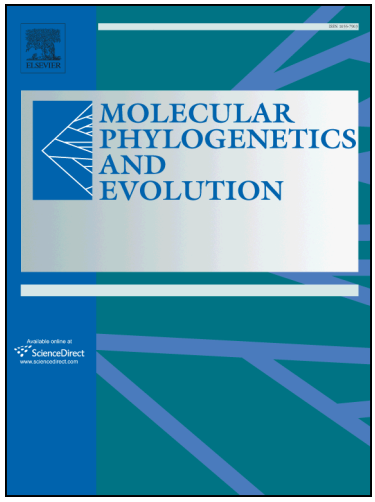

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Multi-locus phylogeny and species delimitation of Australo-Papuan blacksnakes (*Pseudechis* Wagler, 1830: Elapidae: Serpentes)

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Abstract

Genetic analyses of Australasian organisms have resulted in the identification of extensive cryptic diversity across the continent. The venomous elapid snakes are among the best-studied organismal groups in this region, but many knowledge gaps persist: for instance, despite their iconic status, the species-level diversity among Australo-Papuan blacksnakes (*Pseudechis*) has remained poorly understood due to the existence of a group of cryptic species within the *P. australis* species complex, collectively termed "pygmy

mulga snakes". Using two mitochondrial and three nuclear loci we assess species boundaries within the genus using Bayesian species delimitation methods and reconstruct their phylogenetic history using multispecies coalescent approaches. Our analyses support the recognition of 10 species, including all of the currently described pygmy mulga snakes and one undescribed species from the Northern Territory of Australia. Phylogenetic relationships within the genus are broadly consistent with previous work, with the recognition of three major groups, the viviparous red-bellied black snake *P. porphyriacus* forming the sister species to two clades consisting of ovoviviparous species.

Introduction

Molecular phylogenetics has become an increasingly important tool for almost all biological disciplines. While the importance of understanding intrageneric phylogenies is widely accepted (e.g. Barraclough and Nee, 2001), Australia, often referred to as the 'land of the reptile', has lagged behind Northern Hemisphere landmasses in terms of phylogenetic exploration (Beheregaray, 2008). Despite much progress, significant knowledge gaps still remain in snakes.

The highly venomous blacksnakes (*Pseudechis* Wagler, 1830) are widespread across Australia and southern New Guinea and represent one of the most recognisable genera across much of this distribution. The monophyly of the genus had been questioned due to the lack of clear morphological synapomorphies (e.g. Greer, 1997), but hemipenial morphology (Keogh, 1999) and a multilocus molecular phylogeny of Australasian elapids (Sanders et al., 2008) provided substantial evidence supporting the recognition of the genus.

The first published phylogeny of the genus (Mengden et al., 1986) grouped the six species then recognised into 3 major clades: the viviparous *P. porphyriacus* (Shaw, 1794) constituted the sister group to two oviparous clades, one with 19 dorsal scale rows at midbody (*P. colleti* Boulenger 1902, *P. guttatus* De Vis 1905, and *P. papuanus* Peters & Doria, 1878), and its sister clade with 17 scale rows at midbody (*P. australis* (Gray, 1842) and *P. butleri* Smith, 1982). Subsequent mitochondrial (mtDNA) phylogenies (Kuch et al., 2005; Wüster et al., 2005) were largely congruent with that of Mengden et

al. (1986), but also provided evidence of deep divergences within *P. australis*, suggesting that this taxon may represent a multispecies complex, hereafter referred to as the *P. australis* complex (Kuch et al., 2005).

