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Molecular phylogeny and phylogeography of the Australian *Diplodactylus stenodactylus* (Gekkota; Reptilia) species-group based on mitochondrial and nuclear genes reveals an ancient split between Pilbara and non-Pilbara *D. stenodactylus*

Mitzy Pepper [a], Paul Doughty [b], J. Scott Keogh [a,*]

[a] *School of Botany and Zoology, The Australian National University, Canberra, ACT 0200, Australia*
[b] *Department of Terrestrial Vertebrates, Western Australian Museum, 49 Kew Street, Welshpool, WA 6106, Australia*

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Abstract

There is a paucity of research on intra-specific morphological and genetic diversity in Australian arid-zone reptiles, and a number of Australian reptile species have for many years been regarded as "species complexes" that classical morphological analyses could not resolve. We conducted a phylogenetic and phylogeographic study of a widespread species group of Australian geckonid lizards to address two main aims. First, based on a large mitochondrial and nuclear gene data set, we have generated the first molecular phylogeny for the *Diplodactylus stenodactylus* species group (*D. alboguttatus*, *D. damaeus*, *D. maini*, and *D. squarrosus*, *D. stenodactylus*) and multiple outgroups to examine the evolutionary relationships among these arid-zone species and phylogenetic patterns within some species. The edited alignment of 41 individuals comprises 2485 characters (1163 ND2 + tRNAs; 490 16s; 832 RAG-1), and of these 717 (29%) were variable and parsimony informative (568 ND2 + tRNAs; 89 16s; 60 RAG-1). This broad-scale, multi-gene phylogeny has supported previous conjectures on the higher-level phylogenetic relationships among members of the *D. stenodactylus* species-group based on morphology, but also has uncovered hidden diversity within the group with two new species identified. Analysis at this broad level has identified patterns associated with the distribution of the *D. stenodactylus* species group that appear to be influenced by environmental processes operating at large geographic scales. Two major clades within the species group were associated with broad differences in habitat types, with one group largely restricted to the temperate zone of the Southwest Province and another largely restricted to central and northern Western Australia north of Kalgoorlie, in line with the Eremaean Province of the Eremaean Zone and the Northern Province of the Tropical Zone. Second, we have assembled phylogeographic data based on a mitochondrial gene (ND2 + tRNAs) for five species (*Rhynchoedura ornata*, *Diplodactylus maini*, *D. pulcher*, *D. squarrosus*, *D. stenodactylus*) where larger sampling is available, with particular focus on *D. stenodactylus*, which is distributed both in the iconic but little-known Pilbara area of endemism in north-western Australia as well as in other parts of the Australian arid zone. The edited alignment of 95 individuals comprises 1142 characters and of these 601 (53%) are variable and parsimony informative. We found significant intra-specific genetic variation in all five species, highlighting the need for large-scale screening of cryptic species, with sampling specifically targeted at determining the geographic limits of such taxa. In particular, within *D. stenodactylus*, a deep and ancient phylogenetic split distinguishes populations in the Pilbara region from non-Pilbara populations. This split may be the result of broad differences in underlying geological substrate, with the Pilbara clade generally preferring harder soils and the non-Pilbara clade adapted to sand.

Keywords: Pilbara; Australian arid zone; Biogeography; Phylogeography; Reptile; Gekkonidae; Gekkota; *Diplodactylus stenodactylus*; ND2; 16s; RAG-1

* Corresponding author. Fax: +612 6125 5573.
E-mail address: scott.keogh@anu.edu.au (J.S. Keogh).

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1. Introduction

A number of areas of endemism have been described in Australia based on congruent biogeographic patterns of flora and fauna (Fig. 1), including iconic areas such as the Queensland Wet Tropics and Southwestern Australia (Cracraft, 1991;Crisp et al., 1995). Considerable molecular systematic research has been focused on these mesic areas (Joseph et al., 1995;Hugall et al., 2002; Schauble and Moritz, 2001;Stuart-Fox et al., 2001;Hopper and Gioia, 2004;Wong et al., 2004;Moussalli et al., 2005) and in the Australian arid zone (Moritz and Heideman, 1993;Kearney et al., 2003;Chapple and Keogh, 2004;Chapple et al., 2004; Strasburg and Kearney, 2005), but of the major areas of endemism, the Pilbara region in arid northwestern Australia remains virtually unexplored (Cracraft, 1991). The long-term geological stability of the Pilbara, coupled with its geological heterogeneity and phytogeographic complexity relative to the surrounding desert regions suggests that the Pilbara offers rich research potential to generate and to test biogeographic and phylogeographic hypotheses.

The antiquity and complex geological and climatic history of the Pilbara have undoubtedly had a profound influence on the evolutionary history of the flora and fauna there. To date there have been no published inter or intra-specific molecular phylogenetic studies of Pilbara plants, although there is likely to be enormous undiscovered floral diversity in the region (Crisp et al., 2001). The few existing studies on Pilbara fauna indicate high levels of local endemism (Edwards, 1993;Finston and Johnson, 2004). For example, a recent study allocated three members forming a species group of the skink genus *Lerista* to thirteen new species, nine of which are restricted to the Pilbara plateau and its rocky outliers (Smith and Adams, in press), and subsurface geological complexity appears to account for high diversity in subterranean amphipods (Finston and Johnson, 2004;Finston et al., 2004).

The Australian squamate reptile fauna (lizards and snakes) is extremely rich, and the gekkonid Subfamily Diplodactylinae is particularly diverse (Cogger, 2000). The large area and diverse environments of the Australian arid zone may help to explain why this region has a higher diversity of geckos relative to any other continental desert system in the world (Pianka, 1989;Cogger and Heatwole, 1981;Cogger, 1984). This extreme diversity makes geckos a useful model system for addressing biogeographic questions. Ideally, an approach that includes both species-level and intra-specific phylogeographic data from taxa distributed in disparate geographic regions will provide the strongest means of addressing such questions. The Australian *Diplodactylus stenodactylus* species group and *D. stenodactylus* in particular, represent a model system in this regard.

Diplodactylus is the largest and most diverse diplodactyline genus, and it has received some higher-level systematic attention (Kluge, 1967;King and Horner, 1993;Donnellan et al., 1999;Han et al., 2004). Based on morphological data, Kluge (1967) identified and described several Western Australian taxa, which he assigned to the *D. stenodactylus* species group: *D. alboguttatus*, *D. damaeus*, *D. maini*,

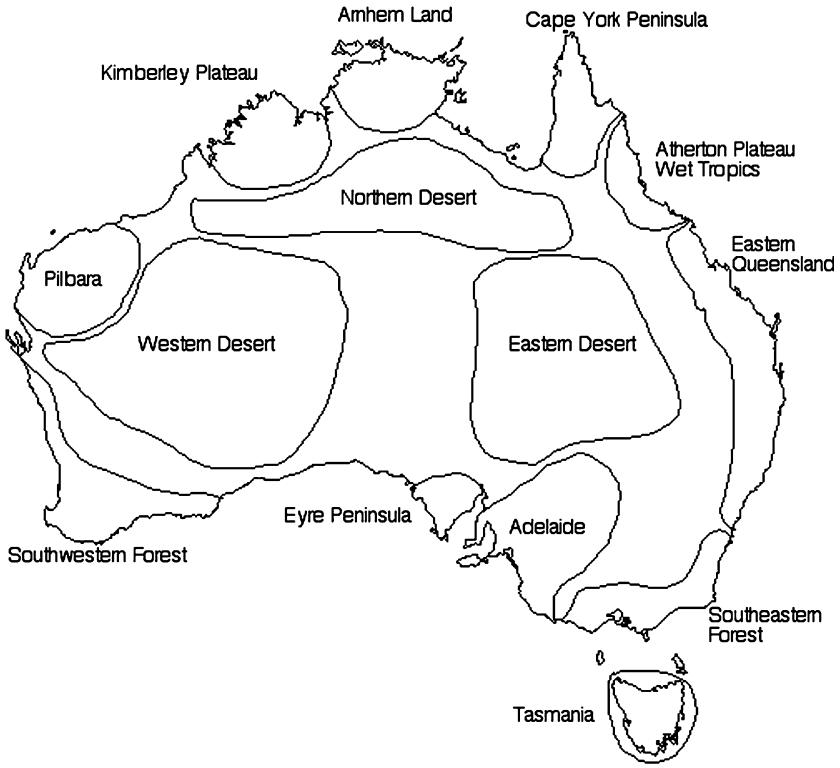

Fig. 1. Map of Australia showing areas of animal endemism (modified from Cracraft, 1991).

D. squarrosus, and *D. stenodactylus*. Other major clades within *Diplodactylus* include the *D. vittatus* and *D. conspicillatus* groups (Kluge, 1967;Storr et al., 1990). In a partial revision of the *D. stenodactylus* group, Storr (1988) described and added *D. immaculatus*, the only non-Western Australian taxon in the species group. Since that time, there has been no comprehensive phylogenetic treatment of the *D. stenodactylus* group. However, based on recognition of a number of shared morphological characteristics, *D. wombeyi* and *D. fulleri* also have been assigned to the *D. stenodactylus* group (Storr et al., 1990). Recent molecular phylogenetic analysis of the gecko genus *Strophurus* and allies (Melville et al., 2004) suggested that *D. stenodactylus* (the only representative of the *D. stenodactylus* group included in their phylogeny) was more closely related to the monotypic *Rhynchoedura ornata* than to other included *Diplodactylus* species.

Clearly, the *D. stenodactylus* species group presents a number of unresolved taxonomic and systematic issues. In addition to recognizing morphological variation within the *D. stenodactylus* group, Kluge (1967) used the presence or absence of particular morphological characters to postulate relationships among the members of the species group. For example, the shared characters of extreme reduction of apical plates, absence of the anterior nasal bone, large numbers of fourth finger and toe lamellae and the long tail led Kluge (1967) to postulate that *D. damaeus* and *D. maini* were sister taxa. Similarly, the presence of an anterior nasal bone, the small numbers of fourth finger and toe lamellae and a moderately long tail common to both *D. stenodactylus* and *D. squarrosus* prompted Kluge (1967) to propose a close relationship between these taxa. A number of characteristics exhibited by *D. alboguttatus* were considered intermediate between *D. damaeus–D. maini* and *D. stenodactylus–D. squarrosus*. Although *R. ornata* shared many characteristics with the *D. stenodactylus* group, Kluge assigned this taxon to a separate genus due to a number of morphological peculiarities, in particular a unique beak-like snout (Kluge, 1967).

