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Hidden species diversity of Australian burrowing snakes (*Ramphotyphlops*)

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The worm-like snakes (Scolecophidia; approximately 400 nominal extant species) have a conservative morphology and are among the most poorly-known terrestrial vertebrates. Although molecular evidence has helped determine their higher-level relationships, such data have rarely been used to discriminate among species. We generated a molecular data set for the continental Australian blindsnakes (genus *Ramphotyphlops*) to determine the concordance of molecular and morphological information in the taxonomic recognition of species. Our dataset included 741 specimens morphologically attributed to 27 nominal *Ramphotyphlops* species. We proposed species hypotheses (SHs) after analysis of sequences from a variable mitochondrial gene (*cytochrome b*) and examined these SHs with additional evidence from a nuclear gene (*prolactin receptor*) and geographical data. Although the nuclear marker was not as fast-evolving and discriminating as the mitochondrial marker, there was congruence among the mitochondrial, nuclear, and geographical data, suggesting that the actual number of species is at least two times the current number of recognized, nominal species. Several biogeographical barriers and complex phytogeographical and geological patterns appeared to be involved in the division of some burrowing snake populations and, by consequence, in their diversification and speciation through isolation. *Biological Journal of the Linnean Society*, 2013, **110**, 427–441.

ADDITIONAL KEYWORDS: cryptic – evolution – integrative taxonomy – Scolecophidia – speciation.

INTRODUCTION

Species inventory and delineation are essential for assessing biodiversity, conservation or biological control projects, as well as more generally understanding the natural world (Wheeler, Raven & Wilson, 2004). However, it is well known that the number of described species on earth is far less than the total (Trontelj & Fišer, 2009). Most of the species yet to be described are assumed to be fungi and 'invertebrate' animals, particularly those living in tropical environments (Pfenninger & Schwenk, 2007)

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Over the last 12 years, the total number of nominal extant species of squamates has increased by 1.7% each year (Pincheira-Donoso *et al.*, 2013) and this value is much lower for mammals and birds (0.1–0.2%) (Wilson & Reeder, 2005; Lepage, 2012; Uetz, Goll & Hallerman, 2013). Cryptic species, two or more distinct species that were classified as a single species as a result of their morphological similarity, are almost evenly distributed among major metazoan taxa and biogeographical regions (Pfenninger & Schwenk, 2007). Therefore, the diversity of many vertebrate groups has yet to be explored (Oliver *et al.*, 2009).

In the present study, we focus on Australian blindsnakes of the genus *Ramphotyphlops* (Scolecophidia, Typhlopidae), whose systematics received little attention until recently (Aplin & Donnellan, 1993; Rabosky *et al.*, 2004; Vidal *et al.*, 2010). Blindsnakes typically are small (approximately 10–30 cm), burrowing species, and feed on social insects (Vidal & Hedges, 2009). They comprise 402 named species segregated in five families: Anomalepididae, Leptotyphlopidae, Typhlopidae, Xenotyphlopidae, and Gerrhopilidae (Vidal *et al.*, 2010; Uetz *et al.*, 2013). The 42 currently recognized Australian scolecophidian species all belong to *Ramphotyphlops*, a genus of 66 species distributed across South and South-east Asia, Australasia, and Melanesia (as far east as Fiji, and across the Caroline Islands from Palau to Pohnpei), and comprise one of the least-known elements of the Australian herpetofauna (Rabosky *et al.*, 2004; Wynn *et al.*, 2012). Previous molecular genetic studies on scolecophidian snakes, including members of the families Typhlopidae and Leptotyphlopidae, have suggested a high level of hidden diversity, as indicated by several recognized species being paraphyletic and polyphyletic, and harbouring deep divergences (Hedges & Thomas, 1991; Aplin & Donnellan, 1993; Rabosky *et al.*, 2004; Hedges, 2008; Adalsteinsson *et al.*, 2009; Kornilios *et al.*, 2012; Marin *et al.*, 2013).

Strong selection associated with a fossorial lifestyle has led to miniaturization, cranial consolidation, and body elongation, which are all common features in burrowing reptiles (Gauthier *et al.*, 2012). Moreover, there are limited characters for which inter- versus intraspecific variations are adequately understood, partly as a result of the lack of interest in scolecophidians (Adalsteinsson *et al.*, 2009). In the present study, we focus on testing and refining species boundaries within the Australian radiation of *Ramphotyphlops* in light of new molecular genetic data.

Although many theoretical species concepts have been discussed over the years, there is general agreement, as supported by genetic data, that species are reproductively isolated from one another (Coyne & Orr, 2004). It is then a practical matter of how to identify and delimit species. If two species occur together in sympatry and are not exchanging genes, then identifying the concordance of two or more characters (coded by unlinked genes) will usually suffice for demonstrating reproductive isolation and delimitation of species. However, whether or not to recognize allopatric populations as full species is a more subjective decision, often requiring additional case-specific information. Padial *et al.*, (2010) discuss two different approaches for distinguishing species: (1) 'cumulation', where only one character set [e.g. mitochondrial (mt)DNA sequences] is used, and (2) 'congruence', where multiple data sets (e.g. DNA sequences and morphology) are used. Rather than alternative approaches, they could also be viewed as a continuum concerning level of evidence (stringency), especially because a single study might diagnose some species using one character set and other species using multiple sets, depending on availability.

In the present study, we have explored species limits in Australian *Ramphotyphlops* using data from only one character set (mtDNA) and multiple sets (mtDNA, nuclear DNA, and geography). We took the general approach of defining preliminary species hypotheses (SHs) using the low stringency approach (mtDNA data set) and then comparing the SHs with additional character sets (Padial *et al.*, 2010; Goldstein & DeSalle, 2011; Yeates *et al.*, 2011; Puillandre *et al.*, 2012b). By adding more specimens ($N = 634$) to our previous molecular genetic dataset (Marin *et al.*, 2013), we built a new dataset. The ABGD (Automatic Barcode Gap Discovery) method (Puillandre *et al.*, 2012a) was used with mitochondrial gene (*cytochrome b*) sequences, and resulting SHs were then compared with variation from a nuclear gene (*prolactin receptor*) and geography. Genetic divergent population in sympatry or divided by recognized barriers was used as additional evidence. The goal was to test the hypothesis: is the number of nominal species a reasonably accurate reflexion of true species diversity? This represents an initial step that could assist a morphology-based taxonomic revision, providing a better delimitation of some Australian *Ramphotyphlops* species.

MATERIAL AND METHODS

Taxon sampling

Ingroup sampling included 741 individuals belonging to 27 nominal Australian *Ramphotyphlops* species (as identified using morphology). The taxa, localities, and GenBank accession numbers of specimens used in the present study are provided in Table S1.

