates used 100 independent tree searches, and 5000 bootstrap replicates to retrieve support values. One vs. two partitions for mtDNA and one vs. four partitions for nDNA did not disrupt topology or change BSS support values substantially.

BI analyses were performed using MrBayes 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012). The mitochondrial dataset was divided into six partitions; ND2, ND2 codon positions (1st, 2nd, 3rd), and tRNAs; and the nuclear dataset into 13 partitions – each gene receiving 4 partitions (whole locus, three codon positions), with the exception of the nuclear intron DYNLL1. One vs. three partitions for mtDNA and four vs. thirteen partitions for

Table 1
List of samples used in this study with appropriate: voucher (museum or field) numbers, locality data, and GenBank accession numbers.

Species	Collection ID	State	Locality	Latitude	Longitude	ND2	DYNLL1	RAG1	Cmos	MXRA5
In group taxa										
Delma australis	SAMA R28215	SA	Dalhousie Ruins	26°31′00″S	135°28′00″E	KP851424	KR697863	KP851230	KR697824	KR697912
Delma australis	SAMA R47178	SA	Peake Station	28°26′10″S	136°07′41″E	KP851425	KR697864	KR697785	KR697825	KR697913
Delma borea	WAM R110606	WA	Tanami Desert	19°34′47″S	128°51′53″E	KT803490	KR697865	KR697787	KR697826	KR697914
Delma borea	WAM R172694	WA	Irvine Island	16°19′21″S	124°02′48″E	KT803491	KR697866	KR697788	KR697827	KR697915
Delma butleri	WAM R141591	WA	5 km WSW Boolathana Homestead	24°35′21″S	113°32′10″E	KP851402	KR697868	KR697789	KP851216	KR697917
Delma butleri	WAM R130986	NSW	19.7 km N Coombah Roadhouse	32°48′58″S	141°36′58″E	KP851401	KR697867	KP851278	KP851211	KR697916
Delma concinna	WAM R96898	WA	Tamala	26°40′00″S	113°47′00″E	KP851406	KR697871	KP851292	KR697829	KR697920
Delma concinna	WAM R141175	WA	Lancelin	30°57′43″S	115°21′53″E	KP851405	KR697870	KR697791	KP851217	KR697919
Delma desmosa	WAM R114555	WA	Sandfire Roadhouse	19°46′00″S	121°05′00″E	KT803492	KR697873	KR697793	KR697831	KR697922
Delma desmosa	WAM R163287	WA	Neale Junction	28°17′23″S	125°49′20″E	KT803493	KR697872	KR697792	KR697830	KR697921
Delma elegans	WAM R110872	WA	Pannawonica	21°40′00″S	115°50′07″E	KT803494	KR697874	KR697794	KR697832	KR697923
Delma elegans	WAM R146640	WA	Pouyouwuncubban	22°08′58″S	119°01′07″E	KT803495	KR697875	KR697795	KR697833	KR697924
Delma fraseri	WAM R96701	WA	Dryandra	32°46′60″S	116°55′00″E	KT803496	–	–	–	–
Delma fraseri	WAM R106203	WA	Nukarni (Presumably)	31°18′03″S	118°12′01″E	KT803497	–	–	–	–
Delma fraseri	WAM R114162	WA	Mount Mcmahon, Ravensthorpe Range	33°32′60″S	120°05′60″E	KT803498	–	–	–	–
Delma fraseri	WAM R114578	WA	Spalding Park, Geraldton	28°45′60″S	114°37′00″E	KT803499	–	–	–	–
Delma fraseri	WAM R114927	WA	Dongara	29°15′00″S	114°55′60″E	KT803500	–	–	–	–
Delma fraseri	WAM R115101	WA	Ken Hearst Park	32°04′60″S	115°52′60″E	KT803501	–	–	–	–
Delma fraseri	WAM R115102	WA	Ken Hearst Park	32°04′60″S	115°52′60″E	KT803502	–	–	–	–
Delma fraseri	WAM R115115	WA	Ken Hearst Park	32°04′60″S	115°52′60″E	KT803503	–	–	–	–
Delma fraseri	WAM R115138	WA	Ken Hearst Park	32°04′60″S	115°52′60″E	KT803504	–	–	–	–
Delma fraseri	WAM R115139	WA	Ken Hearst Park	32°04′60″S	115°52′60″E	KT803505	–	–	–	–
Delma fraseri	WAM R115227	WA	Spalding Park, Geraldton	28°38′60″S	114°37′60″E	KT803506	–	–	–	–
Delma fraseri	WAM R116093	WA	Buller River	28°37′60″S	114°35′60″E	KT803507	–	–	–	–
Delma fraseri	WAM R116845	WA	Ellendale Pool, Greenough River	28°51′60″S	114°58′00″E	KT803508	–	–	–	–
Delma fraseri	WAM R116846	WA	Ellendale Pool, Greenough River	28°51′60″S	114°58′00″E	KT803509	–	–	–	–
Delma fraseri	WAM R116847	WA	Ellendale Pool, Greenough River	28°51′60″S	114°58′00″E	KT803510	–	–	–	–
Delma fraseri	WAM R119061	WA	Spalding Park, Geraldton	28°38′60″S	114°37′60″E	KT803511	–	–	–	–
Delma fraseri	WAM R119240	WA	2 km NE Cape Burney	28°51′05″S	114°37′60″E	KT803512	–	–	–	–
Delma fraseri	WAM R119420	WA	18 km SSW Ravensthorpe	33°44′22″S	119°59′27″E	KT803513	–	–	–	–
Delma fraseri	WAM R120662	WA	Between Lancelin And Ledge Point	31°04′54″S	115°22′16″E	KT803514	–	–	–	–
Delma fraseri	WAM R125962	WA	Narngulu, Geraldton	28°48′60″S	114°40′60″E	KT803515	–	–	–	–
Delma fraseri	WAM R126099	WA	Neerabup NP	31°40′60″S	115°45′00″E	KT803516	–	–	–	–
Delma fraseri	WAM R127540	WA	Esperance	33°51′05″S	121°52′60″E	KT803517	–	–	–	–
Delma fraseri	WAM R129686	WA	Lort River	33°45′00″S	121°13′60″E	KT803518	–	–	–	–
Delma fraseri	WAM R129687	WA	Lort River	33°45′00″S	121°13′60″E	KT803519	–	–	–	–
Delma fraseri	WAM R131927	WA	6.5 km S Condingup	33°48′60″S	122°31′60″E	KT803520	–	–	–	–
Delma fraseri	WAM R132811	WA	Yorkrakine Hill	31°25′60″S	117°31′00″E	KT803521	–	–	–	–
Delma fraseri	WAM R132826	WA	10 km Ne Bindoon	31°18′06″S	116°09′02″E	KT803522	–	–	–	–
Delma fraseri	WAM R132842	WA	10 km Sse Wongan Hills	31°58′18″S	116°45′24″E	KT803523	–	–	–	–
Delma fraseri	WAM R134755	WA	Jilakin Lake	32°40′29″S	118°20′09″E	KT803524	–	–	–	–
Delma fraseri	WAM R135222	WA	Dundas Rock	32°21′60″S	121°45′00″E	KT803525	–	–	–	–
Delma fraseri	WAM R135242	WA	Dundas Rock	32°21′60″S	121°45′00″E	KT803526	–	–	–	–
Delma fraseri	WAM R135503	WA	Redcliffe, Perth Suburb	31°55′60″S	115°57′01″E	KT803527	–	–	–	–
Delma fraseri	WAM R135697	WA	North Bannister, Albany Highway	32°34′60″S	116°27′01″E	KT803528	–	–	–	–
Delma fraseri	WAM R140507	WA	Ca 15 km WNW Cataby	30°42′54″S	115°25′12″E	KT803529	–	–	–	–
Delma fraseri	WAM R141191	WA	Ca 15 km NNE Lancelin	30°59′31″S	115°23′43″E	KT803530	KR697876	KR697796	KR697834	KR697925
Delma fraseri	WAM R141195	WA	Ca 15 km NNE Lancelin	31°01′35″S	115°21′42″E	KT803531	–	–	–	–
Delma fraseri	WAM R146908	WA	Marchagee Track	30°12′18″S	115°32′27″E	KT803532	–	–	–	–
Delma fraseri	WAM R151706	WA	Muchea Air Weapons Range	31°38′39″S	115°55′26″E	KT803533	–	–	–	–
Delma fraseri	WAM R153976	WA	Bindoon Military Training Area	31°09′41″S	116°15′38″E	KT803534	–	–	–	–
Delma fraseri	WAM R153977	WA	Bindoon Military Training Area	31°10′18″S	116°15′48″E	KT803535	–	–	–	–
Delma fraseri	WAM R153998	WA	Bindoon Military Training Area	31°38′39″S	115°55′26″E	KT803536	–	–	–	–
Delma fraseri	WAM R154008	WA	Muchea Air Weapons Range	31°38′32″S	115°550′3″E	KT803537	–	–	–	–