Diplodactylus stenodactylus displays extreme morphological variation across the Australian arid zone. This has caused taxonomic confusion that has been difficult to reconcile (Fry, 1914;Kluge, 1967; Storr, 1988), but also means that this species represents an ideal system for examining phylogeographic structure across adjacent areas of endemism. *D. stenodactylus* (Boulenger, 1896) is a small slender gecko found in a variety of habitats in arid regions of Western Australia, the Northern Territory and South Australia, and extending into southwest Queensland and northwest New South Wales (Wilson and Swan, 2003) (Fig. 1). Variation in scalation, coloration and gross morphology led Kluge (1967) to distinguish western and central Australian populations. Kluge (1967) also identified a single specimen from the Hamersley Range that differed from all other *D. stenodactylus* he examined, further illustrating significant variation and unrecognized diversity within this group. A preliminary morphological analysis of more than 800 specimens from the Western Australian Museum (L.A. Smith, unpublished data) has identified as many as ten different 'morphotypes' of *D. stenodactylus* in Western Australia, with seven of these restricted to the Pilbara. While a number of the Western Australian forms are distinctive and display obvious morphological dissimilarities, the Pilbara morphotypes vary more subtly.

Given the systematic problems in the *D. stenodactylus* group and *D. stenodactylus* itself, a molecular approach was used in this study to evaluate the genetic and geographic variation in these taxa. Here, we present molecular phylogenetic data at two taxonomic levels and use these data to address two different issues. A mitochondrial gene tree is presented for all members of the species group in Western Australia and used to evaluate phylogeographic structure within several species. In particular, we focus on the phylogeography of *D. stenodactylus* to examine the relationship of Pilbara populations to those from the rest of the species' range. Based on this gene tree, representatives of each species and major clades were selected and an additional mitochondrial gene and a nuclear gene were sequenced. This multi-gene data set provides the first molecular assessment of phylogenetic relationships among members of the *D. stenodactylus* species group, and we use this phylogeny to investigate the historical relationships of taxa within this group. We interpret our results in light of an explicitly phylogenetic species concept.

2. Materials and methods

2.1. Taxonomic sampling

Tissue samples were obtained for a total of 95 individuals (Table 1). These samples included all members of the *D. stenodactylus* species group (*D. alboguttatus*, *D. damaeus*, *D. maini*, and *D. squarrosus*, *D. stenodactylus*) except for *D. immaculatus*, for which no tissue samples were available. We also included *D. fulleri* and *D. wombeyi* as putative members of the species group. We sampled *D. stenodactylus* more thoroughly to cover the species' range, with a particular focus on the Pilbara region. Sampling for *D. stenodactylus* is thus most extensive in the Pilbara and is limited only in the southernmost areas of the region due to inaccessibility. *D. stenodactylus* samples from outside the Pilbara are limited in the central and northern inland parts of Australia, however, the most eastern extremity of the species' range is represented by several additional samples. We included 13 samples of *R. ornata* and one sample of *D. savagei* to provide information on phylogenetic relationships of potential close relatives of the *D. stenodactylus* species group (Kluge, 1967). *Diplodactylus pulcher*, a member of the *D. vittatus* species group (Kluge, 1967;King, 1987), was used as the outgroup for all analyses.

2.2. Choice of phylogenetic markers

Two approaches were used to provide phylogenetically useful data at two hierarchical levels. Initially, a 1200-base

Table 1
Locality information for all individuals sampled in this study

Taxon	Lab ID#	Museum	Museum #	Latitude (S)	Longitude (E)	Locality (nearest)
Diplodactylus alboguttatus	Gko 048	WAM	104353	31° 59′ 00″	115° 54′ 00″	Victoria Park, WA
	Gko 049	WAM	119122	25° 52′ 00″	113° 42′ 30″	Dubaut Creek, WA
	Gko 050	WAM	119123	25° 52′ 00″	113° 42′ 30″	Dubaut Creek, WA
	Gko 051	WAM	119200	28° 20′ 00″	115° 13′ 30″	Yuna, WA
	Gko 052**	WAM	121288	31° 57′ 00″	115° 45′ 00″	Bold Park, WA
	Gko 053	WAM	132945	28° 28′ 00″	115° 13′ 00″	East Yuna Nature Reserve, WA
	Gko 054	WAM	132946	28° 28′ 00″	115° 13′ 00″	East Yuna Nature Reserve, WA
	Gko 055**	WAM	135518	26° 23′ 00″	113° 19′ 00″	False Entrance, WA
	Gko 056	WAM	141656	25° 47′ 36″	113° 32′ 24″	Peron Homestead, WA
	Gko 057	WAM	141658	25° 45′ 55″	113° 31′ 43″	Peron Homestead, WA
Diplodactylus damaeus	Gko 060**	WAM	145932	30° 21′ 58″	123° 38′ 03″	Queen Victoria Spring, WA
	Gko 061	WAM	145933	30° 21′ 58″	123° 38′ 03″	Queen Victoria Spring, WA
Diplodactylus fulleri	Gko 062**	WAM	157965	23° 17′ 54″	122° 42′ 06″	Lake Views, WA
	Gko 063	WAM	157967	23° 21′ 07″	122° 40′ 00″	Savory Creek Mouth, WA
	Gko 064	WAM	157982	23° 17′ 54″	122° 42′ 06″	Lake Views, WA
Diplodactylus maini	Gko 065	WAM	77917	32° 37′ 00″	121° 32′ 00″	Kumarl, WA
	Gko 066	WAM	77918	32° 37′ 00″	121° 32′ 00″	Kumarl, WA
	Gko 067**	WAM	96564	30° 15′ 00″	118° 30′ 00″	Bonnie Rock, WA
	Gko 068	WAM	127564	29° 57′ 03″	121° 05′ 40″	Goongarrie, WA
	Gko 069	WAM	127570	29° 57′ 03″	121° 05′ 40″	Goongarrie, WA
	Gko 070	WAM	134140	31° 00′ 06″	118° 41′ 31″	Hyden, WA
	Gko 071	WAM	134141	31° 08′ 52″	118° 28′ 33″	Hyden, WA
	Gko 072	WAM	135201	32° 05′ 45″	121° 48′ 18″	Norseman, WA
	Gko 073	WAM	144700	30° 29′ 00″	119° 30′ 00″	Bungalbin Hill, WA
	Gko 074	WAM	144703	30°25′ 00″	119°34′ 00″	Bungalbin Hill, WA
	Gko 075**	WAM	146937	29° 47′ 05″	117° 20′ 08″	Mount Gibson, WA
	Gko 076	WAM	146950	29° 45′ 26″	117° 19′ 12″	Mount Gibson, WA
	Gko 077	WAM	146961	30° 32′ 24″	121° 14′ 52″	Ora Banda, WA
	Gko 078**	WAM	146962	30° 23′ 50″	121° 07′ 21″	Ora Banda, WA
	Gko 079**	WAM	151211	32° 46′ 26″	121° 26′ 49″	Salmon Gums, WA
	Gko 080	WAM	151215	32° 46′ 26″	121° 26′ 49″	Salmon Gums, WA
Diplodactylus pulcher	Gko 081	WAM	90284	29° 34′ 00″	118° 23′ 00″	Earoo Homestead, WA
	Gko 082**	WAM	134126	31° 08′ 52″	118° 28′ 33″	Hyden, WA
	Gko 083**	WAM	134147	31° 00′ 05″	118° 13′ 60″	Hyden, WA
	Gko 084	WAM	135922	23° 01′ 00″	118° 53′ 00″	Mount Robinson, WA
	Gko 085	WAM	144729	30° 29′ 00″	119° 30′ 00″	Bungalbin Hill, WA
	Gko 087	WAM	152910	30° 04′ 40″	119° 16′ 49″	Koolyanobbing, WA
	Gko 088	WAM	152914	30° 07′ 26″	119° 17′ 21″	Koolyanobbing, WA
	Gko 089	WAM	157560	23° 12′ 10″	118° 50′ 00″	West Angeles, WA
Diplodactylus squarrosus	Gko 091	WAM	112109	29° 17′ 09″	117° 28′ 51″	Paynes Find, WA
	Gko 092	WAM	113183	28° 50′ 00″	117° 40′ 00″	Paynes Find, WA
	Gko 093	WAM	113187	26° 31′ 00″	118° 32′ 00″	Meentheena Homestead, WA
	Gko 094	WAM	119204	25° 32′ 00″	115° 28′ 00″	Carey Downs Homestead, WA
	Gko 096**	WAM	131019	26° 34′ 00″	114° 14′ 00″	Hamelin Homestead, WA
	Gko 097**	WAM	132292	27° 04′ 28″	117° 04′ 44″	Noondie Outstation, WA
	Gko 098	WAM	132293	27° 04′ 28″	117° 04′ 44″	Noondie Outstation, WA
	Gko 099	WAM	141462	25° 49′ 30″	113° 52′ 02″	Faure Island, WA
	Gko 157	WAM	125602	25° 21′ 00″	119° 24′ 00″	Randell Well, WA
Diplodactylus sp. 1	Gko 058**	WAM	77991	26° 00′ 00″	126° 45′ 00″	Warburton, WA
	Gko 059	WAM	77992	26° 00′ 00″	126° 45′ 00″	Warburton, WA
	Gko 310	AM	R139898	16° 01′ 07″	128° 00′ 14″	El Questro Station, WA
	Gko 311**	AM	R140078	14° 09′ 23″	126° 38′ 58″	McGowens Beach, WA
	Gko 312	AM	R140081	14° 09′ 23″	126° 38′ 58″	McGowens Beach, WA
Diplodactylus sp. 2	Gko 150	WAM	121170	30° 17′ 00″	119° 45′ 00″	Bungalbin Hill, WA
	Gko 151**	WAM	121171	30° 17′ 00″	119° 45′ 00″	Bungalbin Hill, WA
	Gko 181**	WAM	141136	27° 56′ 24 ″	120° 25′ 19″	Leinster Downs Station, WA
	Gko 193	WAM	151202	27° 59′ 00″	119° 30′ 00″	Sandstone, WA
	Gko 194	WAM	151203	27° 59′ 00″	119° 30′ 00″	Sandstone, WA
Diplodactylus savagei	Gko 374**	WAM	R161067	21° 03′ 18.70″	116° 15′ 05.2″	DRW07*, WA

Table 1 (*continued*)