Table 1. List of primers used in the present study

Gene	Primer		Reference
cyt b	CS1L	GAAAAACCGCYRTTGTWWTTCAACTA	Adalsteinsson *et al*. (2009)
	LTyph2R	AGYTTGTTTGGGATKGCTCGTAGRAT	Adalsteinsson *et al*. (2009)
	L14910	GACCTGTGATMTGAAAACCAYCGTTGT	Burbrink *et al*. (2000)
	H16064	CTTTGGTTTACAAGAACAATGCTTTA	Burbrink *et al*. (2000)
PRLR	PRLR_f1	GACARYGARGACCAGCAACTRATGCC	Townsend *et al*. (2008)
	PRLR_f2	AAGAGTCRCCCAYATAAAAA	Present study
	PRLR_r3	GACYTTGTGRACTTCYACRTAATCCAT	Townsend *et al*. (2008)
	PRLR_r4	AAGAACYTCTCTGGAGGT	Present study
	PRLR_r5	ATCCATTGGYTTTGYAGACA	Present study

cyt b, *cytochrome b*; *PRLR*, *prolactin receptor*.

Three typhlopid snakes were used as outgroups: *Acutotyphlops subocularis* (Waite) (Vuovo Camp, West New Britain, Papua New Guinea, *cyt b*: JQ910524, *PRLR*: JQ910414), *Ramphotyphlops acuticaudus* (Peters) (Palau, *cyt b*: JQ910543, *PRLR*: JQ910412), and *Ramphotyphlops braminus* (Daudin) (Florida, USA, *cyt b*: JQ910548, *PRLR*: JQ910434).

Molecular genetic markers

A mitochondrial protein coding gene and one nuclear protein coding gene were used. The mitochondrial marker, *cytochrome b* (*cyt b*), is highly variable (intraspecific variation) and thus potentially useful to identify recent speciation events (Burbrink, Lawson & Slowinski, 2000; Adalsteinsson *et al*., 2009). Among the nuclear genes available for squamate phylogenies (Townsend *et al*., 2008), we selected one of the most variable, the *prolactin receptor* (*PRLR*). For this work, 83.9% of the sequences were newly determined; 943 sequences were deposited in GenBank under accession numbers KC489799 to KC490909 and KC493653.

DNA extraction, amplification, and sequencing

DNA extraction was performed with the DNeasy Tissue Kit (Qiagen). Amplification and sequencing was performed using the primers listed in Table 1.

For the two markers, DNA amplification was performed by polymerase chain reaction (PCR) in a final 21-μL volume containing 1 μL of dimethyl sulphoxide, 0.8 μL of dNTP 6.6 mM, 0.12 μL of Taq DNA polymerase (MP Biomedicals or Qiagen), using 2.5 μL of the buffer provided by the manufacturer (100 units mL^{-1}) and 0.32 μL of each of the two primers at 10 pM. Finally, 1 μL of DNA extract was added.

The PCR reactions were performed with the conditions: initial denaturation at 94 °C for 3 min, followed by 40 cycles (3 min at 94 °C, 40 s at 50 °C, 1 min at 72 °C) and a final elongation at 72 °C for 10 min, using a PCR System 2700 thermocycler (Applied Biosystems). Amplification products were visualized on ethidium-bromide stained agarose gels. Sequencing was performed by the National Centre for Sequencing (Genoscope, Evry, France) using the same primers.

The two strands obtained for each sequence were combined using SEQUENCHER, version 4.9 (GeneCodes). Sequence alignment was performed with CLUSTALW2 (default parameters) (Larkin *et al*., 2007), implemented in BIOEDIT (Hall, 1999) and then manually refined with MEGA, version 5 (Tamura *et al*., 2011), using amino acid translations. The absence of stop codons was checked.

Phylogenetic analysis

Cyt b analyses were performed on all the obtained sequences; *PRLR* analyses were performed on haplotypes only to reduce computation time. We built phylogenies using Bayesian and maximum likelihood (ML) methods of inference. Bayesian analyses were performed with MrBayes, version 3.1.2 (Ronquist & Huelsenbeck, 2003) and ML analyses were performed with RAXML, version 7.2.8 (Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008). For both *cyt b* and PRLR, the three-partition strategy (by codon position) was preferred to the one partition strategy (by gene) using standard Bayes factors (Nylander *et al*., 2004). Bayesian analyses were performed by running 50 000 000 generations in four chains, saving the current tree every 1000 generations (until convergence), with the GTR+I+G model applied to each partition (best-fit model inferred by MODELTEST; Posada & Crandall, 1998). Convergence of ESS (effective sample size) values was checked with TRACER, version 1.4.1 (Rambaut & Drummond, 2009) using the default burning (10%). The last 45 000 trees were used to construct a 50% majority rule consensus tree. For the ML analysis, we defined the same partitions and

performed 1000 bootstrap replicates to obtain a bootstrap majority rule consensus tree. Trees were visualized with FIGTREE, version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Species delimitation

The ABGD method (Puillandre *et al.*, 2012a) was employed to statistically detect a barcode gap (i.e. a gap in the pairwise genetic distance distribution, presumably between intraspecific and interspecific distances) from the *cyt b* data set, which is then used to partition the data set (initial partition) into species hypotheses. The resulting inferences are then recursively applied to yield finer partitions (recursive partitions) until no further partitioning is possible. We used the online version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) to analyze the pairwise distance matrix calculated for each dataset with PAUP (Swofford, 2003) under a GTR+I+G model, as inferred by MODELTEST (Posada & Crandall, 1998) using the Akaike information criterion as the best-fitting model of nucleotide substitution for the entire data set. ABGD default parameters were used, with the exception that the relative gap width (X) was set to 1 [except for *Ramphotyphlops nigrescens* (Gray): 0.9], and P_{min} (minimal prior intraspecific divergence) was set to 0.01 to avoid the capture of intraspecific gaps as a result of weak sampling.

The number of putative species (SHs) was first determined using *cyt b*. Then, those *cyt b* SHs were compared with the nuclear gene data to assess concordance or discordance. Major geographical features that can be barriers to gene flow (rivers, mountains, climatic zones) for well-sampled groups were identified and used in further comparisons of the SHs.

PRLR haplotypes

DNASP, version 5 (Librado & Rozas, 2009) was used to determine the haplotypes. For generating haplotype data files, invariable sites were included and sites with gaps or missing data were not considered.

RESULTS

For the *cyt b* gene, the alignment comprised 678 sites, with 386 variable sites among the 741 specimens successfully sequenced (4.6% of missing data). For the *PRLR* gene, the alignment comprised 483 sites, of which 197 were variable among the 583 specimens (0.38% of missing data), 158 specimens were unsuccessfully amplified. The alignments were straightforward for both genes.