Delma fraseri	WAM R154021	WA	Muchea Air Weapons Range	31°38′32″S	115°55′03″E	KT803538	–	–	–	–
Delma fraseri	WAM R154039	WA	Muchea Air Weapons Range	31°38′16″S	115°55′31″E	KT803539	KR697877	KR697797	KR697835	KR697926
Delma fraseri	WAM R154052	WA	Muchea Air Weapons Range	31°38′16″S	115°55′31″E	KT803540	–	–	–	–
Delma fraseri	WAM R154194	WA	Near Kundip	33°40′55″S	120°11′56″E	KT803541	–	–	–	–
Delma fraseri	WAM R154435	WA	Near Kundip	33°40′01″S	120°12′03″E	KT803542	–	–	–	–
Delma fraseri	WAM R156219	WA	Pingrup Area	33°34′04″S	118°49′12″E	KT803543	–	–	–	–
Delma fraseri	WAM R156277	WA	42.6 km Ne Holt Rock	32°24′33″S	119°43′41″E	KT803544	–	–	–	–
Delma fraseri	WAM R157863	WA	Eyre Highway	32°18′52″S	123°32′33″E	KT803545	–	–	–	–
Delma fraseri	WAM R165300	WA	Cape Burney	28°51′06″S	114°38′24″E	KT803546	–	–	–	–
Delma fraseri	WAM R166865	WA	Oakajee	28°34′25″S	114°35′04″E	KT803547	–	–	–	–
Delma fraseri	WAM R169903	WA	Dirk Hartog Island	25°54′07″S	125°06′34″E	KT803548	–	–	–	–
Delma fraseri	WAM R169904	WA	Dirk Hartog Island	25°44′39″S	124°58′60″E	KT803549	–	–	–	–
Delma grayii	WAM R115749	WA	Geraldton	28°45′60″S	114°37′00″E	KT803550	KR697878	KR697798	KR697836	KR697927
Delma grayii	WAM R119065	WA	Yetna	28°36′05″S	114°42′01″E	KT803551	–	–	–	–
Delma grayii	WAM R119655	WA	Wicherina Reserve	28°43′60″S	115°00′00″E	KT803552	–	–	–	–
Delma grayii	WAM R120086	WA	Wicherina Reserve	28°43′60″S	115°00′00″E	KT803553	–	–	–	–
Delma grayii	WAM R120665	WA	Unknown	NA	NA	KT803554	KR697879	KR697799	KR697837	KR697928
Delma grayii	WAM R127635	WA	Unknown	NA	NA	KT803555	–	–	–	–
Delma grayii	WAM R127639	WA	Neerabup	31°41′00″S	115°46′00″E	KT803556	–	–	–	–
Delma grayii	WAM R127664	WA	Unknown	NA	NA	KT803557	–	–	–	–
Delma grayii	WAM R130146	WA	Preston Beach	32°53′24″S	115°39′32″E	KT803558	–	–	–	–
Delma grayii	WAM R131871	WA	8 km S Eneabba	29°52′60″S	115°16′60″E	KT803559	–	–	–	–
Delma haroldi	WAM R108987	WA	Beebingarra Creek	20°25′00″S	118°42′00″E	KT803560	KR697869	KR697790	KR697828	KR697918
Delma hebesa	WAM R132154	WA	Duke of Orleans Bay	33°56′00″S	122°33′00″E	KP851413	KR697880	KR697786	KP851220	KR697929
Delma hebesa	WAM R144237	WA	Bandalup Hill	33°40′29″S	120°23′54″E	KP851415	KR697881	KP851294	KP851221	KR697930
Delma impar	SAMA R43325	ACT	Gungahlin Town	35°13′00″S	149°08′00″E	KT803561	KR697882	KR697800	KR697838	KR697931
Delma impar	SAMA R51504	SA	Hacks Lagoon Conservation Park	37°06′15″S	140°43′45″E	KT803562	KR697883	KR697801	KR697839	KR697932
Delma inornata	AMS R142790	NSW	Ironmines Rd, 6.4 km S Yass, Goulburn	34°42′00″S	149°03′00″E	KT803563	KR697884	KR697802	KR697840	KR697933
Delma inornata	AMS R141999	NSW	Between Nyngan and Nevertire	31°38′00″S	147°20′00″E	KT803564	KR697885	KR697803	KR697841	KR697934
Delma labialis	QM J89155	QLD	Nebo	21°37′27″S	148°07′08″E	KT803565	KR697886	KR697804	KR697842	KR697935
Delma labialis	QM J89591	QLD	Cook	11°43′59″S	142°36′20″E	KT803566	KR697887	KR697805	KR697843	KR697936
Delma mitella	QM J80846	QLD	Tolga, 1.5 W, Atherton	17°12′42″S	145°27′10″E	AY134592	KR697888	KR697806	KR697844	KR697937
Delma molleri	SAMA R30311	SA	Rochester Historic Site	33°42′00″S	138°28′00″E	KT803567	KR697889	KR697807	KR697845	KR697938
Delma molleri	SAMA R36233	SA	Clare	33°42′00″S	138°28′00″E	AY134593	KR697890	KR697808	KR697846	KR697939
Delma nasuta	WAM R164761	WA	Warmun	16°57′26″S	128°14′15″E	KT803568	KR697891	KR697809	KR697847	KR697940
Delma nasuta	WAM R172192	WA	Ilkurlka Roadhouse	28°20′01″S	127°23′50″E	KT803569	KR697892	KR697810	KR697848	KR697941
Delma pax	WAM R134068	WA	Newman	23°05′45″S	118°52′21″E	KT803570	KR697893	KR697811	KR697849	KR697942
Delma pax	WAM R172570	WA	Cummins Range	19°16′56″S	127°06′51″E	KT803571	KR697894	KR697812	KR697850	KR697943
Delma petersoni	WAM R165873	WA	Queen Victoria Spring	29°19′11″S	124°31′28″E	KT803572	KR697895	KR697813	KR697851	KR697944
Delma petersoni	WAM R165874	WA	Queen Victoria Spring	29°14′04″S	124°31′08″E	KT803573	KR697896	KR697814	KR697852	KR697945
Delma plebeia	QM J80132	QLD	Bungil	26°34′41″S	148°54′26″E	KT803574	KR697897	KR697815	KR697853	KR697946
Delma plebeia	QM J89574	QLD	Stanthorpe	28°56′46″S	151°23′37″E	KT803575	KR697898	KR697816	KR697854	KR697947
Delma tealei	WAM R153811	WA	Cape Range NP	22°07′08″S	114°03′44″E	KT803576	KR697899	KR697817	KR697855	KR697948
Delma tealei	WAM R153813	WA	Yardie Homestead Caravan	21°53′37″S	114°00′34″E	KT803577	KR697900	KR697818	KR697856	KR697949
Delma tincta	WAM R164218	WA	Mount Percy	17°40′51″S	124°56′20″E	KT803578	KR697901	KR697819	KR697857	KR697950
Delma tincta	WAM R137953	WA	Kununurra	15°35′20″S	128°58′60″E	KT803579	KR697902	KR697820	KR697858	KR697951
Delma tincta	ANWC R05368	QLD	Shoalwater Bay Army Reserve	22°33′30″S	150°46′55″E	KT803580	KR697903	KR697821	KR697859	KR697952
Delma torquata	QM J83187	QLD	Wongi State Forest	25°25′60″S	152°16′04″E	KP851409	KR697904	KR697822	KR697860	KR697953
Delma torquata	QM J84362	QLD	Tanduringie Creek, W Cooyar Mt.	26°55′53″S	151°45′01″E	KP851410	KR697905	KP851299	KR697861	KR697954
Out group taxa										
Anolis carolinensis	NA	–	NA	NA	NA	–	–	EU402826	AAWZ02015549	–
Aprasia repens	WAM R172989	WA	Jandakot Regional Park at King Rd.	32°13′37″S	115°53′53″E	AY134579	KR697862	KR697784	KR697823	KR697911
Carphodactylus laevis	AMS R143258	QLD	NA	NA	NA	–	–	EF534781	EF534905	–
Lialis burtonis	AMS R151574	NSW	Sturt NP, Olive Downs Homestead	29°03′05″S	141°51′34″E	AY134599	KR697906	GU459540	AF090850	KR697955
Nephrurus amyae	NTM R18299	NT				–	–	JF807385	JF807319	–
Oedura marmorata	SAMAR34209	QLD	Lawn Hill NP	18°35′00″S	138°30′00″E	–	–	FJ571623	FJ571638	–
Ophidiocephalus taeniatus	SAMA 44653	SA	6.5 km SW of Todmorden Station	27°39′26″S	134°39′20″E	AY134601	KR697907	FJ571630	FJ571645	KR697956
Paradelma orientalis	QM J56089	QLD	Peak Downs	23°01′00″S	147°52′60″E	AY134605	KR697908	FJ571626	FJ571642	KR697957