While most species of *Pseudechis* are restricted to specific regions of Australia or New Guinea, the *P. australis* complex is widespread across most of Australia and parts of southern New Guinea. This vast distribution and accompanying morphological variation have rendered this complex the subject of taxonomic confusion and controversy (Smith, 1982). Beside the large, widespread *P. australis* sensu stricto, multiple additional taxa have been described within the complex, but except for *P. butleri*, these additional species, *P. weigeli* (Wells & Wellington, 1987), *P. pailsi* (Hoser, 1998) and *P. rossignolii* (Hoser, 2000), collectively termed "pygmy mulga snakes", were described outside the peer-reviewed scientific literature (e.g. Wells and Wellington, 1987; Hoser 1998). As a result, most have only gained tentative and sporadic recognition by later authors (e.g. Williams et al., 2008; Cogger, 2014). However, several distinctive mitochondrial lineages within the *P. australis* complex appear to correspond to these taxa, suggesting that they may warrant recognition as separate species (Kuch et al., 2005). Nevertheless, there remains considerable uncertainty over the status and nomenclature of these taxa (e.g., Inagaki et al., 2010), not least due to the confusion created by their largely evidence-free naming in the unreviewed hobbyist or self-published literature (see Kaiser et al., 2013, for comments).

Although the deep mitochondrial divergences within the *P. australis* complex are suggestive of taxonomic diversity, mitochondrial data on their own are inadequate for species delimitation (e.g. Leaché and Fujita, 2010) due to the non-recombining, matrilineal mode of inheritance of this locus, and phylogenetic inference on this basis relies on the assumption that the mtDNA gene tree reflects the species tree. Increasingly multiple nuclear markers are being used in combination with mtDNA for species delimitation purposes as well as for phylogenetic reconstruction, due to their biparental inheritance and their evolutionary independence from mtDNA and each other. Advances in multispecies coalescent models (e.g. Heled and Drummond, 2010) has enabled multiple independent loci to be analysed simultaneously within a framework that weights

each locus equally and accounts for gene tree incongruence resulting from incomplete lineage sorting, something that traditional concatenation methods cannot do.

Due to the confusion surrounding the status of the pygmy mulga snakes and other members of the genus, this study aims to (i) clarify species boundaries within the *P. australis* complex, and (ii) employ a multi-locus approach to test past phylogenetic hypotheses for *Pseudechis*. We use an expanded mitochondrial phylogeny to identify candidate species and test these using recently developed Bayesian approaches (Yang and Rannala, 2010, 2014). The phylogenetic relationships among the identified species are then inferred using multispecies coalescent methods.

2. Materials and Methods

2.1. *Tissue collection and extraction*

Pseudechis specimens from all known species were sampled from across Australia and New Guinea (see table 1 and figure 2), and genomic DNA was extracted from tissue, blood or sloughed skin using a Qiagen DNeasy™ Tissue Kit according to the manufacturer's protocol.

2.2. *PCR amplification*

Sequences of two mitochondrial gene fragments (*nadh4* and *cytb*) and partial exon sequences of three single copy protein coding nuclear (nuDNA) genes (*prlr*, *ubn1* and *nt3*) were generated (see supplementary material 1 for primer details). PCRs were carried out in 11ul volumes with the following thermocycling conditions: denature at 94 °C for 2 minutes (min); then 35 (*ubn1, nt3* and *prlr*) or 40 (*nadh4* and *cytb*) cycles of [94 °C for 30 seconds (s); annealing at 48 °C (*cytb, nt3*), 50 °C (*ubn1* and *prlr*), or 54 °C (*nadh4*) for 30 seconds; 72 °C amplification for 45 s]; and a final extension of 72 °C for 5 min. Mitochondrial DNA was Sanger sequenced in the forward direction only and nuDNA in both directions by Macrogen Inc., South Korea.

2.3 *Sequence data preparation*

PHASE v. 2.1.1 (Stephens et al., 2001) was used to reconstruct individual alleles (haplotypes) from diploid nuclear sequences. Input files for PHASE were produced using

the online resource seqPHASE (Flot, 2010). For each locus, PHASE was executed three times at a random starting seed for 1000 iterations, a 10 thinning interval, and 100 burn-in. Each run was examined against the other two to test for mean frequency concordance, and the most similar to zero selected for further analysis. Sites with heterozygous probabilities of ≥0.7 were considered to be correctly called by PHASE, a standard employed in many phylogenetic studies.