Phylogenetic analysis

Based on the *cyt b* analysis, 17 of the 27 nominal species are monophyletic in the *cyt b* tree with moderate support using both methods [ML bootstrap > 75%/posterior probability (PP) > 95] except *Ramphotyphlops diversus* (Waite) (ML bootstrap = 71/PP = 1) (see Fig. S1), and five species, *Ramphotyphlops affinis* (Boulenger), *Ramphotyphlops howi* (Storr), *Ramphotyphlops longissimus* (Aplin), *Ramphotyphlops silvia* (Ingram & Covacevich), and *Ramphotyphlops troglodytes* (Storr), have one representative only. *Ramphotypholops ligatus* (Peters) is polyphyletic in the *cyt b* phylogram (Fig. 1), and *Ramphotyphlops kimberleyensis* (Storr), *Ramphotyphlops leptosoma* Robb, *Ramphotyphlops grypus* (Waite), and *Ramphotyphlops guentheri* (Peters) are polyphyletic in both the mtDNA and nuclear DNA phylograms (Figs 1, 2).

Species delineation

Molecular genetic data

The ABGD method was applied independently to 21 monophyletic groups defined from the *cyt b* phylogeny (Fig. 1) because groups including a limited number of lineages allowed us to avoid problems linked to the heterogeneity of evolution times between lineages. This phenomenon may lead to inter- and intraspecific pairwise distributions overlapping and, by consequence, may prevent the barcode gap detection. These groups corresponded to 17 nominal species that are represented by at least two specimens (16 nominal species and *R. guentheri* lineage 1) plus three *cyt b* clades with non-monophyletic nominal species (clade 1: *R. grypus* lineage 1, *R. leptosoma*, and *R. longissimus*; clade 2: *R. kimberleyensis* and *R. troglodytes*; clade 3: *Ramphotyphlops ganei* (Aplin) and *R. ligatus*) and *R. grypus* lineage 2. Three nominal species and the *R. guentheri* lineage 2 were represented each by only one specimen and were not analyzed with ABGD. Based on the distribution of pairwise genetic distances, ABGD proposed several partitions that varied according to the different a priori thresholds. Apart from the two extreme a priori threshold values ($P = 0.009$ and $P = 0.013$), for which aberrant number of species hypotheses were obtained for some groups (almost every haplotype was considered as a different species hypothesis for $P = 0.009$ and, conversely, all the haplotypes were combined in a single species hypothesis for $P = 0.013$; as described in Puillandre *et al.*, 2012b), all the tested a priori thresholds lead to the same splitting. The only exception is for five groups (*Ramphotyphlops ammodytes* (Montague), *Ramphotyphlops bituberculatus* (Peters), *R. ganei*, *Ramphotyphlops hamatus* (Storr), and *R. ligatus*), for which ABGD proposed two different partitions. We

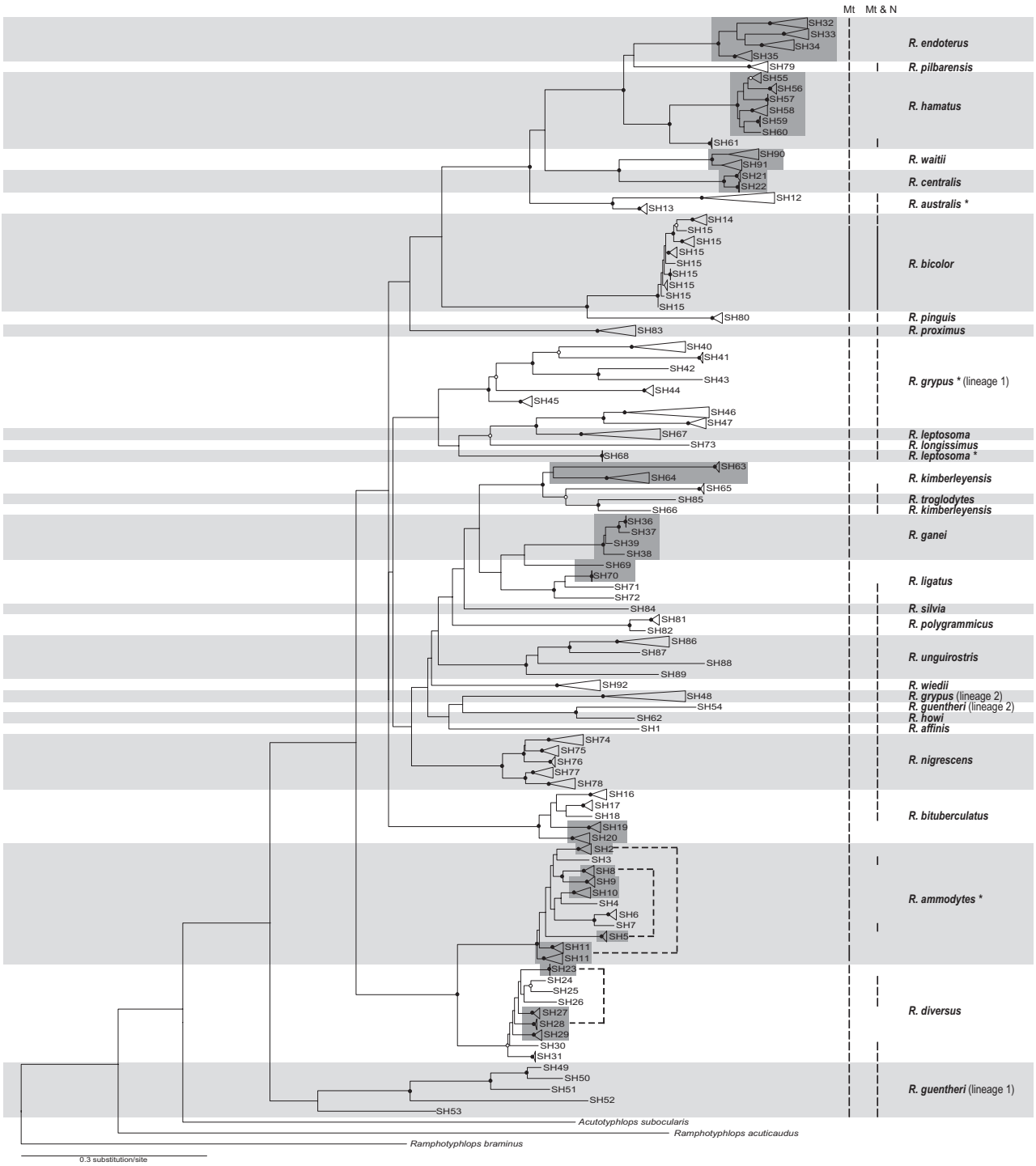

Figure 1. Bayesian inference phylogenetic tree of Australian *Ramphotyphlops* based on analysis of sequences of a mitochondrial protein-coding gene, *cytochrome b*, showing species hypotheses (SHs) obtained with the Automatic Barcode Gap Discovery method. Framed clades share common *prolactin receptor* haplotypes. Dashed lines join SH when they shared a *PRLR* haplotype. Nodes with black circles are supported by posterior probabilities > 95% and Maximum Likelihood (ML) bootstrap probabilities > 75%. Nodes with white circles are supported by posterior probabilities > 90% and ML bootstrap probabilities > 70%. The first set of vertical bars (Mt, mitochondrial data) corresponds to the 92 SHs supported by mitochondrial DNA. The second set of vertical bars (Mt & N, mitochondrial and nuclear data) corresponds to the 56 SHs supported by nuclear and mitochondrial DNA. Asterisks (*) indicate nominal species for which SHs are found in sympatry.