(*continued on next page*)

Table 1 (*continued*)

Species	Collection ID	State	Locality	Latitude	Longitude	ND2	DYNLL1	RAG1	Cmos	MXRA5
Pletholax gracilis	WBJ 2483	WA	Leseur NP	NA	NA	AY134602	KR697909	HQ426315	AY134566	KR697958
Pygopus lepidopodus	WBJ 1206	WA	Leseur NP	NA	NA	AY134603	KR697910	FJ571627	FJ571643	KR697959
Pygopus nigriceps	ERP 29509	WA	Laverton	NA	NA	AY134604	–	FJ571628	FJ571644	–
Python molurus	NA	–	NA	NA	NA	–	–	AEQU010344888	AY099968	–
Sphaerodactylus roosevelti	CAS 198428	–	Puerto Rico, USA	NA	NA	–	–	EF534785	AY172946	–
Sphaerodactylus torrei	JB 34	–	Cuba	NA	NA	–	–	EF534788	EF534913	–
Woodworthia maculata	RAH 292	–	Titahi Bay, New Zealand	NA	NA	–	–	GU459449	JQ945628	–

Abbreviations: The Western Australian Museum (WAM), Australian Museum – Sydney (AMS), South Australian Museum (SAM), Queensland Museum (QM), Museum of Comparative Zoology at Harvard (MCZ), Museum of Vertebrate Zoology at UC Berkeley (MVZ), California Academy of Sciences (CAS), W. Bryan Jennings (WBJ), Jon Boone (JB), Eric R. Pianka (ERP). All ingroup individuals included in nuclear sampling are adults, which have been appropriately identified by museum staff at the institutions in which they were deposited. WAM material was additionally checked by IGB 2015 to ascertain proper species identity, particularly of morphological similar and sympatric species of the northwest Australia clade D.

nDNA returned identical topologies and did not significantly disrupt BSS support values. We executed two parallel (two heated and two cold chain) runs for 200 million generations sampled every 1000 generations, with sampling from the first 20 million generations discarded as burn-in.

2.4. Divergence dating and the multispecies coalescent

Due to the heavy dependence on priors, in instances of limited loci (~10 or less), concatenation of multiple markers has been suggested to perform as successfully as coalescent methods in returning the true species topology (Ogilvie et al., submitted for publication). To test this, we analyzed our nuclear dataset in a coalescent framework via the *BEAST function in BEAST v1.8.1 (Drummond and Rambaut, 2007). Because of inconsistency between mtDNA and nDNA datasets, and a strongly discordant nuclear locus, we used a three gene nuclear dataset for our coalescent species tree and chronogram. The analysis was run for 100,000,000 generations, sampled each 10,000 generations, Tracer v1.6 (Rambaut et al., 2014) was used to identify appropriate burn-in (10%), and remaining trees were summarized in TreeAnnotator v2.1.2 (Rambaut and Drummond, 2014).

We used BEAST 1.8.1 (Drummond and Rambaut, 2007) to estimate divergence times within *Delma*, and did not enforce a topology for this analysis, but did identify ingroup (*Delma*) and outgroup genera (*Aprasia, Lialis, Ophidiocephalus, Paradelma, Pygopus*) as monophyletic. Data were partitioned according to gene and codon position, and substitution and clock rates were unlinked. We implemented a Yule tree prior and uncorrelated relaxed lognormal clocks for all loci. Program specifications and output followed the same procedure as for *BEAST. Following Lee et al. (2009) we applied a floating calibration (exponential prior, mean = 10, offset = 20) for the *Pygopus hortulanus* fossil in the outgroup as a result of uncertain phylogenetic placement. Three additional non-pygopodid calibration points followed published priors in Heinicke et al. (2011) and Agarwal et al. (2014) by applying relative ages to: split between continental and New Zealand diplodactylids (exponential, mean = 17, offset = 16), a fossil sphaerodactylid (exponential, mean = 3, offset = 15), and the most recent common ancestor (TMRCA) of Gekkota (exponential, mean = 20, offset = 110).

2.5. Phylogenetic hypothesis testing

Using the concatenated nuclear dataset in RAxML and the partitioning schemes applied for prior analyses, we enforced topological constraints to test the validity of our hypothesized interspecific relationships as determined by nDNA, against those proposed by our mitochondrial dataset. To identify instances of ancient introgression, we compared per-site log-likelihood results produced in RAxML between these constrained trees and an unconstrained ML tree, and subjected these to the approximately unbiased (AU) and Shimodaira–Hasegawa tests using CONSEL (Shimodaira and Hasegawa, 1999, 2001). To test for recent instances of mitochondrial introgression, we sequenced all tissues of *D. fraseri* and *D. grayii* (n = 63 *D. fraseri*, n = 12 *D. grayii*, total n = 75 individuals), as well as the hypothesized closest sister species *D. petersoni* (n = 3) – previously treated as a subspecies of *D. fraseri*.