Taxon	Lab ID#	Museum	Museum #	Latitude (S)	Longitude (E)	Locality (nearest)
Diplodactylus stenodactylus	Gko 114**	WAM	108799	18° 56′ 26″	128° 47′ 04″	Banana Springs, WA
	Gko 124**	WAM	110591	19° 53′ 55″	128° 51′ 57″	Tanami Desert, WA
	Gko 143**	WAM	114211	16° 24′ 00″	122° 56′ 00″	Cape Leveque, WA
	Gko 147**	WAM	114920	21° 10′ 00″	119° 49′ 00″	Marble Bar, WA
	Gko 174**	WAM	132759	16° 01′ 21″	128° 52′ 07″	Lake Argyle, WA
	Gko 177**	WAM	135319	20° 45′ 16″	117° 04′ 52″	Cape Lambert, WA
	Gko 178**	WAM	139043	19° 48′ 44″	121° 28′ 25″	Mandora, WA
	Gko 205**	WAM	157273	22° 15′ 27″	114° 36′ 00″	Yanrey, WA
	Gko 207**	WAM	157573	21° 44′ 52″	116° 04′ 31″	Pannawonica, WA
	Gko 216**	WAM	158319	22° 39′ 28″	114° 23′ 31″	Giralia, WA
	Gko 302**	AM	R155220	29° 02′ 40″	141° 18′ 26″	Sturt NP, NSW
	Gko 305**	AM	R155245	29° 02′ 40″	141° 18′ 26″	Sturt NP, NSW
	Gko 317**	WAM	R110294	22° 40′ 30.70″	119° 50′ 26.8″	RHNE05*, WA
	Gko 324**	WAM	R158093	21° 23′ 02.70″	117° 03′ 38.8″	PW12*, WA
	Gko 343**	WAM	R159942	20° 55′ 10.86″	117° 51′ 39.3″	DRE10*, WA
	Gko 381**	WAM	R162055	22° 26′ 00.70″	115° 55′ 48.0″	WYE06*, WA
	Gko 383**	WAM	R162205	23° 19′ 09.70″	119° 06′ 05.1″	RHNC02*, WA
Diplodactylus wombeyi	Gko 218	WAM	102488	23° 04′ 47″	115° 47′ 25″	Barlee Range Nature Reserve, WA
	Gko 219	WAM	102489	23° 04′ 46″	115° 47′ 27″	Barlee Range Nature Reserve, WA
	Gko 221	WAM	114916	23° 15′ 00″	119° 53′ 00″	Newman, WA
	Gko 226**	WAM	139429	22° 08′ 07″	115° 41′ 20″	Cane River, WA
	Gko 227**	WAM	154653	23° 22′ 45″	120° 06′ 19″	Newman, WA
Rhynchoedura ornata	Gko 230**	WAM	84540	25° 03′ 00″	128° 40′ 00″	Giles Creek, WA
	Gko 231	WAM	84541	25° 03′ 00″	128° 40′ 00″	Giles Creek, WA
	Gko 232	WAM	84592	29° 04′ 00″	121° 21′ 00″	Leonora, WA
	Gko 333	WAM	100521	29° 50′ 00″	120° 55′ 00″	Menzies, WA
	Gko 234	WAM	102502	23° 06′ 47″	116° 00′ 42″	Barlee Range Nature Reserve, WA
	Gko 235	WAM	110582	19° 54′ 10″	128° 45′ 48″	Tanami Desert, WA
	Gko 236	WAM	110583	19° 54′ 10″	128° 45′ 48″	Tanami Desert, WA
	Gko 237	WAM	112972	17° 03′ 35″	122°43′ 00″	Broome, WA
	Gko 238	WAM	112983	17° 03′ 35″	122° 43′ 00″	Broome, WA
	Gko 239**	WAM	132719	15° 46′ 18″	128° 17′ 17″	Wyndham, WA
	Gko 240**	WAM	144746	30° 28′ 00″	119° 36′ 00″	Bungalbin Hill, WA
	Gko 241	WAM	157554	21° 40′ 26″	115° 53′ 21″	Pannawonica, WA
	Gko 242	WAM	158315	22° 37′ 50″	114° 23′ 33″	Giralia, WA

Lab identification numbers (Lab ID#) were given to each sample and used in the figures. Museum # refers to the voucher specimens held in the Australian Museum (AM) and the Western Australian Museum (WAM). Locality information is given to the nearest named location as provided by the museums. Localities marked with "*" refer to sample sites from the Pilbara Biological Survey 1 (2004) and 2 (2005) (CALM-WA, unpublished report). All samples listed were sequenced for the ND2 + tRNA region while samples marked with "**" refer to those individuals where sequence data also was obtained for 16s and RAG-1.

pair (bp) region of the mitochondrial genome was sequenced that included the entire mitochondrial NADH dehydrogenase subunit 2 (ND2) gene and the flanking transfer RNA (tRNA) genes $tRNA^{Met}$ (partial), and $tRNA^{Trp}$ (entire), $tRNA^{Ala}$ (entire) and $tRNA^{Asn}$ (partial) (hereafter referred to as ND2 + tRNA). This region was sequenced in all 95 animals used in this study, and it formed the basis for preliminary assessment of phylogenetic relationships of species in the *D. stenodactylus* species group.

Additional data were sought for assessment of the species-group relationships because phylogenetic studies comprising multiple unlinked loci can provide more robust conclusions than single gene phylogenies (Nichols, 2001;Ballard and Whitlock, 2004). In addition, including loci that evolve at different rates can facilitate phylogenetic accuracy at both the deeper branches and tips of the tree (Hare, 2001).

Therefore, based on the initial ND2 + tRNA data set, a subset of 41 individuals representing all species and major clades was sequenced for an additional mitochondrial gene that evolves at a slower rate than the ND2 + tRNA region (a 550-bp portion of 16s rRNA) and an unlinked and slowly evolving nuclear gene (a 800-bp portion of RAG-1). The ND2 + tRNA and 16s genes have been used successfully in many phylogenetic studies of other squamate reptiles at comparable taxonomic levels, including diplodactyline geckos and related groups (Jennings et al., 2003;Melville et al., 2004;Strasburg and Kearney, 2005). The RAG-1 gene has been used recently in numerous vertebrate phylogenetic studies, including reptiles (e.g., Townsend et al., 2004).

2.3. DNA extraction and polymerase chain reaction (PCR)

Tissue samples consisted of either liver or toes, which were frozen at −70 °C and subsequently stored in 100% ethanol. Genomic DNA was extracted from approximately 1 mm^3 of tissue using EDNA HiSpEx tissue kit (Chaga)

following the manufacturers protocols. PCR amplification was performed using a Corbett PC-960C cooled thermal cycler and negative controls were run for all amplifications. Amplification of the mitochondrial DNA fragments was conducted with an activation step at 94 °C for 4 min followed by 8 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 20 s (stepping down 5 °C every 2 successive cycles), and extension at 72 °C for 90 s, with a further 35 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 30 s, and extension at 72 °C for 45 s, followed by a final extension step at 72 °C for 5 min. Amplification of nuclear genomic DNA was conducted using an activation step at 94 °C for 4 min, 14 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 25 s (stepping down to 55 °C after 2 cycles and 50 °C for the last 10 cycles), followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 25 s and extension at 72 °C for 90 s, with a final extension step at 72 °C for 5 min. PCR amplification was performed in a 40 μl reaction mix consisting of 4.0 μl of 10×PCR buffer, 2.4 μl of 50 mM $MgCl_2$, 2.0 μl dNTP's (10 mM), 1.0 μl of both forward and reverse primers (10 pmol), 26.4 μl double distilled water, 0.2 μl platinum Taq (5 U/μl) and 1 μl of template DNA diluted 1:3. Approximate concentration of amplification products was determined after each extraction and amplification step by electrophoresis in a 2% agarose gel stained with 5.0 μl ethidium bromide and visualized under ultra-violet light.

Various primer combinations were used to amplify from genomic DNA, and PCR and sequencing primers used in this study are listed in Table 2. The ND2 + tRNA region was amplified and sequenced in three overlapping fragments, with the forward primer L4437 and the reverse primer $tRNA^{Asn}$ starting in the tRNA regions tRNA-methionine and tRNA-asparagine, respectively, and L4882 an internal primer. Regions of the 16s gene and the RAG-1 gene were both sequenced using two overlapping fragments. All PCR products were gel purified using UltraClean™15 DNA purification kit (MoBio laboratories Inc.) and procedures specified by the manufacturer.

Cycle-sequencing reactions were performed in reaction volumes of 20 μl, consisting of 1.0 μl BigDye (Applied Biosystems), 4.5 μl of 5× sequencing buffer, 1.0 μl Primer (3.2 pm), 12.5 μl doubly distilled water, and 1.0 μl purified PCR product. PCR sequencing cycles were performed using a Corbett PC-960C cooled thermal cycler, with a denaturation step at 94 °C for 5 s, annealing at 50 °C for 10 s, and extension at 60 °C for 4 min, for 25 cycles. To precipitate sequence products and to remove all unincorporated nucleotides, 80 μl of 75% isopropanol was added to each sample and left for 30 min at room temperature. Precipitated DNA were pelleted and washed twice in 150 μl of 70% isopropanol. Pellets were dried before being dissolved in 20 μl of HiDi formamide and run on an ABI 3100 autosequencer. Sequences were edited and assembled using Sequencher 3.0 (Genes Codes Corporation) and aligned in ClustalX (Thompson et al., 1997). Protein-coding regions of ND2 and RAG-1 were translated into amino acid sequences using the vertebrate mitochondrial and universal genetic codes, respectively, and were compared to *Gekko gecko* translations on GenBank to check for stop codons and frame shifts.

2.4. Phylogenetic analyses

We ran two sets of phylogenetic analyses: one set including all 95 individuals for the ND2 + tRNA data set, and the other set with 41 individuals for all three gene regions. For the combined data set we first ran a total of 10,000 unweighted parsimony bootstrap replicates on each individual data set to get an assessment of phylogenetic resolution and branch support provided by each data set alone. We then performed a partition-homogeneity test in PAUP* to determine whether the three individual data sets were heterogeneous with regard to phylogenetic signal. We used maximum likelihood, parsimony and Bayesian approaches to analyse both the ND2 + tRNA data set based on wider sampling of individuals and the combined data set based on a subset of taxa. Prior to ML analyses, the computer program ModelTest 3.06 (Posada and Crandall, 1998) was used to select the most appropriate model of molecular evolution. We used the ModelTest estimates of the empirical nucleotide frequencies, substitution rates, gamma distribution, and proportion of invariant sites in the ML analyses implemented in PAUP*4.0b10 (Swofford, 2002). Heuristic parsimony analyses also were implemented with the computer program PAUP*. We used TBR branch swapping and ran the parsimony analyses five times from random starting points and with random sequence addition to confirm that overall tree space was well searched. Bayesian analyses were run with the computer program MrBayes (v3.0b4; Huelsenbeck and Ronquist, 2001). We allowed all

Table 2
Amplification and sequencing primers (5′–3′) used in this study

mtDNA region	Primer name	Sequence	Source
$tRNA\text{-}^{Met}$	L4437	5′-AAGCTTTCGGGGCCCATACC-3′	Macey et al. (1998a,b)
ND2	L4882	5′-CAACATGACAAAAAATCGCCCC-3′	Macey et al. (2000) (modified)
$tRNA\text{-}^{Asn}$	$tRNA\text{-}^{Asn}$	5′-CTAAAATRTTRCGGGATCGAGGCC-3′	Read et al. (2001)
16s	L2510	5′-CGCCTGTTTATCAAAAACAT-3′	Palumbi (1996)
16s	H3056	5′-CTCCGGTCTGAACTCAGATCACGTAGG-3′	Palumbi (1996)
RAG-1	L-RAG-1b	5′-TTCCAGCCAYTGCATGCTCT-3′	Townsend et al. (2004)
RAG-1	H-snRAG-1	5′-ATTGCCAATGTCACAGTGCA-3′	Townsend et al. (2004)

parameters to be estimated from the data during the run. We used the default value of four Markov chains per run and also ran the full analysis four times to make sure overall tree space was well sampled and to avoid getting trapped in local optima. We ran each analysis for a total of 2,000,000 generations and sampled the chain every 100 generations, resulting in 20,000 sampled trees. Log-likelihood values reached a plateau after approximately 100,000 generations (1000 sampled trees); to ensure that we discarded the full burn-in phase, we discarded the first 5000 trees and used the last 15,000 trees to estimate Bayesian posterior probabilities.