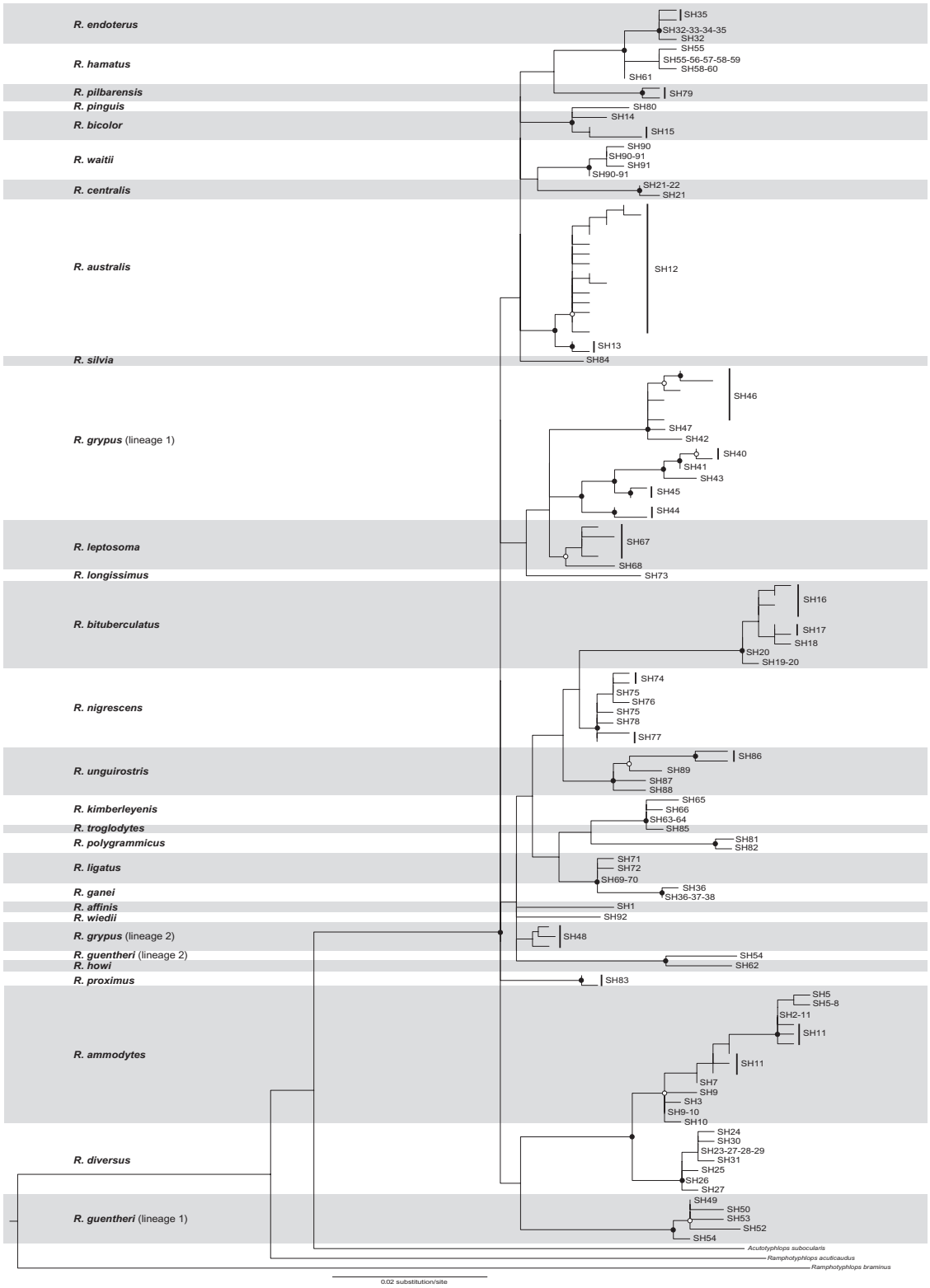
R. endoterus
R. hamatus
R. pilbarensis
R. pinguis
R. bicolor
R. waitii
R. centralis
R. australis
R. silvia
R. grypus (lineage 1)
R. leptosoma
R. longissimus
R. bituberculatus
R. nigrescens
R. unguirostris
R. kimberleyenis
R. troglodytes
R. polygrammicus
R. ligatus
R. ganei
R. affinis
R. wiedii
R. grypus (lineage 2)
R. guentheri (lineage 2)
R. howi
R. proximus
R. ammodytes
R. diversus
R. guentheri (lineage 1)
SH35
SH32-33-34-35
SH32
SH55
SH55-56-57-58-59
SH58-60
SH61
SH79
SH80
SH14
SH15
SH90
SH90-91
SH91
SH90-91
SH21-22
SH21
SH12
SH13
SH84
SH46
SH47
SH42
SH40
SH41
SH43
SH45
SH44
SH67
SH68
SH73
SH16
SH17
SH18
SH20
SH19-20
SH74
SH75
SH76
SH75
SH78
SH77
SH86
SH89
SH87
SH88
SH65
SH66
SH63-64
SH85
SH81
SH82
SH71
SH72
SH69-70
SH36
SH36-37-38
SH1
SH92
SH48
SH54
SH62
SH83
SH5
SH5-8
SH2-11
SH11
SH11
SH7
SH9
SH3
SH9-10
SH10
SH24
SH30
SH23-27-28-29
SH31
SH25
SH26
SH27
SH49
SH50
SH53
SH52
SH54
Acutotyphlops subocularis
Ramphotyphlops acuticaudus
Ramphotyphlops braminus
0.02 substitution/site

Figure 2. Maximum likelihood (ML) phylogenetic tree of Australian *Ramphotyphlops* based on the analysis of a nuclear protein-coding gene, *prolactin receptor*. Species hypotheses (SHs) shown here were obtained with the Automatic Barcode Gap Discovery method using *cytochrome b*. Nodes with black circles are supported by posterior probabilities > 95% and ML bootstrap probabilities > 75%. Nodes with white circles are supported by posterior probabilities > 90% and ML bootstrap probabilities > 70%.

only used initial partitions because they were stable on a wide range of values contrary to the recursive partitions (results not shown); the initial partitions are also supposed to more closely match the groups described by taxonomists (Puillandre *et al*., 2012a).