3. Results

3.1. A basal split and reciprocal monophyly among Pygopodidae genera

PartitionFinder (Lanfear et al., 2012) assigned varying nucleotide substitution models based on nuclear loci and codon positions

Table 2
Primers used for PCR amplification and sequencing.

Gene	Primer name	Sequence	Primer reference
ND2	MetF1 L4437	5′-AAGCTTTCGGGCCCATACC-3′	Macey et al. (1997)
	ND2F17	5′-TGACAAAAAATTGCNCC-3′	Macey et al. (2000)
	TRPR3 H5540	5′-TTTAGGGCTTTGAAGGC-3′	Macey et al. (1997)
	CO1R1	5′-AGRGTGCCAATGTCTTTGTGRTT-3′	Macey et al. (1997)
16S	16ScL2189	5′-GTMGGCCTAAAAGCAGCCAC-3′	Reeder (1995)
	16SbH2920	5′-GCGCTGTTATCCCTAGGGTAACTTG-3′	Reeder (1995)
RAG1	RAG1 396	5′-TCTGAATGGAAATTCAAGCTGTT-3′	Groth and Barrowclough (1999)
	RAG1 F700	5′-GGAGACATGGACACAATCCATCCTAC-3′	Bauer et al. (2007)
	RAG1 R700	5′-TTTGTACTGAGATGGATCTTTTTGCA-3′	Bauer et al. (2007)
	RAG1 397	5′-GATGCTGCCTCGGTCGGCCACCTTT-3′	Groth and Barrowclough (1999)
MXRA5	MXRA5 PF2	5′-AAYATTTTGGCAAAAGTCCGWGGRA-3′	This study
	MXRA5 PR2	5′-GCTTKGGTCTYYTGAACCTATTTGG-3′	This study
DYNLL1	DYNLL1 ex1.F	5′-TGATCAAGAATGCGGATATGTCTGAG-3′	Fujita et al. (2010)
	DYNLL1 F312	5′-CCCATGAGYGACTGAAGCAAC-3′	This study
	DYNLL1 R1224	5′-TCAAACCACCTCAGTAACTTGCT-3′	This study
	DYNLL1 ex2 R	5′-TCTTCCCACAATACAGTGCCAAGTAG-3′	Fujita et al. (2010)
MOS	MOS G73	5′-GCGGTAAAGCAGGTGAAGAAA-3′	Saint et al. (1998)
	MOS G74	5′-GTMGGCCTAAAAGCAGCCAC-3′	Saint et al. (1998)

(Supplementary Table 1). Both mtDNA and nDNA datasets unequivocally support the monophyly of all currently recognized genera (BSS and BPP = 100), as well as the sister-genus relationship between *Paradelma* and *Pygopus*, and the basal split between *Delma* and all remaining pygopodid genera. Remaining intergeneric relationships are generally poorly supported by both mtDNA and nDNA datasets.

The monophyly of *Delma* remains highly supported (BSS and BPP = 100) across the two datasets, as is the monophyly of most currently recognized species. To date, Jennings et al.'s (2003) mitochondrial tree has been widely accepted as the accurate representation of interspecific relationships within *Delma*, and our matrilineal history of *Delma* reflects those findings with strong support. Our concatenated nDNA dataset, however, provides a strongly supported incongruent topology.

3.2. Phylogenetic analyses of nuclear data

Bayesian and maximum likelihood analyses of four nuclear loci analyzed independently and as a single concatenated nuclear dataset, are congruent in returning strong support for the monophyly of *Delma*, as well as the basal split between the *D. australis* group and all other delmas. Three of four nuclear loci (DYNLL1, RAG1, MOS) return largely concordant interspecific relationships, however phylogenetic signal from MXRA5 provides a largely unresolved topology of relationships within *Delma*. Despite incongruence in phylogenetic reconstructions between MXRA5 and remaining nuclear loci, we continued to include this marker in all analyses, as part of a total evidence assessment. The four gene concatenated data provide high support for most species groups and all currently recognized species, however, deeper relationships among species groups receive only moderate support. RAG1 and DYNLL1 provide the most well resolved phylogenies, exclusive of the concatenated data.

Ultimately, we have placed confidence in the interspecific topology returned by the coalescent species tree (CST) approach in *BEAST, which broadly agrees with both ML and BI analyses. Our CST largely mirrors that produced by RaxML and MrBayes in identifying three distinct clades (A, B, D, Fig. 1), a single non-monophyletic but morphologically similar species group (C), and three enigmatic range-limited species: *D. concinna, D. labialis*, and *D. mitella*. The basal split of the *D. australis* group (*D. australis*, *D. hebesa*, *D. torquata*) from the rest of the genus is strongly echoed (1.0*BEAST BPP/95RAxML BS/1.0MrBayes BPP) across datasets, regardless of the inconsistent position of *D. concinna*. Exclusive of the *D. australis* group, *D. mitella* is identified as the sister taxon to the remaining delmas (0.80/78/1.0). There is high support (1.0/99/1.0) for a broadly distributed 'inornate' group comprising *D. butleri*, *D. grayii*, *D. haroldi*, *D. inornata*, and *D. nasuta*. The group C, several species of large, stout-bodied delmas which stretch across southern and western Australia, are recognized as a non-monophyletic group, united by similar morphology. Nuclear data does support the monophyly (1.0/99/0.96) of a largely northwestern Australian group composed of *D. borea, D. desmosa, D. pax, D. tealei*, and *D. tincta*. *Delma elegans* is generally associated with this group despite varied amounts of support (0.50/83/0.70) in our analyses. The position of *D. labialis* remains enigmatic *D. labialis* (–/48/0.68). Interspecific relationships within the northwest clade mostly receive poor support, and are not well addressed in this group, and this matter is complicated by considerable morphological similarity, making morphological assessment difficult.

3.3. Phylogenetic analyses of mitochondrial data

Although mtDNA data also provide high support for the monophyly of *Delma*, as well as the basal split (exclusive of *D. concinna*) between the *D. australis* group and remaining delmas, the mtDNA topology is discordant with nDNA data (Fig. 1). Species groups are well supported, as well as interspecific relationships, but positions of the range-limited species *D. concinna* (62 BS/0.79 BPP) and *D. labialis* (50/0.55) basal to other delmas are given low support. There remains support (100/1.0) for the northwestern Australian clade of *D. borea, D. desmosa, D. elegans, D. pax*, *D. tealei*, and *D. tincta*. A geographically proximate east/southeastern group composed of *D. impar, D. mitella, D. molleri*, and *D. plebeia* are returned as a monophyletic group with strong support (99/1.0) for interspecific relationships. As in the nDNA results, *Delma butleri, D. haroldi*, and *D. nasuta* are returned within the same species group (100/1.0), however, *D. inornata* and *D. grayii* are instead allied with *D. petersoni*, and *D. fraseri*. This group stretches along the southern coast of Australia from extreme southern Queensland along the southern and western coasts up to Shark Bay, Western Australia. High support (91/1.0) unites *D. fraseri* and *D. grayii* as sister taxa, with moderate support (71/0.90) for *D. petersoni* as sister species to this pair. Both *D. fraseri* and *D. grayii* remain strongly supported as monophyletic (BSS/BPP = 100/1.0), with no shared mitochondrial haplotypes (Supplementary Fig. 1).