It was not possible to perform non-parametric ML bootstrap analyses on either the ND2 + tRNA data set or the combined multi-gene data set due to the large number of taxa and the size of the data sets. Therefore for both data sets we used the results of 10,000 "fast" unweighted non-parametric parsimony bootstrap replicates and Bayesian posterior probabilities to assess branch support. In keeping with commonly used convention, we consider branches supported by parsimony bootstrap values greater than or equal to 70% (Hillis and Bull, 1993) and posterior probability values greater than or equal to 95% (Wilcox et al., 2002) to be worthy of discussion.

3. Results

3.1. ND2 + tRNA data set based on 95 individuals

After the exclusion of unalignable regions in the tRNAs, the edited alignment comprised 1142 characters and of these 601 (53%) were variable and parsimony informative. Following alignment, protein-coding mtDNA sequences were translated into amino acid sequences using the vertebrate mitochondrial genetic code. No premature stop codons were observed or any other sign of paralogous sequences; therefore, we concluded that all sequences obtained were mitochondrial in origin.

Both the hierarchical likelihood-ratio tests and the Akaike information criterion in Modeltest supported the general time reversible (GTR) plus invariant sites (I) plus gamma shape (G) model as the best-fit substitution model for the ND2 + tRNA data set and gave a $-\ln L = 12979.60$. The estimated parameters were as follows: nucleotide frequencies $A = 0.3651$, $C = 0.3710$, $G = 0.0511$, and $T = 0.2128$; substitution rates $A \leftrightarrow C$ 0.2897, $A \leftrightarrow G$ 8.8795, $A \leftrightarrow T$ 0.3686, $C \leftrightarrow G$ 0.2793, $C \leftrightarrow T$ 3.1554, $G \leftrightarrow T$ 1.0000; proportion of invariant sites = 0.3672; gamma shape parameter = 0.9301. The Bayesian analysis produced parameter estimates that were very similar to those produced by ModelTest (not shown).

The ML analysis in PAUP* using the above parameters and the Bayesian analysis yielded very similar trees (ML $-\ln L = 12962.58$, Bayesian $-\ln L$ was slightly higher at 13067.11) that also were very similar to the strict consensus of 768 most-parsimonious trees (Length = 2510; CI = 0.4096; HI = 0.5909; RI = 0.8947; RC = 0.3664). Fig. 2 shows the Bayesian tree with parsimony bootstraps and Bayesian posterior probabilities on nodes, as well as the ML phylogram.

We discuss higher-level phylogenetic relationships based on the combined data set below. Here, we outline intra-specific phylogeographic patterns only (Fig. 2). Several taxa sequenced in this study exhibit high intra-specific Jukes–Cantor genetic distances for the ND2 + tRNA data set and show significant phylogeographic structure; *R. ornata* (18.2%), *D. maini* (9.0%), *D. pulcher* (8.3%), *D. squarrosus* (8.7%), and *D. stenodactylus* (15.0%). *R. ornata* comprises two distinct parts, one represented by a single individual from near Wyndam in the Kimberley in the extreme north-east of Western Australia (Gko 239) and the other one comprising the rest of the individuals from Western Australia, clade A (Fig. 2). *Diplodactylus maini* comprises extremely well supported eastern and western clades (ND2 + tRNA bootstrap values 100, posterior probabilities 100) that are in close geographic proximity to one another, but are separated by a drainage divide (Fig. 2). An eastern clade A is distributed along the western margin of the Nullarbor Plain, within the Yilgarn region of internal drainage basins, while a eastern clade B occurs in another set of internal drainage basins on the western side of this drainage divide in the vicinity of Lake Moore. *Diplodactylus pulcher* comprises two clades separated by a geographic distance of more than 800 km (Fig. 2). Clade B comprises a population in the Hamersley Range in the southern Pilbara, while clade A is distributed in southwestern Western Australia east of Kalgoorlie. *Diplodactylus squarrosus* comprises two extremely well supported clades (ND2 + tRNA bootstrap values 99 and 100, posterior probabilities 100) that correspond to a coastal population on the low-lying Cainozoic sandplains near Shark Bay, clade B, and an inland population that occurs on the Proterozoic sandplains, hills and ridges from Canning Hill in the south to Mt. Murchison in the north, clade A (Fig. 2).

Our sampling for phylogeographic structure is best for *D. stenodactylus*, and our results show that this species displays perhaps the most interesting phylogeographic pattern. *D. stenodactylus* comprises two major clades that correspond geographically to the Pilbara, clade A, (bootstrap value 100, posterior probability 100) and non-Pilbara populations, clade B, (bootstrap value 100, posterior probability 100). The eastern distributional limit of the Pilbara clade A and the western distributional limit of clade B are remarkably close together and correspond to the sharp border of the Pilbara region in the west and the sandy desert to the east.

3.2. Combined multi-gene data set based on 41 individuals

After the exclusion of unalignable regions, the edited alignment comprised 2485 characters (1163 ND2 + tRNA; 490 16s; 832 RAG-1), and of these 717 (29%) were variable and parsimony informative (568 ND2 + tRNA; 89 16s; 60 RAG-1). Following alignment, protein-coding mtDNA

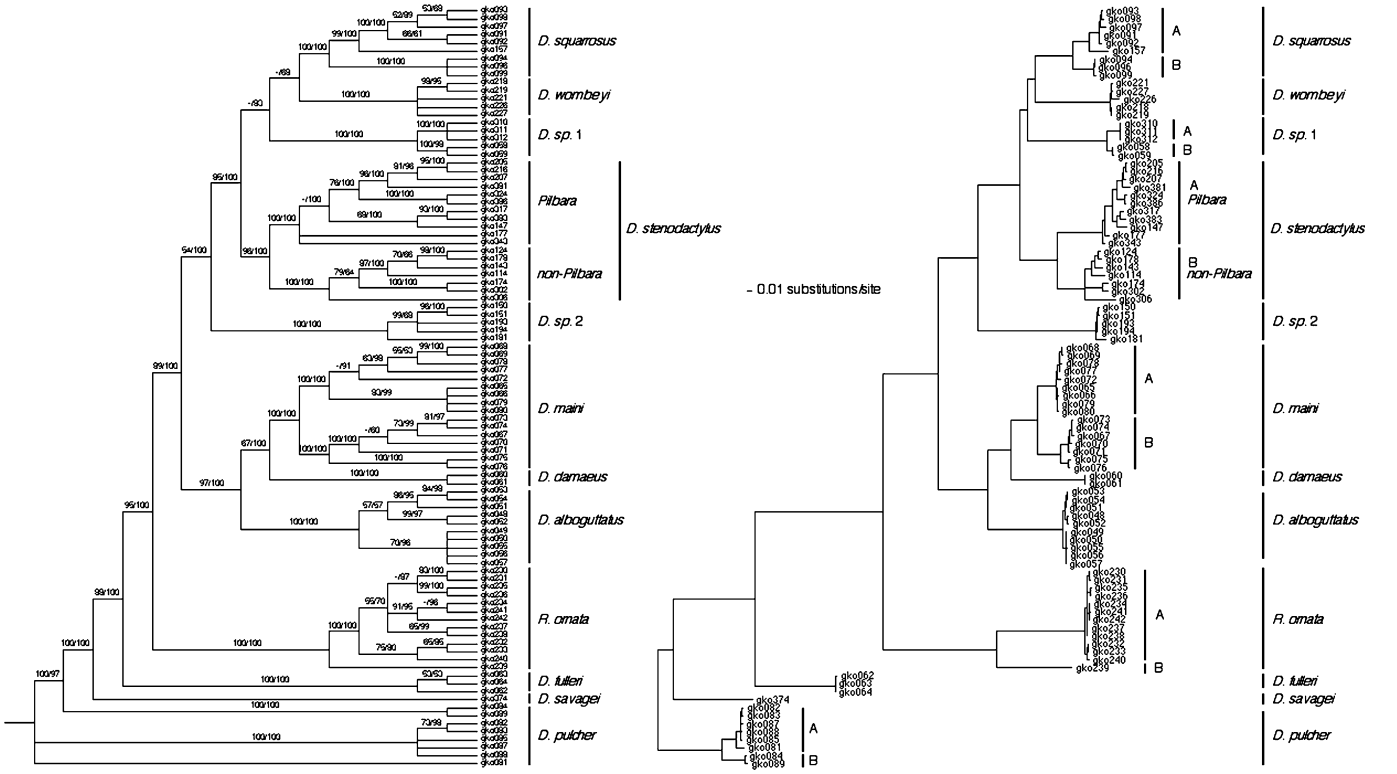

Fig. 2. Summary of phylogenetic results of the fine-scale ND2 + tRNA data set of *Diplodactylus stenodactylus* based on parsimony, maximum likelihood and Bayesian analyses. (Left) Bayesian tree. Values above the node refer to unweighted parsimony bootstraps and Bayesian posterior probabilities, respectively. (Right) Maximum likelihood phylogram based on the GTR + G + I model generated from ModelTest. See text for details.

sequences were translated into amino acid sequences using the vertebrate mitochondrial genetic code. No premature stop codons were observed in the ND2 data set, and the conserved portions of the 16s data set aligned perfectly with published gecko data, therefore, we concluded that all ND2 and 16s sequences obtained were mitochondrial in origin. As expected, ND2 + tRNA was the most variable and RAG-1 the least variable. Inter-specific and inter-clade genetic distances for representative taxa for each individual gene and for the combined data set are shown in Tables 3 and 4. Ranges of intra-specific genetic distances and intra-clade genetic distances for the individual genes and for the combined data set are shown in Table 5. Results of the 10,000 unweighted parsimony bootstraps for each individual gene are shown in Fig. 3. As expected, the ND2 + tRNA data provided the highest level of resolution at the tips of the tree, and also provided good resolution for major clades, while the 16s data set showed support for major clades but poor support for structure between these clades. The RAG-1 data set provided strong resolution and support for major clades but poor support at the tips of the tree. A partition-homogeneity test in PAUP* could not reject the null hypothesis that the data were homogeneous with regard to overall phylogenetic signal ($P > 0.01$). Therefore the rest of the results are based on analysis of the combined data.