Considering all possible initial partitions allowed the delineation of 92 partitions or *cyt b* SHs (Table 2). SHs containing more than one specimens are monophyletic and highly supported with three exceptions: SH 55 (ML bootstrap = 70/PP = 0.93), SH 90 (ML bootstrap = 43/PP = 0.79) and SH 91 (ML bootstrap = 61/PP = 0.53) (Fig. S1). SH 15 and SH 11 are polyphyletic (Fig. 1).

In a few cases, when ABGD splits one nominal species into several *cyt b* SHs, the divergence between these SHs is similar to the divergence between pairs of nominal species. For example, the genetic distance (*p*-distance) between *Ramphotyphlops guentheri* lineage 2 (SH 54) and *R. howi* (SH 62) is 0.10, whereas it is 0.12 among SHs 86–89 within the group of specimens identified morphologically as *Ramphotyphlops unguirostris* (Peters).

The 92 *cyt b* SHs displayed 121 different *PRLR* haplotypes. Among them, 56 SHs are defined by their own haplotypes (i.e. their haplotypes are not shared with any other *cyt b* SHs) (Fig. 2; Table 2; Fig. S2): *R. affinis* (SH 1), *R. ammodytes* (SH 3, 7), *Ramphotyphlops australis* (Gray) (SH 12–13), *Ramphotyphlops bicolor* (Peters) (SH 14–15), *R. bituberculatus* (SH 16–18), *R. diversus* (SH 24–26, 30–31), *R. grypus* (SH 40–48), *R. guentheri* (SH 49–54), *R. hamatus* (SH 61), *R. howi* (SH 62), *R. kimberleyensis* (SH 65–66), *R. leptosoma* (SH 67–68), *R. ligatus* (SH 71–72), *R. longissimus* (SH 73), *R. nigrescens* (SH 74–78), *Ramphotyphlops pilbarensis* (Aplin & Donnellan) (SH 79), *Ramphotyphlops pinguis* (Waite) (SH 80), *Ramphotyphlops polygrammicus* (Schlegel) (SH 81–82), *Ramphotyphlops proximus* (Waite) (SH 83), *R. silvia* (SH 84), *R. troglodytes* (SH 85), *R. unguirostris* (SH 86–89) and *Ramphotyphlops wiedii* (Peters) (SH 92). Among them, SH 15 (*R. bicolor*) was the only non-monophyletic SH in the *cyt b* ABGD analysis; however, it is represented by unique *PRLR* haplotypes. Five species include only one sample. The 33 remaining *cyt b* SHs share *PRLR* haplotypes, the SHs within a nominal species that are sharing haplotypes are phylogenetically closely related.

Geographical data

Geographical distributions of mtDNA clades for several well sampled species are congruent with landscape features (Fig. 3). Three main climatic zones are recognized across the Australian continent (Fig. 4). The monsoon tropic zone in northern Australia receives heavy rainfall during the summer and dates from the Late Eocene/Early Oligocene (Greenwood, 1996; Pole & Bowman, 1996; Alexandre *et al*., 2004). The mesic zone, which includes the Wet Tropics rainforest in the far north-east, extends south along the Great Dividing Range of the eastern coast, with an isolated region in the south-west. It is the oldest Australian biome, originating from the forests of the Mesozoic that were widespread until the Early Miocene (Hill, 1994; Schodde, 2006). The arid zone in central and western Australia is much younger, with origins from the Early Pliocene (Fujioka *et al*., 2005, 2009; Byrne *et al*., 2008).

In the monsoon tropic zone of northern Australia, four major phylogeographical barriers have been identified: the Daly River Drainage Barrier (Ford, 1978), the Victoria River Drainage Barrier (Joseph & Omland, 2009), the Ord Arid Intrusion (Ford & Blair, 2005; Bowman *et al*., 2010), and the East–West Kimberley Divide (Potter *et al*., 2012). These barriers are congruent with the distributions of *R. guentheri*, *R. kimberleyensis*, and *R. unguirostris* (Fig. 3), with SHs restricted to each side of the barriers. The geographical repartition of mitochondrial lineages of *R. diversus* can be partly explained by these geographical barriers, and by the boundary between the arid and monsoon regions (Fig. 3). For *Ramphotyphlops waitii* (Boulenger), the boundary between the south-west mesic zone and the arid zone is congruent with distributions of SHs 90 and 91, respectively (Fig. 3). Further south, two rivers (Darling River, River Murray) and the Flinders Ranges are congruent with the three major mtDNA lineages of *R. bituberculatus* (SHs 16, 19, and 20) (Fig. 3) and, to a lesser degree, with those of *R. bicolor* (not shown). SH 14 and SH 15 (*R. bicolor*) are distributed on each side of the Flinders Ranges, except three specimens of the SH 15 located within the eastern SH 14 geographical zone. The separation by recognized barriers of allopatric SHs is additional evidence for SHs 19 and 20 (*R. bituberculatus*; separated by Darling River and River Murray), SHs 63–64

Table 2. Recapitulative table of results for Australian *Ramphotyphlops*

Species names	Number of sequences		SHs (cumulative approach)	First partition	Alternative partition	*PRLR*		SHs (congruent approach)	Geography
	cyt b	*PRLR*				Number of haplotypes	Shared haplotype		
Ramphotyphlops affinis	1	1	SH1	x		1		SH1	–
Ramphotyphlops ammodytes	9	5	SH2	x	x	1	SH11		Sympatry
	1	1	SH3	x	x	1		SH3	
	1	0	SH4	x	x	0			
	11	3	SH5	x	x	2	SH8		
	4	1	SH6	x	x	0			
	1	1	SH7	x		1		SH7	
	2	2	SH8	x	x	1	SH5		
	2	2	SH9	x		2	SH10		
	4	3	SH10	x		2	SH9		
	58	32	SH11	x		7	SH2		
Ramphotyphlops australis	135	116	SH12	x		14		SH12	Sympatry
	6	3	SH13	x		2		SH13	
Ramphotyphlops bicolor	17	11	SH14	x		1		SH14	Allopatry
	26	20	SH15	x		2		SH15	
Ramphotyphlops bituberculatus	25	20	SH16	x	x	4		SH16	Allopatry
	4	4	SH17	x		2		SH17	
	1	1	SH18	x		1		SH18	
	11	10	SH19	x	x	1	SH20		
	14	11	SH20	x	x	2	SH19		
Ramphotyphlops centralis (Storr)	3	3	SH21	x		2	SH22		–
	2	2	SH22	x		1	SH21		
Ramphotyphlops diversus	3	2	SH23	x		1	SH27–28–29		Allopatry
	1	1	SH24	x		1		SH24	
	1	1	SH25	x		1		SH25	
	1	1	SH26	x		1		SH26	
	4	4	SH27	x		2	SH23–28–29		
	4	4	SH28	x		1	SH23–27–29		
	2	2	SH29	x		1	SH23–27–28		
	1	1	SH30	x		1		SH30	
	2	1	SH31	x		1		SH31	
Ramphotyphlops endoterus	76	58	SH32	x		2	SH33–34–35		Allopatry
	7	6	SH33	x		1	SH32–34–35		
	3	3	SH34	x		1	SH32–33–35		
	10	9	SH35	x		3	SH32–33–34		
Ramphotyphlops ganei	2	2	SH36	x	x	2	SH37–38		–
	1	1	SH37	x		1	SH36–38		
	1	1	SH38	x		1	SH36–37		
	1	0	SH39	x		0			
Ramphotyphlops grypus	10	8	SH40	x		2		SH40	Sympatry
	4	4	SH41	x		1		SH41	
	1	1	SH42	x		1		SH42	
	1	1	SH43	x		1		SH43	
	10	5	SH44	x		2		SH44	
	21	12	SH45	x		2		SH45	
	28	22	SH46	x		6		SH46	
	2	2	SH47	x		1		SH47	
	4	4	SH48	x		2		SH48	