3.4. Divergence dating

We used the four gene concatenated nuclear dataset to build a Bayesian time-tree using the program BEAST (Drummond and Rambaut, 2007). The resulting topology (Fig. 2) is largely concordant with our mixed method (ML, BI) and coalescent concatenated nDNA species tree topologies, but provides higher support at a number of previously weakly or moderately supported clades. Monophyly of the northwestern Australia group (*D. labialis, D. elegans, D. desmosa, D. pax, D. borea, D. tincta, D. tealei*) (BPP = 1.0) and its position nested within the stout-bodied southern Australia group (*D. fraseri, D. impar, D. molleri, D. petersoni, D. plebeia*) are returned with high support (BPP = 97). *Delma concinna* is strongly supported as the sister species to all other delmas. The broadly distributed 'inornate' group remains well supported (BPP = 100). Despite monophyly of the northwest Australia group, interspecific relationships within this clade remain poorly resolved.

The crown group split within Pygopodidae appears to have occurred approximately 25 Mya, following the basal Carphodactylidae–Pygopodidae split ca. 60 Mya reported elsewhere (Lee et al., 2009). A basal split between the *D. australis* group and the rest of *Delma* – following the divergence of *D. concinna* – appears to have occurred in a similar timeframe as the split of *D. mitella*, 15 Mya. Several short branches with associated moderate support values indicate relatively rapid speciation events between *D. labialis* and the northwest Australia group, and again between *D. borea* and remaining members of the group. The relatively long branch leading to *D. elegans* may identify it as an isolated lineage, a Pilbara endemic relic, similar to those found within other pygopodoidean groups (Oliver et al., 2010a, 2014). Within-group divergences appear clocklike in timing, with new speciation events occurring each 1.5–2.5 million years. The majority of speciation events appear less than 10 million years in age.

3.5. Coalescent species tree

Results from our *BEAST analysis (not figured) are largely concordant with those produced by BEAST, ML, and BI. There remains high support for all recognized species groups – *D. australis* group (1.0 BPP) and 'inornate' group (1.0), and the 'northwest Australia' group (1.0) and the large-bodied southern group (C) within which they are nested (1.0). The phylogenetic position of *D. concinna* remains uncertain, as it is weakly placed (0.52) as sister taxon to all remaining delmas with the exception of the *D. australis* group. *Delma elegans* is only weakly affiliated (0.50) with the remaining 'northwest Australia' group, and the current position of *D. labialis* remains unsupported.

3.6. Testing phylogenetic hypotheses

Congruence between nuclear loci across phylogenetic analyses allowed us to subject three species groups, as well as three independent instances of potential mitochondrial introgression, to ML tests of monophyly via AU and SH tests in CONSEL. Results are reported in Table 3. Both AU and SH tests were unable to reject the monophyly of species groups A, B, and D. Several relationships

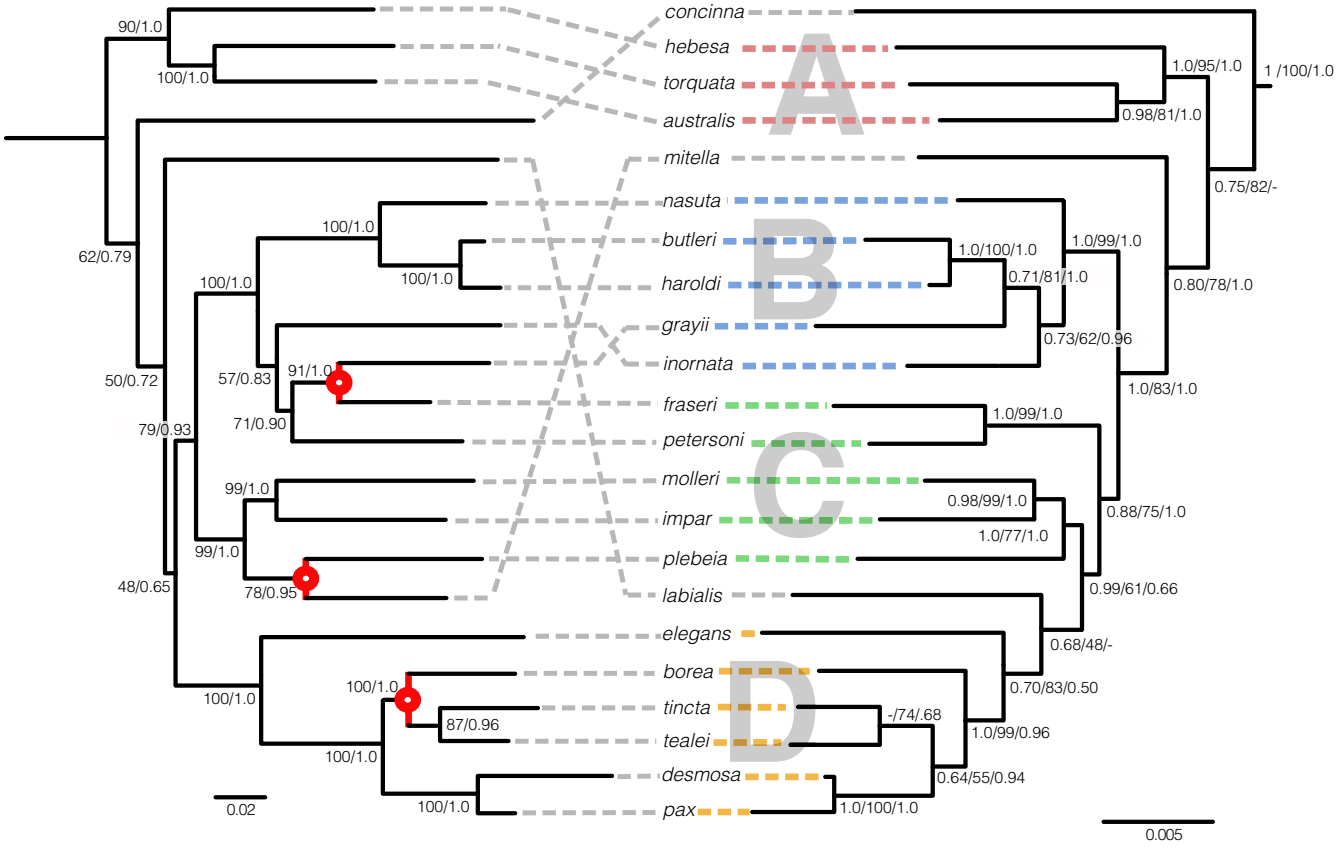

Fig. 1. Discordant simplified interspecific phylogenies of *Delma* as inferred by mitochondrial (left) and nuclear (right) datasets. Support values indicated at nodes of mitochondrial tree are BSS/BPP. Grey dashes on the mtDNA tree identify phylogenetic position and highlight the discordance of mitochondrial and nuclear phylogenies. Red nodes indicate phylogenetic relationships strongly supported by mtDNA, however rejected in the nDNA dataset by AU and SH tests. Support values indicated at nodes of nuclear tree are BPP(MrBayes)/BSS(RAxML)/BPP(*BEAST). Clades A, B, D are denoted by red, blue, and gold dashed lines, respectively. Group C is denoted by green dashed lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inferred from mitochondrial data and supported through ML and BI analyses: 1. *D. fraseri* + *D. grayii*; 2. *D. mitella* + *D. plebeia*; and 3. *D. borea*+(*D. tincta* + *D. tealei*), were rejected by both AU and SH (1, 2), or by AU tests alone (3). Additionally, investigation into introgression of the sympatric *D. fraseri* and *D. grayii* indicates reciprocal monophyly, with no shared mitochondrial haplotypes.