The optimal partitioning strategy and evolutionary substitution models (for Bayesian inference (BI) were selected based on results using PartitionFinder (Lanfear et al., 2012) (supplementary material 2), from a total set of six possible partitions for mtDNA analyses comprising (codon positions 1, 2 and 3 from *nadh4* and *cytb*); and from four possible partitions for each locus for complete dataset analyses. The optimal partitioning strategy and evolutionary substitution models were identical across all optimality criteria (AIC, AICc and BIC).

2.4. *Mitochondrial phylogeny*

Acanthophis rugosus and Oxyuranus scutellatus were used as outgroups in the mtDNA analyses based on their relationships with *Pseudechis* (sister genus and a more distant outgroup respectively) (Sanders et al., 2008). All sequences were aligned using CodonCode Aligner v. 3.7.1 and checked for any unexpected indels or stop codons using MEGA5 (Tamura et al., 2011).

Maximum likelihood (ML) and BI methods were employed to infer phylogenetic relationships of the mitochondrial haplotypes. The dataset was first partitioned into four pertinent partitions based on suggestions from PartitionFinder (*nadh4* codon position (cp) 1; *nadh4* cp2 and *cytb* cp1; *nadh4* cp3 and *cytb* cp2; and *cytb* cp3). For ML, Randomized Axelerated Maximum Likelihood (RAxML) v.7.2.2 (Pfeiffer and Stamatakis, 2010) was used to estimate the ML tree under the GTR-gamma model and assess clade support using 500 bootstrap replicates using the GTR-CAT model. RAxML was run through the CIPRES Science Gateway v.3.1 (Miller et al., 2010). Data generated in this study was also compared with the *nadh4* sequence data from Kuch et al. (2005) and obtained through GenBank (see supplementary material 5 for further information).

MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) was implemented for BI and was run for 10^7 generations, with two independent parallel runs each with one cold and three heated chains each. Trees were sampled every 1000 generations. Convergence and sufficient parameter sampling was assessed using Tracer v.1.5 (available from http://beast.bio.ed.ac.uk/Tracer) by examining effective sample size of each parameter.

2.5. *Species delimitation*

To visualise patterns of haplotype sharing between candidate species, we constructed median-joining networks using the software Network v.4.6.11 (Fluxus-engineering.com).

The reversible-jump Markov Chain Monte Carlo (rjMCMC) method of Yang & Rannala (2010), implemented through the software BPP v.3 (Rannala and Yang, 2003; Yang and Rannala, 2014), was used to test whether the candidate species suggested by the mitochondrial gene tree are supported by the nuclear gene sequences. This method tests alternative speciation hypotheses while accommodating uncertainty in the species tree. Posterior probabilities are determined for different speciation models by collapsing (no speciation) or expanding (speciation) nodes of the species tree using rjMCMC sampling.

In any Bayesian analysis, the results may be strongly influenced by the priors. The key priors for Bayesian species delimitation are the ancestral population size (θ) and root age (τ_0). Both can affect the posterior probabilities for speciation models (Yang & Rannala, 2010). Since we lacked adequate prior knowledge of these parameters, we assessed the impact of different combinations of priors on the posterior speciation probabilities. We considered four different combinations of priors, both being assigned a gamma G(α, β) distribution, consisting of four combinations of small or large ancestral population sizes and shallow or deep divergences. Large and small ancestral population size estimates were modelled as $\theta \sim G(1,10)$ and $\theta \sim G(2,2000)$, respectively, and deep and shallow divergences as $\tau_0 \sim G(1,10)$ and $\tau_0 \sim G(2,2000)$ respectively (following the scheme of Leaché & Fujita, 2010). Priors assuming large ancestral population sizes and shallow divergences are more conservative in terms of numbers of species estimated (Yang & Rannala, 2010).