Both the hierarchical likelihood-ratio tests and the Akaike information criterion in Modeltest supported the complex general time reversible (GTR) plus invariant sites (I) plus gamma shape (G) model as the best-fit substitution model for the combined data set and gave a $-\ln L = 16709.89$. The estimated parameters were as follows: nucleotide frequencies $A = 0.3333$, $C = 0.3066$, $G = 0.1418$, $T = 0.2183$; substitution rates $A \leftrightarrow C$ 2.2329, $A \leftrightarrow G$ 10.8681, $A \leftrightarrow T$ 2.5729, $C \leftrightarrow G$ 0.2697, $C \leftrightarrow T$ 17.4611, $G \leftrightarrow T$ 1.0000; proportion of invariant sites = 0.5087; gamma shape parameter = 0.5920. The Bayesian analysis produced parameter estimates that were very similar to those produced by ModelTest (not shown). The ML analysis in PAUP* using the above parameters and the Bayesian analysis both yielded the same optimal tree (ML $-\ln L = 16488.91$, Bayesian $-\ln L$ was slightly higher at 16522.83) and this tree also was identical to the single most-parsimonious tree (Length = 2770; CI = 0.4502; HI = 0.5498; RI = 0.7414; RC-0.3338). Fig. 4 shows the unweighted parsimony tree with parsimony bootstraps and Bayesian posterior probabilities on nodes, as well as the ML phylogram.

Overall, the phylogeny is well resolved and supported by very high bootstrap values and posterior probabilities (Fig. 4). The monophyly of each species is supported by extremely high bootstrap values of 100% and posterior probabilities of 100. This also is evident in each of the individual gene trees (bootstrap values 96–100% in ND2 + tRNA, 85–100% in 16s, and 75–100% in RAG-1) except for *D. wombeyi* which is unresolved based on RAG-1 (Fig. 3). The combined data set and individual gene trees also identified two previously unknown but well supported

Table 3
Genetic distances for representatives of each clade in the multi-gene phylogeny (Fig 4)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	gko082. *D. pulcher*	—	0.05561	0.05557	0.06029	0.06461	0.08265	0.07839	0.07328	0.08569	0.06316	0.07804	0.08964	0.08964	0.09196	0.09291	0.09664	0.08964	0.08045	0.08271
2	gko374. *D. savagei*	0.17891	—	0.07114	0.06469	0.08504	0.08508	0.08308	0.07323	0.09639	0.08285	0.08745	0.09668	0.09669	0.09904	0.10559	0.09903	0.09907	0.10149	0.11097
3	gko062. *D. fulleri*	0.22892	0.21153	—	0.05792	0.07351	0.08044	0.08523	0.07776	0.08560	0.07303	0.06675	0.07798	0.07800	0.08029	0.08536	0.07800	0.08259	0.07572	0.08029
4	gko239. *R. ornata*	0.30516	0.27592	0.29125	—	0.06902	0.06486	0.07185	0.07113	0.07020	0.05894	0.06938	0.08067	0.07378	0.07605	0.08075	0.07379	0.07151	0.07156	0.08070
5	gko052. *D. alboguttatus*	0.33478	0.29985	0.28214	0.24799	—	0.08019	0.05779	0.05730	0.08965	0.08446	0.05982	0.07322	0.07319	0.07544	0.08975	0.07549	0.07547	0.08925	0.09614
6	gko058. D. sp. 1	0.31000	0.28138	0.27795	0.28418	0.23999	—	0.08019	0.06640	0.04028	0.03992	0.05527	0.07330	0.07110	0.06883	0.07308	0.05983	0.06656	0.05983	0.06655
7	gko060. *D. damaeus*	0.29908	0.29475	0.27217	0.26380	0.17043	0.24718	—	0.05320	0.09725	0.08463	0.07340	0.08961	0.08966	0.09210	0.10765	0.08960	0.09198	0.10131	0.10375
8	gko067. *D. maini*	0.29863	0.26970	0.26599	0.27082	0.18135	0.24244	0.14704	—	0.08244	0.07699	0.05967	0.07312	0.07761	0.07536	0.08733	0.07761	0.07533	0.08675	0.09365
9	gko096. *D. squarrosus*	0.28804	0.29229	0.26748	0.26221	0.23136	0.17412	0.21746	0.22879	—	0.05302	0.07246	0.07012	0.06301	0.06057	0.06259	0.05528	0.05527	0.05005	0.05806
10	gko226. *D. wombeyi*	0.29739	0.28687	0.27965	0.28590	0.24771	0.17942	0.22881	0.23708	0.15548	—	0.06289	0.07723	0.07496	0.07259	0.07587	0.06547	0.06309	0.05351	0.05594
11	gko151. D. sp. 2	0.29009	0.29227	0.28243	0.26135	0.21165	0.22471	0.22669	0.22210	0.20273	0.21266	—	0.05104	0.05769	0.05547	0.06117	0.05102	0.05323	0.05538	0.05980
12	gko324. *D. stenodactylus*. P	0.31604	0.29860	0.28717	0.27361	0.21612	0.19390	0.24422	0.25262	0.17924	0.17690	0.21857	—	0.01658	0.01448	0.02428	0.01867	0.02078	0.03782	0.04432
13	gko205. *D. stenodactylus*. P	0.32399	0.31025	0.29863	0.27984	0.22189	0.19279	0.25503	0.25261	0.18803	0.19010	0.22204	0.03527	—	0.00205	0.01989	0.01448	0.01032	0.03353	0.04215
14	gko381. *D. stenodactylus*. P	0.30944	0.30636	0.29735	0.27360	0.21958	0.19503	0.24780	0.25021	0.18805	0.18128	0.22204	0.03980	0.02540	—	0.01769	0.01240	0.00824	0.03139	0.03998
15	gko147. *D. stenodactylus*. P	0.31869	0.29604	0.29863	0.26741	0.21958	0.18832	0.24900	0.24064	0.18362	0.18457	0.21856	0.04710	0.04986	0.04986	—	0.01321	0.01095	0.03542	0.03777
16	gko383. *D. stenodactylus*. P	0.32132	0.30505	0.29479	0.27984	0.21727	0.19170	0.25020	0.24780	0.18142	0.18457	0.22552	0.05170	0.04710	0.05262	0.03980	—	0.01032	0.03139	0.03998
17	gko343. *D. stenodactylus*. P	0.30817	0.30117	0.29097	0.27736	0.21040	0.17730	0.24183	0.23710	0.17054	0.18018	0.21512	0.04435	0.04435	0.05078	0.04802	0.04527	—	0.02925	0.03353
18	gko124. *D. stenodactylus*. NP	0.29235	0.30993	0.28467	0.26721	0.22064	0.19258	0.23939	0.23941	0.15864	0.16811	0.20583	0.12732	0.13967	0.13760	0.12731	0.13141	0.12426	—	0.01867
19	gko306. *D. stenodactylus*. NP	0.29875	0.30773	0.31033	0.26712	0.24311	0.19169	0.24193	0.24755	0.17902	0.16938	0.20609	0.13667	0.14498	0.14394	0.13978	0.14498	0.13565	0.07609	—

For ease of comparison with other studies, the commonly used Jukes and Cantor (1969) genetic distances for the 16s data set are shown above the diagonal and the ND2 + tRNA data set are shown below the diagonal. For *D. stenodactylus* "P" refers to Pilbara and "NP" refers to non-Pilbara.

Table 4
Genetic distances for representatives of each clade in the multi-gene phylogeny (Fig 4)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	gko082. *D. pulcher*	—	0.01341	0.01713	0.02458	0.03345	0.02715	0.02345	0.02458	0.02481	0.02481	0.02581	0.02957	0.02832	0.02958	0.02963	0.02963	0.02962	0.02842	0.02838
2	gko374. *D. savagei*	0.14432	—	0.02319	0.03569	0.04207	0.03831	0.03332	0.03192	0.03591	0.03341	0.03695	0.04075	0.03951	0.04079	0.04082	0.04082	0.03958	0.03707	0.03956
3	gko062. *D. fulleri*	0.20436	0.20263	—	0.02567	0.03575	0.03074	0.02578	0.02692	0.02961	0.02836	0.02942	0.03317	0.03193	0.03319	0.03323	0.03323	0.03326	0.03203	0.03197
4	gko239. *R. ornata*	0.35196	0.33649	0.31967	—	0.03448	0.02448	0.02451	0.02567	0.02205	0.02208	0.02319	0.02692	0.02569	0.02694	0.02695	0.02695	0.02698	0.02575	0.02570
5	gko052. *D. alboguttatus*	0.38430	0.39612	0.32626	0.26988	—	0.02951	0.01221	0.01459	0.02833	0.02582	0.02820	0.03195	0.03200	0.03197	0.03200	0.03200	0.03205	0.02955	0.03075
6	gko058. D. sp. 1	0.37268	0.37956	0.31722	0.31669	0.24784	—	0.01956	0.02075	0.00851	0.00606	0.00848	0.00725	0.00725	0.00725	0.00725	0.00725	0.00725	0.00726	0.00603
7	gko060. *D. damaeus*	0.35597	0.38189	0.31428	0.28912	0.13812	0.24807	—	0.00604	0.01838	0.01837	0.01830	0.02200	0.02203	0.02201	0.02203	0.02203	0.02206	0.02084	0.02079
8	gko067. *D. maini*	0.34529	0.35237	0.30927	0.29529	0.15056	0.24197	0.11037	—	0.01832	0.01710	0.01948	0.02319	0.02321	0.02321	0.02321	0.02321	0.02324	0.01951	0.02197
9	gko096. *D. squarrosus*	0.34912	0.40708	0.32324	0.30366	0.23972	0.13300	0.22466	0.22803	—	0.00607	0.00974	0.01092	0.01092	0.01093	0.01092	0.01092	0.01093	0.00971	0.00970
10	gko226. *D. wombeyi*	0.32968	0.36961	0.31408	0.30077	0.24889	0.13136	0.22790	0.23375	0.11546	—	0.00731	0.00849	0.00849	0.00850	0.00849	0.08049	0.00850	0.00606	0.00728
11	gko151. D. sp. 2	0.33139	0.37903	0.32035	0.28446	0.19887	0.19321	0.21635	0.20915	0.17635	0.17753	—	0.01090	0.01091	0.01090	0.01091	0.01091	0.01092	0.01092	0.00969
12	gko324. *D. stenodactylus*. P	0.37858	0.40723	0.35073	0.30626	0.21429	0.16168	0.24666	0.25657	0.14591	0.14613	0.18217	—	0.00000	0.00000	0.00000	0.00000	0.00000	0.00605	0.00603
13	gko205. *D. stenodactylus*. P	0.38670	0.41847	0.35835	0.30758	0.22062	0.16022	0.25858	0.25978	0.15129	0.15514	0.18799	0.02073	—	0.00000	0.00000	0.00000	0.00000	0.00606	0.00603
14	gko381. *D. stenodactylus*. P	0.37184	0.41665	0.35941	0.30554	0.21627	0.16055	0.24874	0.25201	0.14896	0.14730	0.18647	0.02254	0.01249	—	0.00000	0.00000	0.00000	0.00606	0.00603
15	gko147. *D. stenodactylus*. P	0.39152	0.41548	0.37092	0.31094	0.22797	0.15579	0.26154	0.25464	0.14781	0.15272	0.19135	0.02896	0.02953	0.02895	—	0.00000	0.00000	0.00606	0.00603
16	gko383. *D. stenodactylus*. P	0.39080	0.42065	0.35825	0.31529	0.21725	0.15398	0.25111	0.25367	0.14262	0.14641	0.19113	0.03003	0.02653	0.02895	0.02245	—	0.00000	0.00606	0.00603
17	gko343. *D. stenodactylus*. P	0.36757	0.40838	0.35064	0.30682	0.20859	0.14496	0.24225	0.23979	0.13342	0.14118	0.18098	0.02635	0.02401	0.02674	0.02632	0.02443	—	0.00607	0.00604
18	gko124. *D. stenodactylus*. NP	0.34209	0.40854	0.33163	0.28407	0.22218	0.15053	0.24322	0.24059	0.11955	0.12482	0.17168	0.08530	0.09155	0.08953	0.08531	0.08558	0.07972	—	0.00363
19	gko306. *D. stenodactylus*. NP	0.35148	0.42237	0.36117	0.29360	0.24890	0.15465	0.25167	0.25238	0.13874	0.12977	0.17115	0.09628	0.10018	0.09891	0.09718	0.09981	0.09085	0.04496	—