4. Discussion

Comprehensive species level sampling of the genus *Delma* reflects the complicated story of Australian climatic history and interspecific interaction, indicating a lack of reproductive isolation. Incongruence between molecular data detected by this study highlight the necessity of integrative approaches for phylogenetic reconstruction. Mitochondrial loci, particularly ND2, have been used consistently for estimation of interspecific relationships and identifying morphologically cryptic lineages in squamates (Townsend et al., 2004; Jackman et al., 2008; Portik et al., 2011). Here, investigation into intraspecific maternal lineages of *fraseri* and *grayii* offer valuable insight into geographic isolation and divergent lineages. In contrast, the independently evolving genetic history of individual nuclear loci, and their slower mutational rates mean few loci are consistently employed in non-overlapping phylogenetic studies. We have however used the well-established nuclear exons RAG1 and MOS to provide continuity, MXRA5, and the fast evolving nuclear intron DYNLL1 to provide much needed resolution at the tips of our *Delma* phylogeny. Although MXRA5 fails to contribute a well-supported phylogenetic reconstruction, GC content and substitution rates and sites of MXRA5 appear inconsistent with directional selection, suggesting that topological inconsistencies are likely the result of a lack of phylogenetic informativeness, and not ILS. In light of the move of phylogenetics toward Next Generation Sequencing methods, we recognize the continued necessity for highly informative nuclear loci like RAG1, which are able to provide accurate phylogenetic reconstructions at the inter- and intraspecific level, and provide a legacy of pre-existing data.

4.1. Addressing discordance in Delma

Current theory suggests that individual gene-tree discordance is generally the result of: ILS, horizontal gene transfer (including hybridization), and gene duplication and extinction (Maddison, 1997; McGuire et al., 2007). Despite the ability to identify possible causes, rectifying incongruence between mitochondrial and nuclear data remains difficult. Instances of mito-nuclear discordance are most often recognized in events of recent conflict, apparent as shared mitochondrial haplotypes, and are commonly attributed to sex-biased dispersal, sympatry, and selection (Toews and Brelsford, 2012).

Instead, we find *Delma* to be similar to other groups such as *Sceloporus* (Leaché, 2009, 2010), Cordylidae (Stanley et al., 2011), *Thomomys* (Belfiore et al., 2008), and Neoaves (Poe and Chubb, 2004), in that difficulty associated with resolving some interspecific relationships among *Delma* may be the result of rapid radiation, and subsequent introgression events, in comparatively young lineages. Our BEAST analysis of pygopodids suggest an increase in speciation events between 10 and 5 Mya, much younger than those proposed by Jennings et al. (2003). Rapid molecular diversification as the result of movement bias via sex-based dispersal, or directional selection such as a selective sweep (Rato et al., 2010, 2011) or sexual selection (Panhuis et al., 2001) can confound results when ancestral polymorphism is conserved by independent and conflicting gene trees (Maddison, 1997; Belfiore et al., 2008). These problems are exacerbated when branches are short and wide, suggesting short generations with relatively large effective population size (Maddison, 1997). While increased sampling across and within species may help to assuage the issues associated with rapid increases in speciation rates by providing greater phylogenetic resolution via a broader picture of genetic diversity, a basic violation of the molecular clock remains. Clock-like evolutionary rates may speed up (directional selection) or slow down (stabilizing selection) depending upon external pressures, requiring careful investigation and application of appropriate evolutionary models (Lemmon and Moriarty, 2004), and even then, polytomies may persist. Rapid speciation appears particularly evident in the northwest Australia group, in which all speciation events (exclusive of *D. elegans*) occurred <15 Mya.

4.2. Discordant phylogenetic relationships

Our molecular sampling comprises all currently recognized species of *Delma*, as well as broad intraspecific sampling across several species, and builds upon previous molecular phylogenetic study of this group (Jennings et al., 2003; Maryan et al., 2007, 2015). Whereas discordance between trees of explosive radiations (those with short, wide branches) often results in a number of poorly supported nodes, a phylogenetic comb, bush, or polytomy (Poe and Chubb, 2004; Kelly et al., 2009; Stanley et al., 2011), phylogenetic reconstruction of *Delma* displays clades which receive strong, conflicting support across mitochondrial and nuclear datasets, similar to conflict found by Leaché (2010). Although conflict between mitochondrial and nuclear signals (mito-nuclear discordance) is often ignored or left unaddressed, a recent review (Toews and Brelsford, 2012) compiled 126 recent cases in animal systems in which there is strong discordance between the two datasets. In these situation, it can be difficult to tease apart the specific cause of such incongruity. So, if provided two strongly supported, yet conflicting phylogenies, trouble comes when we are forced to decide which is the most accurate representation of the true evolutionary history of the group.

Differences in the biology and evolution of mitochondrial and nuclear markers make them useful for studies at varying phylogenetic depths (Leaché and McGuire, 2006; Leaché, 2009; Fisher-Reid and Wiens, 2011; Kubatko et al., 2011). While the rapid mutational rate of mtDNA is useful in identifying independently evolving lineages, it is hampered by the singularity of its maternally inherited history. Conversely, because of slower evolutionary rates, nuclear genes are often more informative deeper in the phylogeny, and because of recombination, often prove valuable in studies of population genetics. As an exercise in identifying mitochondrial introgression at a fine scale, we focused on the previously hypothesized sister-species *D. fraseri* and *D. grayii,* which are sympatric across the entirety of the range of *D. grayii*. Despite sympatry over substantial geographic distance, and documented syntopy (Jennings et al., 2003), *D. fraseri*, *D. grayii*, and *D. petersoni* all remained reciprocally monophyletic with no shared mitochondrial haplotypes among species, suggesting no recent introgression events. Incongruity with the nuclear dataset, and well supported sister relationships between *D. fraseri* and *D. petersoni* instead suggest an older hybridization event between *D. fraseri* and *D. grayii*, which succeeded the split of *D. fraseri* and *D. petersoni*, and is not visible in this phylogeographic level. Mitochondrial introgression between these sympatric species has subsequently been obfuscated by the rapid mitochondrial mutation rate and subsequent divergent evolutionary lineages. We hypothesize similar ancestral reticulations have occurred between *D. mitella* and *D. plebeia*, and between *D. borea* and TMRCA of *D. tincta* and *D. tealei*. AU and SH tests reject the mitochondrially assessed monophyly of these relationships, instead, supporting relationships as diagnosed by nuclear data.