We used the clades found in the mitochondrial phylogeny as candidate species for species delimitation. We tested the following candidate species using this procedure: the universally recognised taxa *P. porphyriacus, P. colletti, P. guttatus, P. papuanus, P. butleri,* and *P. australis* sensu stricto, and four contentious taxa from the *P. australis* complex: the New Guinea pygmy mulga snake, *P. rossignolii* (Hoser, 2000), the Kimberley form described as *Cannia weigeli* Wells & Wellington (1987), the SW Queensland form described as *Pailsus pailsei* Hoser (1998) and a Northern Territory form of unclear nomenclatural status, here referred to as NT dwarf form (see Kaiser et al., 2013). BPP3 was run twice for each combination of tau and theta priors, using 100,000 generations, a burnin of 20,000 generations and a sampling frequency of 2.

2.6 *Multilocus coalescent species tree*

A species level phylogeny was produced using all sampled loci based on the results of the mtDNA analysis and the Bayesian species delimitation analysis using *BEAST (Heled and Drummond, 2010). The MCMC chain ran for 3×10^8 generations with sampling every 30,000 generations. The first 10% of reads were discarded as burn-in. The dataset was partitioned by locus and following preliminary runs that detected significant variation in evolutionary rate for all genes except *prlr* relaxed molecular clocks were used for all partitions apart from *prlr* where a strict clock was used. Evolutionary rates of the nuclear loci were estimated relative to the mitochondrial locus. A Yule-process prior was used for the species tree in conjunction with a piecewise linear and constant-root population-size model. Convergence and adequate sampling (ESS $\geq$ 200) of all parameters was verified using Tracer.

3. Results and Discussion

3.1. *Gene variability and mitochondrial phylogenetic relationships*

Within *Pseudechis, nadh4* (698bp) had 232 variable sites of which 159 were parsimony informative*; cytb* (709bp) had 214 variable sites of which 148 were parsimony informative*; prlr* (538bp) had 16 variable sites of which 11 were parsimony informative; *ubn1* (486bp) had 7 variable sites of which 3 were parsimony informative; and *nt3* (584bp) had 19 variable sites of which 13 were parsimony informative. The low

variability present in the nuclear datasets is unsurprising and is consistent with the recent radiation of the Australian elapid fauna (Sanders et al., 2008), yet despite this they provided phylogenetic signal in haplotype assemblages conforming to splitting between some of the major clades.

The mitochondrial dataset revealed the same major clades presented in Wüster et al. (2005) and the relationships within the *P. australis* complex presented by Kuch et al. (2005) (Fig. 1; supplementary material 5). In particular, the four mtDNA clades within the *P. australis* complex identified by Kuch et al. are represented in our mitochondrial gene tree, and provide the basis for the designation of candidate species for delimitation.

3.2 Species delimitation

Networks of nuclear haplotypes (Fig. 2) revealed patterns of shared alleles broadly consistent with the mitochondrial gene tree: *P. porphyriacus* group, *P. australis* group, and *P. papuanus* group all possess unique alleles. However, the constituent species of these clades frequently share alleles. *Pseudechis colletti* and *P. guttatus* share alleles and both of these also share alleles with *P. papuanus* in *ubn1*. Similarly, the sympatric *P. australis* and *P. butleri* share alleles with each other in *ubn1*. Since sharing of alleles occurs across the Torres Strait and between clearly distinct species that are sympatric, and thus likely to be largely reproductively isolated, it seems more likely that this represents the sharing of common ancestral alleles rather than the result of ongoing gene flow.