For ease of comparison with other studies, the commonly used Jukes and Cantor (1969) genetic distances for the RAG-1 data set are shown above the diagonal. Below the diagonal are maximum likelihood genetic distances estimated using the model of best fit selected by ModelTest 3.0 based on all three gene regions (GTR + G + I model). For *D. stenodactylus* "P" refers to Pilbara and "NP" refers to non-Pilbara.

Table 5
Ranges of intra-specific genetic distances and intra-clade genetic distances for *D. stenodactylus*

Taxon	ND2 + tRNAs	16s rRNA	RAG-1	Combined
D. alboguttatus	0–0.0177 ($n = 10$)	0–0.0104 ($n = 2$)	0 ($n = 2$)	0–0.0109 ($n = 2$)
D. damaeus	0 ($n = 2$)	0 ($n = 1$)	0 ($n = 1$)	0 ($n = 1$)
D. fulleri	0–0.0044 ($n = 3$)	0 ($n = 1$)	0 ($n = 1$)	0 ($n = 1$)
D. maini	0–0.0894 ($n = 16$)	0–0.0292 ($n = 4$)	0–0.0036 ($n = 4$)	0–0.0561 ($n = 4$)
D. pulcher	0–0.0829 ($n = 8$)	0–0.0146 ($n = 2$)	0–0.0012 ($n = 2$)	0–0.0452 ($n = 2$)
D. squarrosus	0-0.0873 ($n = 9$)	0–0.0354 ($n = 3$)	0–0.0012 ($n = 3$)	0–0.0563 ($n = 3$)
D. wombeyi	0–0.0195 ($n = 5$)	0–0.0065 ($n = 2$)	0–0.0073 ($n = 2$)	0–0.0127 ($n = 2$)
D. sp. 1	0-0.0388 ($n = 5$)	0–0.0041 ($n = 2$)	0–0.0012 ($n = 2$)	0–0.0189 ($n = 2$)
D. sp. 2	0–0.0232 ($n = 5$)	0–0.0041 ($n = 2$)	0 ($n = 2$)	0–0.0117 ($n = 2$)
D. savagei	0 ($n = 1$)	0 ($n = 1$)	0 ($n = 1$)	0 ($n = 1$)
D. stenodactylus Pilbara	0–0.0517 ($n = 11$)	0–0.0243 ($n = 11$)	0–0.0024 ($n = 11$)	0–0.0053 ($n = 11$)
D. stenodactylus non-Pilbara	0–0.0922 ($n = 7$)	0–0.0187($n = 7$)	0–0.0048 ($n = 7$)	0–0.0512 ($n = 7$)
D. stenodactylus (Pilbara–non-Pilbara)	0.1246–0.1499 ($n = 18$)	0.0293–0.0444 ($n = 18$)	0.0048–0.0073 ($n = 18$)	0.0774–0.1023 ($n = 18$)
R. ornata	0–0.1818 ($n = 13$)	0–0.0404 ($n = 3$)	0–0.0048 ($n = 3$)	0–0.1347 ($n = 3$)

ND2 + tRNA distances are based on the fine-scale data set for which more individuals were sampled while the16s rRNA and RAG-1 distances are from the multi-gene phylogeny data set. For ease of comparison with other studies, Jukes and Cantor (1969) genetic distances are shown for the three individual gene regions. ML genetic distances are shown for the combined data and are based on the model of best fit from ModelTest (GTR + I + G).

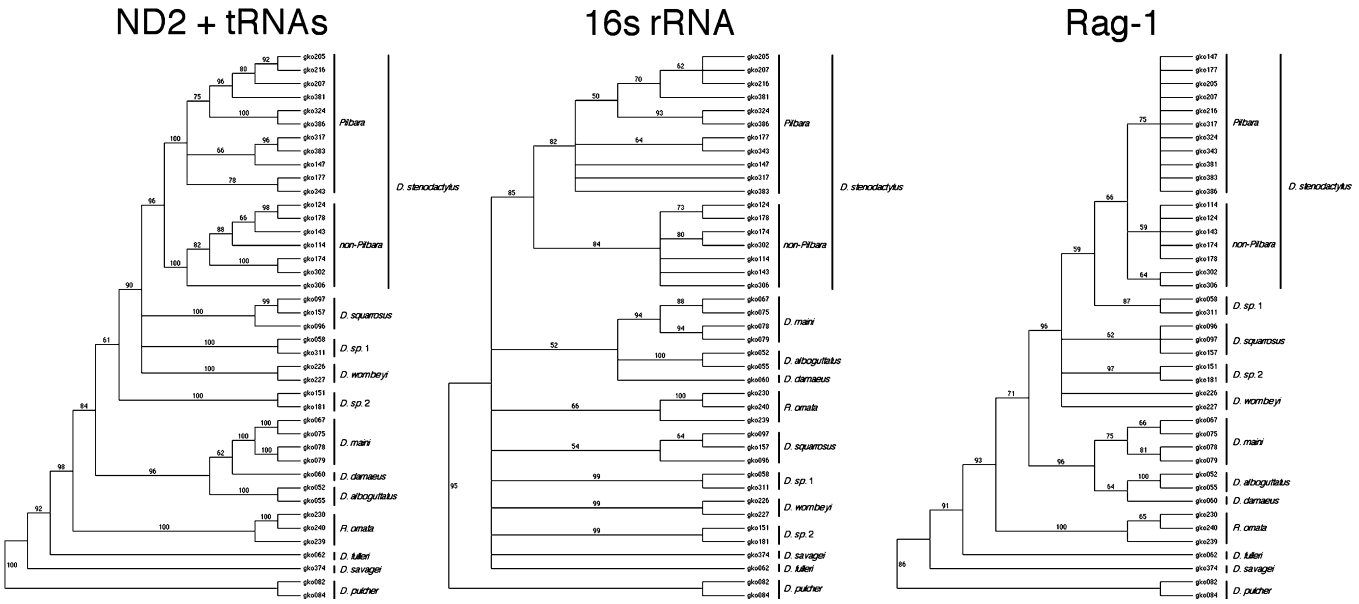

Fig. 3. Individual gene trees for the two mitochondrial DNA regions and the nuclear gene region used in the multi-gene analyses of the *Diplodactylus stenodactylus* species group. The trees show the results of 10,000 unweighted parsimony bootstrap replicates.

clades (bootstrap values 100%, posterior probabilities 100). These taxa were identified as *D. stenodactylus* based on morphology, but in the combined multi-gene data set they exhibit long terminal branch lengths and show large ML genetic distances from all representative *D. stenodactylus* ranging from 14.5 to 16.2% in one taxon to 24.2–26.2% in the second taxon (see Tables 3 and 4 for Jukes–Cantor genetic divergences for individual genes). These genetic distances are greater than those observed between *D. wombeyi* and *D. stenodactylus* based on the same molecular data. We refer to these populations as *Diplodactylus* sp. 1 and 2 pending further study.

This phylogeny has confirmed that *R. ornata* is a closely related sister taxon to Kluge's (1967) *D. stenodactylus* group (see below) (bootstrap value 100%, posterior probability 100). Despite morphological similarities, *D. fulleri* is more distantly related to other *D. stenodactylus* group members than *Rhynchoedura*. Based on the combined data set, genetic distances from members of the *D. stenodactylus* species group range from 27.0 to 35.2% for *R. ornata* and 30.9–37.1% for *D. fulleri* (Tables 3 and 4 also show Jukes–Cantor genetic distances for the individual genes).

Kluge (1967) placed *D. alboguttatus*, *D. damaeus*, *D. maini*, *D. squarrosus*, and *D. stenodactylus* in a "*D. stenodactylus*" species group. This group was supported by extremely high bootstrap values (99%) and posterior probabilities (100) in the combined tree and also is supported in two of the three gene trees (ND2 + tRNA and RAG-1). There was strong support for two major clades within the *D. stenodactylus* group that correspond to southwestern Western Australian species with coastal populations extending to Shark Bay (bootstrap value 100%, posterior