Ramphotyphlops guentheri	1	1	SH49	x			1				SH49		Allopatry
	1	1	SH50	x			1				SH50		
	1	1	SH51	x			1				SH51		
	1	1	SH52	x			1				SH52		
	1	1	SH53	x			1				SH53		
	1	1	SH54	x			1				SH54		
Ramphotyphlops hamatus	20	18	SH55	x	x		2	SH56–57–58–59					Allopatry
	9	8	SH56	x			1	SH55–57–58–59					
	5	4	SH57	x			1	SH55–56–58–59					
	10	8	SH58	x			2	SH55–56–57–59–60					
	3	3	SH59	x			1	SH55–56–57–58					
	1	1	SH60	x			1	SH58					
	2	2	SH61	x	x		1				SH61		
Ramphotyphlops howi	1	1	SH62	x			1				SH62		–
Ramphotyphlops kimberleyensis	4	4	SH63	x			1	SH64					Allopatry
	2	1	SH64	x			1	SH63					
	3	3	SH65	x			1				SH65		
	1	1	SH66	x			1				SH66		
Ramphotyphlops leptosoma	12	12	SH67	x			4				SH67		Sympatry
	2	2	SH68	x			1				SH68		
Ramphotyphlops ligatus	1	1	SH69	x	x		1	SH70					Allopatry
	3	3	SH70	x	x		1	SH69					
	1	1	SH71	x			1				SH71		
	1	1	SH72	x			1				SH72		
Ramphotyphlops longissimus	1	1	SH73	x			1				SH73		–
Ramphotyphlops nigrescens	6	6	SH74	x			2				SH74		Sympatry
	3	2	SH75	x			2				SH75		
	4	3	SH76	x			1				SH76		
	8	6	SH77	x			2				SH77		
	4	4	SH78	x			1				SH78		
Ramphotyphlops pilbarensis	30	29	SH79	x			2				SH79		–
Ramphotyphlops pinguis	2	2	SH80	x			1				SH80		–
Ramphotyphlops polygrammicus	4	4	SH81	x			1				SH81		–
	1	1	SH82	x			1				SH82		
Ramphotyphlops proximus	6	3	SH83	x			2				SH83		–
Ramphotyphlops silvia	1	1	SH84	x			1				SH84		–
Ramphotyphlops troglodytes	1	1	SH85	x			1				SH85		–
Ramphotyphlops unguirostris	4	4	SH86	x			2				SH86		Allopatry
	1	1	SH87	x			1				SH87		
	1	1	SH88	x			1				SH88		
	1	1	SH89	x			1				SH89		
Ramphotyphlops waitii	24	17	SH90	x			3	SH91					Allopatry
	10	5	SH91	x			3	SH90					
Ramphotyphlops wiedii	3	3	SH92	x			1				SH92		–

Geographical patterns (sympatry and allopatry) are globally and visually assessed for each morphologically-defined species.
cyt b, *cytochrome b*; *PRLR*, *prolactin receptor*; SH, species hypotheses.