Although our sampling in this study is limited, our nuDNA data provides additional evidence on species limits within the genus, and in particular within the contentious *P. australis* complex. Bayesian species delimitation provided strong support for most of the candidate species, irrespective of tau and theta priors (Table 2). Overall the highest posterior support was for a 10 species model, thus supporting the status of all candidate species. The universally recognised taxa *P. porphyriacus*, *P. butleri*, *P. papuanus* and *P. australis* were consistently supported by speciation probabilities ≥ 0.99. The same is also true of the previously contentious taxon *P. rossignolii* from New Guinea. *Pseudechis colletti* and *P. guttatus* were somewhat less supported, with speciation probabilities of 0.89 in analyses where priors assumed a large ancestral

population size. The contentious taxon *P. pailsi*, from western Queensland, received similar support as *P. colletti* and *P. guttatus*. We regard all of these taxa as separate species based on the evidence presented here. The status of the Kimberley (*P. weigeli*) and NT forms was less certain: analyses with priors based on assumptions of large ancestral populations provided little support for separate species status for the two taxa, whereas analyses based on assumptions of small ancestral populations provided stronger support. Nevertheless, in all analyses, separate species status for both *P. weigeli* and the NT dwarf form was the option receiving the highest support. While we acknowledge the need of more data to fully resolve the status of *P. weigeli* and the NT pygmy mulga as separate species, we consider both to be distinct for coalescent species tree inference.

3.3 Multilocus phylogeny

The nuclear genes used here have provided novel evidence on the phylogeny of *Pseudechis*. The multilocus species tree (Fig. 1) is largely congruent with the mitochondrial phylogeny. The three major subclades of *Pseudechis* (Mengden et al., 1986; Wüster et al., 2005) and the relationships among them remain strongly supported. Within the *P. australis* complex, our data were unable to resolve relationships between the four main groups: *P. australis* sensu stricto, *P. butleri*, *P. rossigolii*, and the Australian pygmy mulga clade.

There was strong support for the sister species relationship of the Australian *P. colletti* with *P. guttatus*, with the Papuan *P. papuanus* forming their closest relatives. Within the strongly supported clade of species with 17 scale rows, our data revealed four major subclades: *P. australis* sensu stricto, *P. butleri*, *P rossignolii* and an Australian pygmy mulga clade containing *P. pailsi*, *P. weigeli* and the NT dwarf form (species A: Fig. 1). However, we were unable to resolve the relationships between these four subclades in the *P. australis* complex.

The nuDNA revealed a large amount of variation within *P. australis* sensu stricto (Fig. 2), which was not revealed by mtDNA alone (Kuch et al., 2005; Wüster et al., 2005; Fig. 1). This could be attributed to aridification changes (which occurred in the Pliocene from arid to mesic to arid) (see Byrne et al., 2008) or glacial cycling causing populations to retract to different refugia therefore separating populations. This would contradict the

theory of a single rapid range expansion during the Pleistocene (Kuch et al., 2005) as a sole explanation for the phylogeographic pattern observed, suggesting instead multiple instances of range expansion by several populations from different refugia after glacial cooling. The lack of variability in mt- compared to nuDNA may have been due to secondary introgression and fixation, possibly as a result of a selective sweep. Although this pattern has been commonly observed in other taxa and its occurrence in reptiles is documented reasonably well in lizards (e.g., Rato et al., 2010, 2011), only limited data is available for snakes (Barbanera et al., 2009). Alternatively, the observed pattern is also consistent with past diversification followed by more recent genetic drift or a short bottleneck (as per Kuch et al., 2005), resulting in reduced greater reduction of mtDNA haplotype diversity compared to nuDNA allele diversity due to the lower effective population size of the former. This further underscores the problems with solely utilising mtDNA in phylogenetic studies.

In conclusion, our multilocus phylogeny largely confirmed previous mitochondrial (Kuch et al., 2005; Wüster et al., 2005) and morphological phylogenies (Mengden et al., 1986) of *Pseudechis*, supporting the existence of three major clades within the genus. Our new nuclear data confirm the validity of separate species across several lineages that have previously been revealed by mtDNA phylogeographic analyses, but whose status had remained contentious due to a lack of other corroborating evidence. Based on the current body of evidence, our results suggest that 10 species of *Pseudechis* should be recognized pending further analysis.