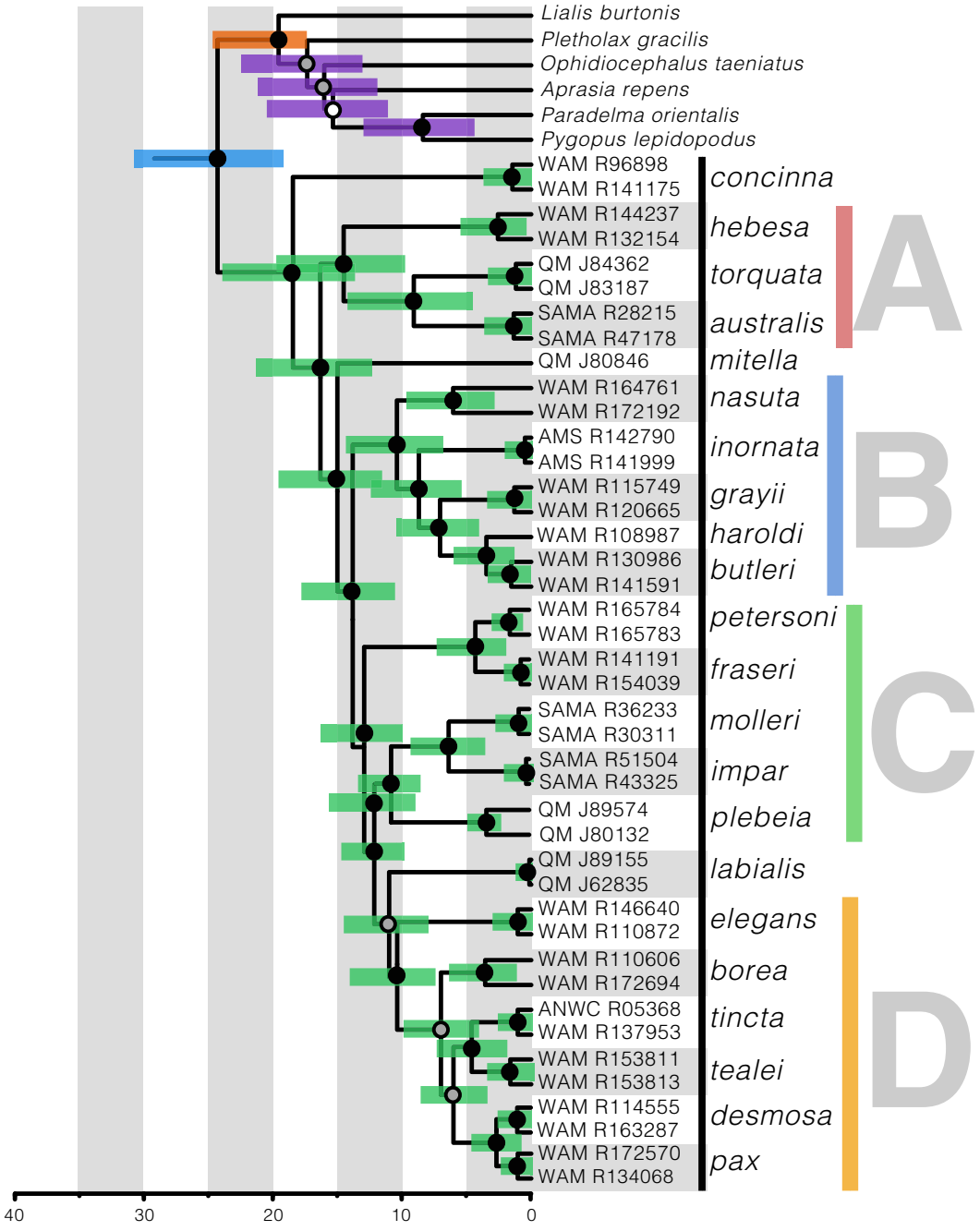

Fig. 2. Bayesian time-tree of divergence dates among Pygopodidae, inferred from the concatenated nuclear dataset (DYNLL1, RAG1, MOS, MXRA5) using BEAST. Circles at nodes indicate BPP support values, black >0.95, grey >0.80, and white <0.80. Node bars represent 95% credibility intervals, green bars designating the in group taxa *Delma*, purple representing outgroup pygopodids, blue – the crown split of pygopodids, and orange bar indicating confidence interval for the split between *Paradelma* and *Pygopus*, where we placed the *Pygopus hortulanus* fossil calibration point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Approximately Unbiased (AU) and Shimodaira–Hasegawa (SH) tests of monophyly and alternative (mitochondrial) topologies using the three-loci concatenated nuclear dataset. Bold typeface indicates *P*-values <0.05, suggesting the rejection of proposed monophyly.

Constraint	ln Likelihood	Δ ln Likelihood	P–AU test	P–SH test
Unconstrained	−6724.123917	–	–	–
australis group (A)	−6724.049506	0.074411	0.551	0.978
'inornate' group (B)	−6724.199507	−0.075590	0.519	0.926
northwest Australia group (D)	−6724.199507	−0.075590	0.519	0.926
Fraseri + grayii	−6787.571486	−63.447569	**2e−04**	**0.003**
mitella + plebeia	−6767.751552	−43.627635	**4e−04**	**0.022**
borea+(tincta + tealei)	−6739.741228	−15.617311	**0.027**	0.322

Attempts to accurately define the theoretical 'species' have resulted in varied success, however, accepting that introgression can and does happen is essential to appreciating discrete taxonomic units. Mayr (1996) suggested that as long as two species return to their differing evolutionary histories – i.e. bounce back to their original lineages – they should continue to be treated as distinct taxa, regardless of the "inefficiency of their isolating mechanisms." This does not however address the concept of mitochondrial capture, and continued linkage through matrilineal history. We can identify several instances in which introgression has likely occurred: (1) between the sympatric *D. fraseri* and *D. grayii*; (2) between the currently allopatric *D. mitella* and *D. plebeia*; and (3) between the broadly sympatric *D. borea* and *D. tincta*. The monophyly of all *Delma* species associated with ancient hybridization events presented here supports Mayr's (1996) claim that closely related and sympatric species do often reproductively interact with one another across an evolutionarily timescale. It is only now, through genetic methods that we are able to recognize these events, many of which have left no other artifacts.

4.3. Morphological species groups

The decision to accept the relationships within *Delma* as resolved by nDNA is validated by morphological and biogeographic evidence. Several phylogenetic similarities exist between our nDNA topology and Kluge's (1976) morphological assessment. Prior use of the mtDNA topology for *Delma* is largely the result of a previously uninformative nuclear dataset. However, interspecific relationships as inferred by nDNA are unsurprising when viewed in light of general morphology. The inability to reject monophyly of nDNA relationships as determined by AU and SH tests adds credence to our hypotheses. We do however recognize