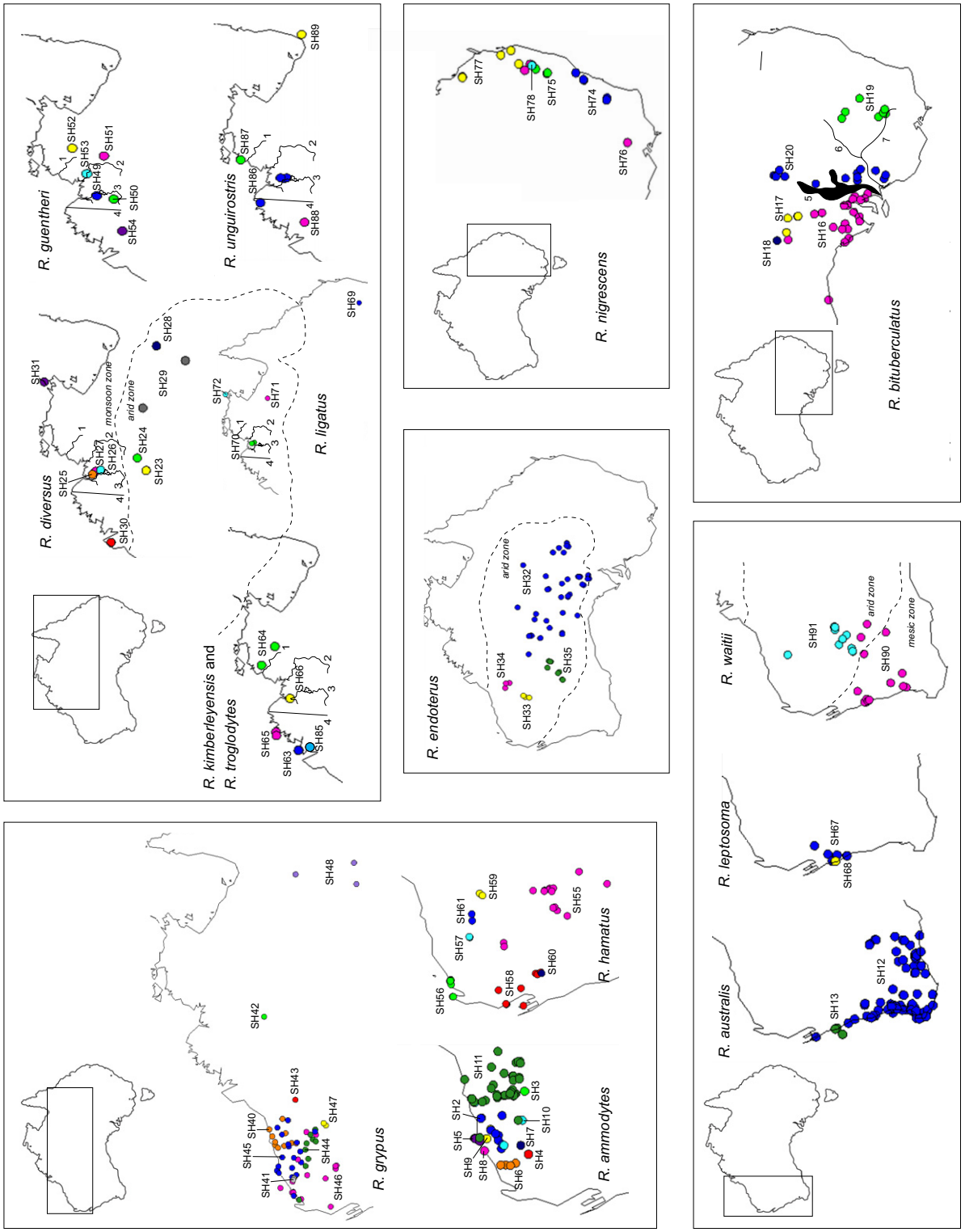

Figure 3. Sample locations of Australian *Ramphotyphlops* species for which geographical patterns are discussed. Recognized biogeographical barriers: 1, Daly River; 2, Victoria River; 3, Ord Arid Intrusion; 4, East–West Kimberley Divide; 5, Flinders Ranges; 6, Darling River; 7, Murray River; SHs, species hypotheses.

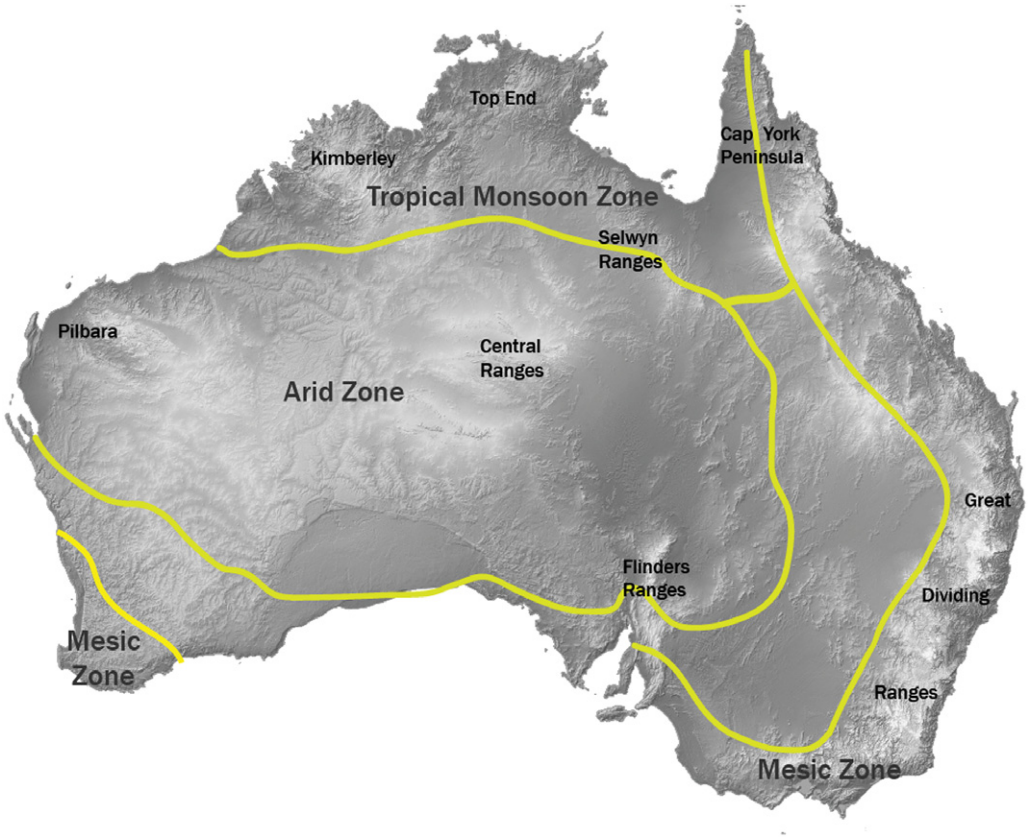

Figure 4. Map of the three main climatic zones and mountainous regions of the continental Australia. Modified from Fujita *et al*. (2010); Byrne *et al*. (2011); and Pepper *et al*. (2011).

(*R. kimberleyensis*; separated by four major phylogeographical barriers: the Daly River Drainage Barrier, the Victoria River Drainage Barrier, the Ord Arid Intrusion, and the East–West Kimberley Divide) and SHs 69–70 [*R. ligatus*; separated by the Victoria River Drainage Barrier and also a huge distance (approximately 2600 km)] (Fig. 3).

Other species display different distribution patterns. Instead of observing geographically restricted mtDNA lineages, some species harbour deep genetic divergences [0.73 between SHs 7 and 8 (*R. ammodytes*) and 0.137 between SHs 40 and 45 (*R. grypus*)] among geographically close and/or sympatric clades. This pattern is clearly observable for *R. ammodytes* and *R. grypus*, each with many SHs in the rocky Pilbara region, and, to a lesser degree, for *R. australis* and *R. leptosoma* that are restricted to the west central coast, and for *R. nigrescens* on the east coast. Divergent genetic lineages (a SH with distinct nuclear haplotype) found in sympatry reinforce SHs because, despite geographical proximity, there is no evidence of gene flow. However, because many *PRLR* haplotypes are shared among the SHs of *R. ammodytes*, it might be more parsimonious to consider these SHs (2, 4–6 and 8–11) as a single species rather than a complex of several species with incomplete lineage sorting or hybridization.

Conversely, the four *Ramphotyphlops endoterus* (Waite) SHs occur across the arid zone in four allopatric mtDNA lineages but without recognized barriers between them. In this case, as with the seven *R. hamatus* SHs (distributed across the rocky Pilbara zone and the eastern part of the arid zone), geographical data do not suggest a consolidation of SHs.

In several cases, there is strong congruence among mitochondrial, nuclear, and geographical data, leading us to recognize 56 robust putative species using *cyt b* and *PRLR* haplotypes and 61 putative species when adding geographical (sympatry or allopatry with barriers) data only (Table 2). In the least conservative scheme (*cyt b* only), there are 92 putative species (Table 2).