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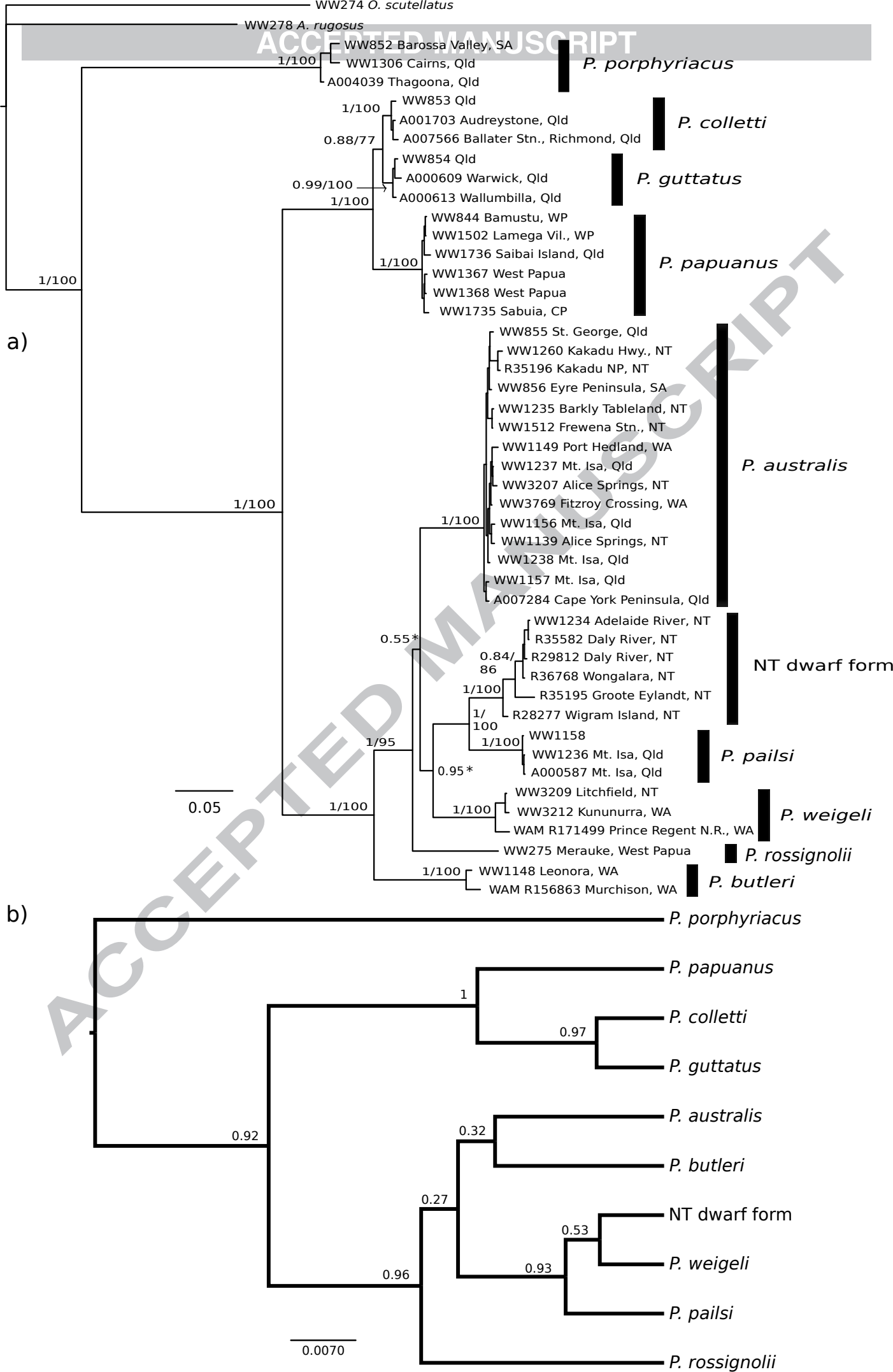
Table 1. Sample information and sequences generated in this study. Abbreviations in accession number refer to: NT = Northern Territory Museum; QM = Queensland Museum; WAM = Western Australia Museum; SAM = South Australian Museum. Locality abbreviations are as follows: NT = Northern Territory; QLD = Queensland; WA = Western Australia; SA = South Australia; PNG = Papua New Guinea and within PNG CP = Central Province and WP = Western Province.