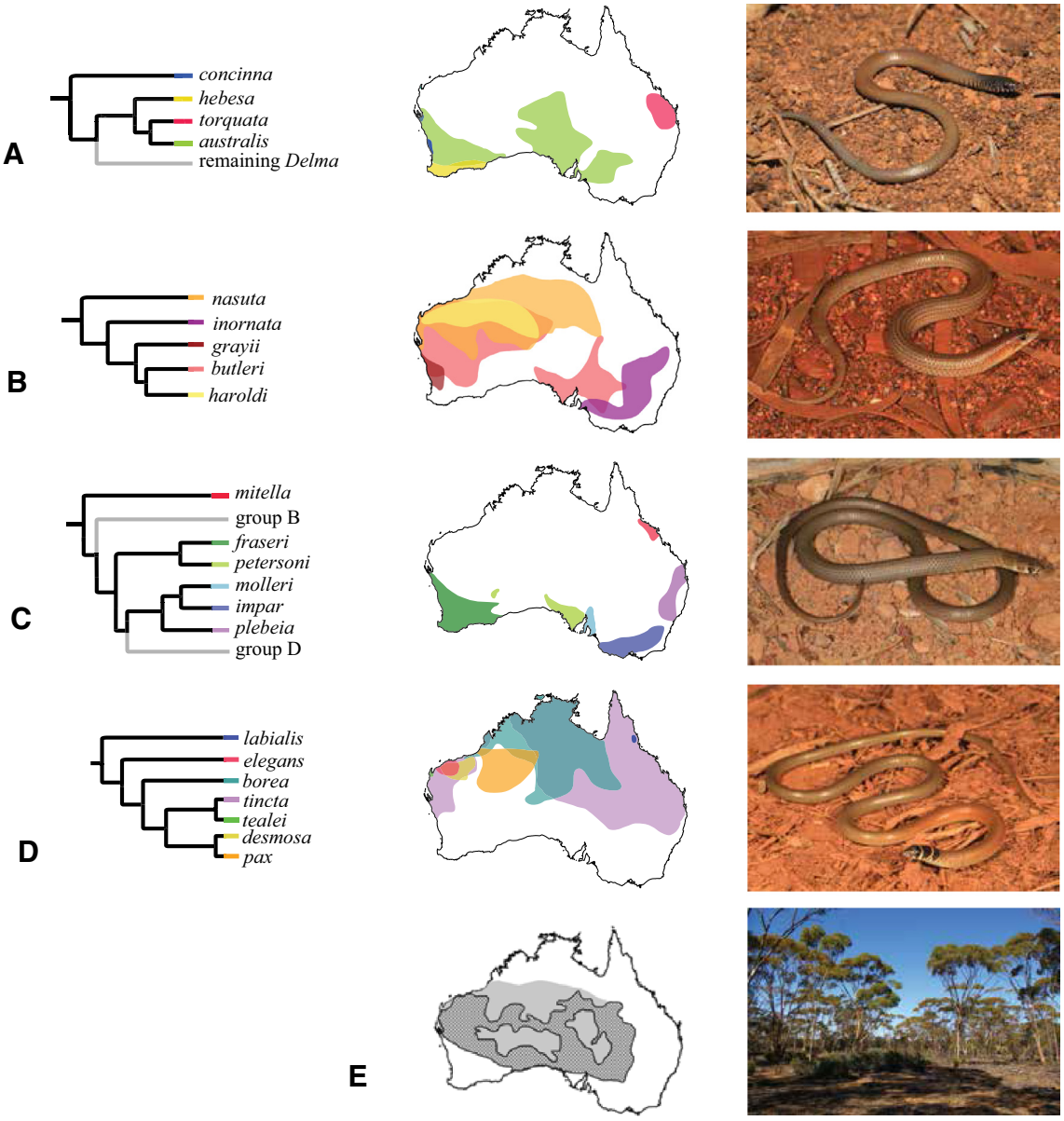

Fig. 3. Geographic distributions and interspecific relationships within major species groups: (A) *D. australis* group and *D. concinna*, image of *Delma hebesa* from Fitzgerald River National Park, WA; (B) 'inornate' group, and image of *Delma nasuta* from Pannawonica, WA; (C) *D. mitella*, and morphologically similar *D. fraseri* and *D. plebeia* groups, image of *Delma fraseri* from Kalbarri, WA; (D) northwestern Australia group and *D. labialis*, image of *Delma borea* from El Questro Station, WA. (E) shows extent of the Australian arid zone in grey, and proposed Miocene shrubland corridors adapted from Cracraft (1986), with image of potential vegetative corridor, *Acacia* and *Eucalyptus* canopy with tussock grass and shrubby understory from near Kellerberrin, WA. All photos courtesy of Ryan Ellis.

a non-monophyletic southern Australian species group C, united by large size, stout bodies, and considerable facial and nuchal banding which decreases in intensity with age. Nested within group C is the northwest Australian group, clade D, a group which generally reach smaller adult sizes, and retain strong head and nuchal banding, reminiscent of juveniles of group C. Conserved morphological characters suggest the possibility of some degree of paedomorphism within the northwest Australian clade, and hint at a large, strongly banded ancestral condition for groups C and D. These hypotheses, however, would require substantial morphological and osteological assessment to validate. In contrast, members of the inornate clade B are relatively large, but exhibit longer, sharper snouts, and as a rule, lack the broad prominent banding of the previous groups. As suggested by the common names patternless delma (*D. inornata*) and unbanded delma (*D. butleri*), these species lack the nuchal banding found in groups C and D. While patterning and coloration are variable within members of the *D. australis* clade, they are distinct in the combination of small adult size, comparatively brief snouts, and short tails (Fig. 3).

4.4. Revising interspecific relationships within Delma and Conclusion

This study represents the only investigation of pygopodid relationships to date to include a multilocus nuclear dataset in attempt to resolve the phylogenetic affinities of the genus *Delma*. We identify four diverse species groups A–D, as well as several enigmatic species – *D. concinna, D. mitella, D. labialis* – which do not conform to any single group based on morphology, distribution, or molecular results. Broadly disjunct distributions and seemingly associated morphological variation within the *D. australis* clade encourages future study. The Pilbara and western Kimberley regions are ares of high delma endemism (Fig. 3), and may potentially harbor species not yet described. Although species in the far north remain largely sheltered, many southern and east coast species are range restricted, and three – *D. impar, D. labialis, D. torquata* – are listed as Vulnerable by the International Union for Conservation of Nature (IUCN, 2012).

4.5. Speciation in an aridifying landscape

Speciation patterns and divergence dates within *Delma* as inferred by nDNA are highly concordant with the proliferation of other arid biota as Australia became increasingly arid in the Late Miocene 20–6 Mya (Byrne et al., 2008). The absence of pygopodids from closed-forest systems and abundance in semi-arid regions across Australia suggests adaptations to aridity similarly found across a number of Australian lineages. The increase in species richness between 10 and 5 Mya coincides with increased aridification and expanding xeric habitat across the continent. It is difficult to assess if aridification caused speciation by fragmenting formerly widespread populations into allopatric populations tied to suitable habitat. Or, if the expansion of arid biomes opened new ranges and niches for pygopodids, however, it is likely that both processes have shaped current phylogenetic diversity within *Delma*.

Despite drought-tolerance, hyperarid dunefields and deserts of much of central Australia are inhospitable to most *Delma* species. Many delmas appear to be strongly associated with *Triodia* spinifex, tussock grass, and related dense, low vegetation. The paucity of *Delma* records from hyper-arid regions such as Channel Country, Gibson Desert, Little Simpson Desert, Nullarbor Plain, and the Simpson Strzelecki Dunefields coincides with the lack of suitable habitat and groundcover (Pianka, 1969, 2010; Wilson and Swan, 2013). As an effect of desertification, important corridors (Fig. 3) between more preferable habitat may have mediated dispersal events, particularly in species with broadly distributed, disjunct ranges – e.g. *D. butleri*, *D. tincta* (Cracraft, 1986). These events have been documented in fairy-wrens (Schodde and Weatherly, 1982; Ford, 1987; Driskell et al., 2003) as well as in the squamates *Ctenophorous scutulatus, Egernia depressa, Ctenotus leonhardii*, and *Eremiascincus richardsonii* (Pianka, 1972). Additionally, speciation as a result of isolation by distance and allopatry, via corridor-mediated dispersal has been suggested as the most likely scenario for another Australian squamate group *Tympanocryptis* (Shoo et al., 2008). As these corridors were largely temporally restricted, they served as a collapsing bridge to suitable habitat, and remain difficult to accurately date. Members of group C appear the best example of this scenario. These species (*D. fraseri*, *D. petersoni*, *D. impar*, *D. molleri*, *D. plebeia*) are confined to subtropical and temperate sclerophyllous habitats, which became repeatedly fractured by arid zone expansion. In contrast, arid adapted species of the 'northwest Australia' clade D, appear to have diversified and expanded during this period, aided by increasing available habitat.

Because of the generalist habits, both dietary and habitat preferences, of many delmas, identifying direct causes of speciation events is difficult. The rapid radiation of species richness within this group is most likely the result of considerable expansion of xeric biomes, but may also be attributable to rapid growth and subsequent shrink of mesic and rainforest habitats 10–2.5 Mya. Proliferation of temperate biomes and closed forest systems in this period may have reduced or fractured available habitat, resulting in allopatric divergence events. Upon succeeding reduction of this mesic Pleistocene expansion, the potential for secondary contact between previously separated species may have caused the mitochondrial reticulations we see in *Delma* today. Strongly discordant mitochondrial and nuclear topologies we present for *Delma* underscore the substantial morphological conservatism of this genus, and acknowledge instances of hybridization between divergent species. Pygopodids represent a uniquely Australian lineage of limbless squamates, rich in ecological diversity. Further investigation of broadly distributed *Delma* species, as well as other pygopodids, may continue to yield insight into patterns of speciation within Australian squamates, and extend our understanding of Australian fauna as a whole.

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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.2015.10.005.

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