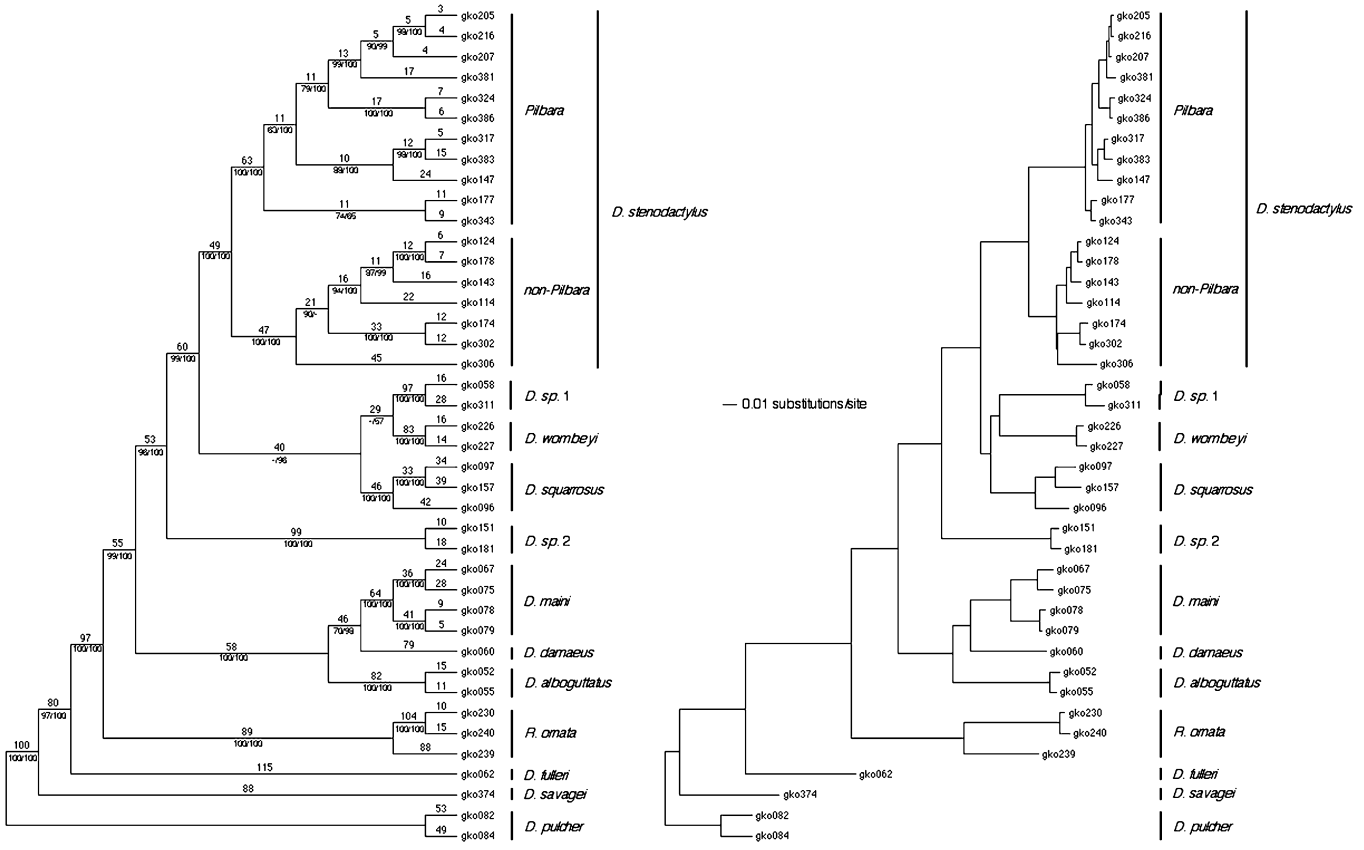

Fig. 4. Summary of phylogenetic results of the multi-gene data set based on parsimony, maximum likelihood and Bayesian analyses of the *Diplodactylus stenodactylus* species group. All analyses recover the same topology. (Left) Single most-parsimonious tree with unweighted branch lengths indicated above the node. Values below the node refer to unweighted parsimony bootstraps and Bayesian posterior probabilities, respectively. (Right) Maximum likelihood phylogram based on the GTR + G + I model generated from ModelTest. See text for details.

probability 100; *D. maini*, *D. damaeus* and *D. alboguttatus*) and central and northern Western Australian species all with distributions north of Kalgoorlie (bootstrap value 96%, posterior probability 100; *D. stenodactylus*, *D. squarrosus*, *D. wombeyi*, and *Diplodactylus* sp. 1 and 2). The maximum ML genetic distance between a representative of each of these two major groups is 26.2% (*D. stenodactylus* Gko 147 and *D. damaeus* Gko 060) while inter-specific ML genetic distances within the *D. stenodactylus* group range from 11.0 to 26.2%.

The phylogeny based on the combined data did not provide strong support for the relationships among members of the clade comprising *D. stenodactylus*, *D. squarrosus*, *D. wombeyi*, and *Diplodactylus* sp. 1. However, *Diplodactylus* sp. 2 formed a well supported sister species (bootstrap value 96%, posterior probability 100) to this clade.

Like the ND2 + tRNA data set, the combined multi-gene data set shows considerable intra-specific genetic variation in *D. stenodactylus* and strong support for a deep phylogenetic split (bootstrap value 100%, posterior probability 100) within the species, supporting the existence of a Pilbara clade and a non-Pilbara clade. The Pilbara clade comprises populations strictly from the Pilbara region in Western Australia (bootstrap value 100%, posterior probability 100), and the non-Pilbara clade comprises populations from central and northern Western Australia and New South Wales (bootstrap value 100%, posterior probability 100) (Fig. 5). The ML genetic distances between these two major clades range from 7.74 to 10.22% (Table 5).

4. Discussion

We presented two well-resolved molecular phylogenies using genes that evolve at different rates, and we use these trees to investigate several taxonomic and biogeographic questions in the *D. stenodactylus* group. The broad-scale, multi-gene phylogeny has supported previous conjectures on the higher-level phylogenetic relationships among members of the *D. stenodactylus* species group that were based on morphology, but also has uncovered hidden diversity within the group. The finer-scale phylogeny made use of a faster-evolving gene (ND2 + tRNA) and has provided the basis for an examination of phylogeographic structure within several species in the group. In particular, large geographic sampling of *D. stenodactylus* has enabled a more detailed phylogeographic assessment of the species in Western Australia.

The following discussion is structured around two main themes that point to different features of evolution among *Diplodactylus* geckos. Because the combined multi-gene

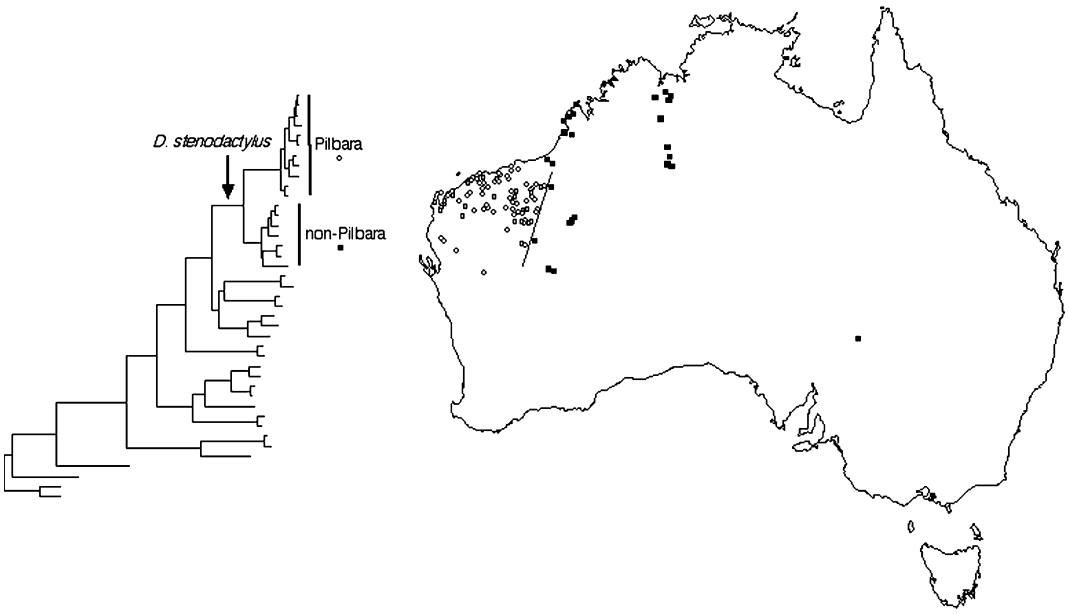

Fig. 5. Multi-gene maximum likelihood phylogram from Fig. 4 illustrating the distribution of the two major *D. stenodactylus* clades within and outside the Pilbara region.

phylogeny and the ND2 + tRNA phylogeny agree on deeper level structure, we first outline the higher-level phylogenetic relationships in species within the *D. stenodactylus* species group based on the multi-gene phylogeny and comment on their taxonomic implications. We then use the finer-scale ND2 + tRNA phylogeny to investigate phylogeographic patterns within several taxa that display high levels of intra-specific divergence. In particular, the phylogeography of *D. stenodactylus* is considered with an emphasis on the Pilbara region in Western Australia.

4.1. Multi-gene phylogeny of the D. stenodactylus group

Based on morphological data, Kluge (1967) described the *D. stenodactylus* group with the members *D. alboguttatus*, *D. damaeus*, *D. maini*, *D. squarrosus*, and *D. stenodactylus*. Since then, *D. fulleri* and *D. wombeyi* also have been assigned to the group based upon recognition of a number of shared morphological characteristics (Storr et al., 1990). Kluge (1967) also concluded that while a number of unique characters prevented placement within *Diplodactylus*, the monotypic *R. ornata* had close affinities to the *D. stenodactylus* group.

Phylogenetic analysis using both mtDNA and nDNA data revealed high levels of sequence divergence among taxa with high bootstrap values and Bayesian posterior probabilities supporting clades. In addition, the combined multi-gene phylogeny and individual gene trees provided strong support for two new species, here called *Diplodactylus* sp. 1 and 2. Both taxa were identified as *D. stenodactylus* based on morphology but are highly divergent from *D. stenodactylus* in the recovered molecular phylogeny. Both new taxa also are well supported in the ND2 + tRNA phylogeny. *Diplodactylus* sp. 1 is represented by five individuals collected from Warburton in central Western Australia and the Bonaparte Archipelago in the Kimberley region of Western Australia, but further sampling is required to determine the limits of this taxon's range and any morphologically distinguishing characteristics. It is interesting to note that the latitudinal extremes of this species' range suggest a tolerance of vast ecological and climatic conditions. *Diplodactylus* sp. 2 also is represented by five individuals collected over a much narrower range corresponding to a sandplain that extends from Bungalbin Hill to Leinster in southwestern Western Australia. We will describe these new species elsewhere.

The combined multi-gene phylogeny also provides strong support for a number of inter-specific relationships. The parsimony, ML and Bayesian trees all strongly support monophyly of Kluge's *D. stenodactylus* group (bootstrap value 99, posterior probability 100) and have confirmed the placement of *D. wombeyi* within the species group. However, the phylogeny does not support recognition of *D. fulleri* as a member of the *D. stenodactylus* group as defined by Kluge (1967), and ML genetic distances suggest closer affinities to members of the *D. vittatus* species group (Kluge, 1967), represented by *D. pulcher* and *D. savagei* in this phylogeny. A molecular phylogeny based on the same ND2 + tRNA region by Melville et al. (2004) showed that *D. stenodactylus* (representing the *D. stenodactylus* group in their phylogeny) and *R. ornata* form a sister clade to other *Diplodactylus* species sampled in their study, rendering *Diplodactylus* paraphyletic. In our study, two unlinked gene trees (ND2 + tRNA and RAG-1) as well as the combined multi-gene phylogeny each provide robust support for the close sister-taxon relationship of *R. ornata* to the *D. stenodactylus* species group, which lends additional support to the paraphyly of *Diplodactylus*. The most-parsimonious conclusion we can draw based on genetic evidence gathered to date is that *Rhynchoedura* does not warrant recognition

as a separate genus and should be synonymised with *Diplodactylus* pending a broad-scale phylogeny of *Diplodactylus* and related taxa including diverse sampling of all the relevant species groups.