DISCUSSION

The concordance of independent genes (nuclear and mitochondrial) is generally considered to represent valuable evidence for species delimitation (Knowlton,

2000; Barberousse & Samadi, 2010). In our data set, 56 of the 92 *cyt b* SHs are robustly defined by an independent nuclear gene. The other *cyt b* SHs shared nuclear haplotypes, although the fact that these SHs remain closely related in the *cyt b* phylogram suggests a lack of variability of the *PRLR* gene at this scale, or a nuclear gene flow. To clarify this situation, the use of other more variable nuclear markers is needed. Analyzing more specimens per nominal species should also be useful. Indeed, the probability to find rare and shared haplotypes is higher for species that are densely sampled (several localities and several specimens per localities) even if some nominal species appear to be genetically uniform. For example, the nine SHs of the nominal species *R. grypus* are defined by specific haplotypes over the 59 specimens analysed with the nuclear marker. Concerning *R. pilbarensis*, even when the number of nuclear sequences was relatively high ($N = 29$), only two different haplotypes were found. If we analyze the *cyt b* SHs on an individual basis, the number supported by different independent sources of information increases to 61 when the geographical data are considered. Therefore, from the 27 nominal species of Australian blindsnakes investigated in the present study, our results support at least 56 (most conservative) and up to 92 (least conservative) species. Nine nominal species were not subdivided. The identification of new morphological characters should also help to discriminate among the different proposed hypotheses. The visceral anatomy traits may be useful because they allowed the discrimination of some leptotyphlopid species (Adalsteinsson *et al.*, 2009).

The early diversification of Australian *Ramphotyphlops* was probably driven by the development of the arid zone approximately 20 Mya (Marin *et al.*, 2013). As the arid zone expanded in central Australia, mesic-adapted lineages were confined to the east coast and south-west, to the northern monsoonal tropics, and also to Pilbara in the arid zone, which likely served as refugia (Martin, 2006; Byrne *et al.*, 2008, 2011). Occupation of areas such as the Pilbara and Kimberley (northern monsoonal tropics), which have humid refugia but are seasonally very arid, may have allowed time for this lineage of snakes of wet-tropical origin to acquire adaptations to resist seasonally dry conditions, which then pre-adapted them for the truly arid conditions further inland (Fujita *et al.*, 2010).

Several biogeographical barriers within these refugia appear to be involved in a later, < 8 Mya (Marin *et al.*, 2013), allopatric diversification of species (*R. bicolor*, *R. bituberculatus*, *R. diversus*, *R. guentheri*, *R. kimberleyensis* *R. ligatus*, and *R. unguirostris*) (Fig. 3). They are more subject to isolation as a result of their fossorial lifestyle (Vidal *et al.*, 2010). Similar patterns of diversification were recently found for rock wallabies in the monsoon tropic zone (Potter *et al.*, 2012) and for beaked geckos in the eastern arid zone and south-east mesic zone (Pepper *et al.*, 2011). Some other SHs of our *Ramphotyphlops* dataset display close and/or sympatric geographical distributions in the rocky Pilbara region. This region is characterized by geological heterogeneity, complex phytogeography, and long-term geological stability (Pepper, Doughty & Keogh, 2006), leading to a mosaic of habitat types (Doughty *et al.*, 2011). This may explain the sympatric distribution observed for *R. ammodytes*, *R. grypus*, and *R. hamatus*. Assuming that each SH may be restricted to a particular habitat, they should have evolved independently through ecological diversification. By contrast to the results for *R. grypus* and *R. hamatus*, nuclear haplotypes of *R. ammodytes* are shared between SHs. This could reflect a more recent speciation event or, more probably, could be linked to the fact that *R. ammodytes* is one species. One other interesting geographical pattern concerns *R. endoterus*, which is consistent with a sandy desert expansion out of the west. Several different genetic groups (SHs 33–35) have differentiated in the west, although only a single lineage (SH 32) has expanded eastward into the younger central and eastern desert areas, a pattern seen in other arid zone nonsnake taxa (Moritz & Heideman, 1993; Kearney *et al.*, 2006). *Ramphotyphlops hamatus* shows a similar though less expansive pattern. SHs of *R. endoterus* share a common nuclear haplotype, reflecting a recent speciation event or a single species with a polymorphic mitochondrial marker.

Overall, our results suggest that the current complement of nominal Australian *Ramphotyphlops* species is less than the total, with the true species diversity ranging between 207% and 341% of the currently described species. However, even though our dataset is large (approximately 740 specimens), the sampling is limited for some taxa, and adding more specimens (especially for species with a single sequenced specimen) may help to more accurately delimitate the current species. On a larger taxonomic scale, these new species (29–65) represent an increase of 7–16% of the entire scolecophidian species diversity. Currently, 402 scolecophidian species are described and, at the same time as acknowledging that extrapolations of hidden biodiversity from limited surveys are subject to sampling errors (Gray, 2002), taken at face value, our results suggest that between 834 and 1370 scolecophidian species may exist, mostly hidden from current taxonomy. If true, that would be an exceptional increase in the number of reptile or vertebrate species.

The morphological conservativeness of blindsnakes may be responsible for this hidden diversity because potentially informative characters have been reduced or eliminated as an outcome of their burrowing lifestyle (Hedges & Thomas, 1991; Thomas & Hedges, 2007). The limited knowledge on the morphological characters useful for the discrimination of blindsnakes is also likely responsible. Fortunately, in other cases where genetic analysis has revealed hidden species of scolecophidians, nontraditional morphological characters always have been found and used to diagnose the species (Hedges & Thomas, 1991; Aplin & Donnellan, 1993; Rabosky *et al*., 2004; Thomas & Hedges, 2007). For this reason, we suspect that most of all of the putative new species revealed in the present study will be diagnosed morphologically and named.

These results also have implications for conservation because accurate taxonomic data are critical for determining basic parameters of protection, such as distributions and threat levels (Rondinini *et al*., 2006). Also, nominal species already considered endangered or threatened may comprise several species, each of which is often rarer than their 'parent species', making them more susceptible to extinction (Hedges & Conn, 2012). For these species, taxonomic revisions are urgent. Without published descriptions, these species are essentially 'off the conservation radar' and therefore are not considered in conservation plans (Hedges & Conn, 2012).

Conclusions

Morphological conservativeness and a limited knowledge of useful morphological discriminant characters appear to have prevented the recognition of numerous Australian blindsnake species (*Ramphotyphlops*). Using several lines of independent evidence, including mtDNA, nuclear DNA, and geography, we found that at least 56 species exist, which is twice the currently recognized number of species. This is consistent with the results of previous smaller-scale studies of scolecophidians conducted elsewhere in the world, suggesting that the proportion of species of these burrowing snakes yet to be described is greater than is typical for terrestrial vertebrates.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Phylogenetic tree of Australian *Ramphotyphlops* based on the analysis of sequences of *cytochrome b* (*cyt b*).

Figure S2. Phylogenetic tree of Australian *Ramphotyphlops* based on the analysis of sequences of prolactin receptor (*PRLR*).

Table S1. Taxa, localities, and accession numbers of the specimens used in the present study.