In his systematic review of the *D. stenodactylus* group, Kluge (1967) speculated on the relationships among taxa based on the presence or absence of a number of shared characteristics. He suggested that *D. alboguttatus*, *D. damaeus*, and *D. maini* were closely related based on a number of morphological characters, and we also found strong support for a close relationship among these taxa. These three species all have ranges in the temperate zone of the Southwest Province (Beard, 1990) concordant with a recognized area of faunal and floral endemism (Cracraft, 1991;Crisp et al., 1995). In addition, these species share a number of ecological similarities, whereby all shelter in insect and spider holes and favor sandy to heavy soils with open vegetation of *Eucalyptus*, *Acacia*, and *Triodia* (Storr et al., 1990;Wilson and Swan, 2003).

We also found support for another monophyletic group within the *D. stenodactylus* group comprising *D. squarrosus*, *D. stenodactylus*, and *D. wombeyi* plus the new taxa *Diplodactylus* sp. 1 and 2. *Diplodactylus squarrosus*, *D. stenodactylus*, and *D. wombeyi* have distributions in central and northern Western Australia north of Kalgoorlie, in line with the Eremaean Province of the Eremaean Zone and the Northern Province of the Tropical Zone (Beard, 1990). These species occur on many soil types but favor heavier to rocky soils (Storr et al., 1990;Wilson and Swan, 2003). *Diplodactylus* sp. 1 also appears to be associated with the mountainous and rocky area of the Warburton Ranges of central Western Australia as well as the rugged ranges in the Kimberley, while *Diplodactylus* sp. 2 has so far been collected only on sandplains (L.A. Smith, personal communication).

While the phylogenetic relationships within the clade comprising *D. squarrosus*, *D. stenodactylus*, *D. wombeyi*, *D. sp.* 1 and *D. sp.* 2 were fully resolved, there were poor bootstraps and Bayesian posterior probabilities on the nodes. However, the phylogeny did provide extremely strong support for the close sister-taxon relationship between *D. sp.* 2 and the rest of the clade. This also is supported by geographical and ecological data in that *D. sp.* 2 is the only member of this clade to favor sandplains, and also has the most southern distribution relative to other members of the clade.

4.2. Phylogeography of *Rhynchoedura ornata*, *Diplodactylus maini* and *D. pulcher* and *D. squarrosus*

Overall, the fine-scale ND2 + tRNA phylogeny is well resolved and recovered essentially the same higher-level relationships as the phylogeny based on the multi-gene data set, although with generally lower bootstrap support. In this section, we focus on phylogeographic patterns of four species due to the greater number of individuals sampled within each taxon, and the considerable genetic diversity and phylogeographic structure that was revealed.

Rhynchoedura ornata comprises two distinct parts, one represented by a single individual from near Wyndam in the Kimberley in the extreme northeast of Western Australia (Gko 239) and the other one comprising the rest of the individuals from Western Australia, clade A (Fig. 2). The lack of differentiation of most *R. ornata* sampled (including several from the Kimberley region) coupled with an extremely large Jukes–Cantor genetic distance of 18.0% for the ND2 + tRNA data set that separates the Wyndam individual from Gko 240 suggests that *R. ornata* from most of the state is a single species, but that a cryptic species from northern Australia occurs just over the border in the Northern Territory. Further sampling of *R. ornata* across its continent-wide range is warranted to determine the number and distributions of possible cryptic species within this taxon.

We recovered two extremely well supported clades within *D. maini* (ND2 + tRNA bootstrap values 100, posterior probabilities 100). These clades have a relatively close geographical proximity to one another but are separated by a drainage divide identified on the Australian River Basins data set (Geoscience Australia, 1997). The eastern clade is distributed along the western margin of the Nullarbor Plain, within the Yilgarn region of internal drainage basins. The western clade occurs in another set of internal drainage basins on the eastern side of this drainage divide in the vicinity of Lake Moore. Water barriers are known to be important in restricting the movement of reptiles in the Australian continent (Cogger and Heatwole, 1981), and persistent water barriers would have existed in the southwest during the wetter periods of the Miocene. The Jukes–Cantor genetic distance between a representative from each of these major clades (Gko 065 and Gko 075) is 7.8% based on the ND2 + tRNA data set. However, there are only minor differences in morphology and coloration between the two clades, which seems to correspond to background coloration.

Diplodactylus pulcher is characterized by a deep phylogenetic split between two main clades that are separated by a geographic distance of more than 800 km. One clade comprises a population in the Hamersley Range in the southern Pilbara, while the other is distributed in southwestern Western Australia east of Kalgoorlie, though the species range is known to extend between these areas (Storr et al., 1990). The Jukes–Cantor genetic distance between a representative from each clade (Gko 083 and Gko 089) is 8.1% based on the ND2 + tRNA data set. There also was evidence for considerable genetic structure within the southwestern clade.

Diplodactylus squarrosus also displays significant phylogeographic structure and is composed of two extremely well supported clades (ND2 + tRNA bootstrap values 99 and 100, posterior probabilities 100) that correspond to a coastal population on the low-lying Cainozoic sandplains near Shark Bay, and an inland population that occurs on the Proterozoic sandplains, hills and ridges from Canning Hill in the south to Mt. Murchison in the north. The Jukes–Cantor genetic distance between a representative from each

clade (Gko 093 and Gko 094) is 8.3% based on the ND2 + tRNA data set.

Despite the small number of samples representing each of the species, we found significant intra-specific genetic variation in *R. ornata*, *D. maini* and *D. pulcher* and *D. squarrosus*. The deep phylogeographic structure observed within each of these species highlights the need for large-scale screening of cryptic species, with sampling specifically targeted at determining the geographic limits of such taxa (Donnellan et al., 1999). For example, based on only a single individual, our results suggest that a cryptic species exists in the currently recognized taxon *R. ornata*. Whether the formal recognition of species within *D. maini*, *D. pulcher*, and *D. squarrosus* is warranted requires further morphological and genetic analyses.

4.3. Phylogeography of D. stenodactylus

The phylogeographic pattern evident in *D. stenodactylus* is of particular interest. The ND2 + tRNA phylogeny provides strong support for a deep phylogenetic split within *D. stenodactylus* that also was recovered in the 16s gene tree and the combined multi-gene phylogeny. The two distinct clades correspond geographically to populations in the Pilbara region and populations to the east of the Pilbara, including central and northern Western Australia and extending into northeastern NSW and southwestern Queensland (we call these the "Pilbara" and "non-Pilbara" clades, respectively) (Fig. 5). This split is concordant with the morphological data of Kluge (1967), who described major geographic differences in morphology between western ("Population A"), and central and northern ("Population B") populations of *D. stenodactylus.* The Jukes–Cantor genetic distance between these clades is 12.4–15.0% based on the ND2 + tRNA data set and is well above species-level differences in other reptiles using a mitochondrial gene that evolves at a similar rate (ND4, Scott and Keogh, 2000;Keogh et al., 2003, 2005). There currently is only a single gecko fossil from a very distantly related group with which to calibrate a molecular clock for the *D. stenodactylus* group. While the intrinsic level of error associated with single fossil calibrations precluded the use of molecular dating in this study, a rough mitochondrial calibration of 1.3–2% sequence divergence per million years (Wilson et al., 1985;Zamudio and Greene, 1997) suggests the two clades separated during the late Oligocene to middle Miocene approximately 16–27 Ma. The lower end of this range is favored as the 1.3% calibration has been well supported by numerous other studies (Macey et al., 1998a,b, 2000;Weisrock et al., 2005).

An examination of current landscape features coupled with knowledge of past geomorphological events may provide insights into possible mechanisms that have caused the phylogenetic split between the Pilbara and non-Pilbara populations. The boundary separating the clades is remarkably sharp and falls on the eastern boundary of the Pilbara and the sandy deserts to the east of the Pilbara (Fig. 5). The boundary also is followed by the present-day courses of the highly seasonal De Gray and Oakover rivers. While the combination of high sequence divergence and small geographic distances is not uncommon, there are no obvious persistent physical barriers along the boundary between these clades. However, the present lack of physical barriers does not preclude their existence at some time in the past. The unique tectonic history and topographical architecture of the Pilbara have produced an intricate drainage network, and erosion of less-resistant rock types have shaped the courses of a number of river systems (Bunting et al., 1974). These river systems should have been significantly more prominent during Australia's frequent and extensive fluvial periods in creating barriers to migration.

In addition to the drainage boundary, a major boundary in substrate type corresponds to the geographical boundary between the Pilbara clade (west) and the non-Pilbara clade (east). Most of the area covered by the sandy deserts directly to the east of the boundary coincides with outcrops of sedimentary rocks laid down in a succession of marine basins that existed intermittently from the Palaeozoic onwards (Beard, 1969), the last of which was during the global sea-level highstand of the Cretaceous (~100 Ma) (Haq et al., 1987). The longitudinal dune systems of the Great and Little Sandy Deserts are believed to have formed relatively recently, in association with arid climates during the glacial/inter-glacial cycles of the Pleistocene (van Vreeswyk et al., 2004). Conversely, the areas west of the boundary are underlain by extremely ancient rocks comprising the Precambrian cratons of Western Australia (Geological Survey of Western Australia, 1990). In the Pilbara, these Archean to Proterozoic igneous and sedimentary rocks (ironstones and cherts) are expressed as outcrops at the surface, whereas further south they are overlain by Proterozoic sedimentary rocks that also have given rise to sandplains (Beard, 1969).

Ecological factors such as habitat specificity widely have been invoked as mechanisms for diversification, and zones of "unsuitable habitat" are thought to limit the distribution of arid-zone reptiles (Pianka, 1969a,b, 1981, 1989). The deep split within *D. stenodactylus* may be an example of the control exerted by habitat differences. The Pilbara and non-Pilbara clades clearly are associated with very different types of underlying substrate (rocks and sand, respectively), and in addition to climatic factors, the vegetation of these areas is strongly influenced by the topography and soils these different substrates have produced (Beard, 1969).

In summary, the phylogenetic agreement between the three genes used in this study provides robust support for the relationships among members of the *D. stenodactylus* species group (Kluge, 1967). In addition, the population-scale mitochondrial phylogeny has a number of well-supported phylogeographic implications for several species. Considerable genetic structure within the *D. stenodactylus* species group and the identification of cryptic lineages highlights the need for large-scale revisionary studies across this and other *Diplodactylus* species groups. Analysis at both

broad and fine scales also has identified patterns associated with the distribution of the *D. stenodactylus* group that appear to be influenced by environmental processes operating at different geographic scales. At the biogeographic scale, the two major clades within the *D. stenodactylus* species group may be related to differences between Eremaean-Tropical, and Southwestern faunas. At the landscape scale, the deep phylogenetic split within *D. stenodactylus* may be the result of broad differences in underlying geological substrate with the Pilbara clade generally preferring harder soils and the non-Pilbara clade adapted to sand. The next logical step is much more extensive sampling of *D. stenodactylus* throughout its range so that we can integrate geological and phytogeographic data in finer detail.

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