			Locus				
Species	**Accession number**	**Locality**	**NADH4**	**CYTB**	**PRLR**	**UBN1**	**NT3**
NT dwarf form	NT R28277	Wigram Island, English Company Islands, NT	*				
NT dwarf form	NT R29812	Oolloo Road, Daly River, NT	*	*	*		*
NT dwarf form	NT R35195	Umbakumba, Groote Eylandt, NT	*	*			
NT dwarf form	NT R35582	Daly River, NT	*		*		*
NT dwarf form	NT R36768	Wongalara, NT	*	*	*		*
NT dwarf form	WW1234	Adelaide River, NT	*	*	*		*
P. australis	NT R35196	Kapalga, Kakadu NP, NT	*	*	*	*	*
P. australis	QM A007284	Heathlands Resource Reserve, Cape York Peninsula, QLD		*	*	*	*
P. australis	WW1139	Alice Springs, NT	*	*	*	*	*
P. australis	WW1149	Port Hedland, WA	*	*		*	*
P. australis	WW1157	Mt. Isa, QLD	*	*	*		*
P. australis	WW1235	Barkly Tableland, NT	*	*			*
P. australis	WW1237	Mt. Isa, QLD	*	*			*
P. australis	WW1238	Mt. Isa, QLD	*	*	*		*
P. australis	WW1260	Kakadu Hwy, NT	*	*			*
P. australis	WW1512	Frewena Station, NT	*	*			*
P. australis	WW3207	Alice Springs, NT	*	*	*	*	*
P. australis	WW855	St George, QLD	*	*	*	*	*
P. australis	WW856	Eyre Peninsula, SA	*	*	*	*	*
P. butleri	WAM R156863	Murchison, WA	*		*	*	*
P. butleri	WW1148	Leonora, WA	*	*	*		*
P. colletti	QM A001703	Audreystone, QLD	*	*	*		
P. colletti	QM A007566	Ballater Stn, 123 km SE Richmond, QLD	*				

P. colletti	WW853	QLD	*	*	*	*	
P. guttatus	QM A000609	Warwick, QLD	*		*	*	*
P. guttatus	QM A000613	Wallumbilla, QLD	*		*	*	*
P. guttatus	WW854	QLD	*	*	*	*	*
P. pailsi	QM A000587	Dajarra Road, Mt. Isa, QLD		*	*	*	*
P. pailsi	WW1158	Captive specimen	*	*	*	*	*
P. pailsi	WW1236	Mt. Isa, QLD	*	*	*	*	*
P. papuanus	WW1367	Papua, Indonesia	*	*	*	*	
P. papuanus	WW1368	Papua, Indonesia	*	*	*	*	*
P. papuanus	WW1502	Iamega Village, WP, PNG	*	*	*		
P. papuanus	WW1735	Sabuia, CP, PNG	*	*	*	*	*
P. papuanus	WW1736	Saibai Island, QLD	*	*	*	*	*
P. papuanus	WW844	Bamustu, WP, PNG	*	*	*	*	*
P. porphyriacus	QM A001270	Twin Falls, Paluma, QLD			*	*	*
P. porphyriacus	QM A004039	Karrabin-Rosewood Road, Thagoona, QLD	*	*	*		*
P. porphyriacus	WW852	Borossa Valley, SA	*	*	*	*	*
P. rossignolii	WW275	Merauke, Indonesia	*	*	*	*	*
P. rossignolii	WW4238	Captive specimen			*	*	*
P. rossignolii	WW4239	Captive specimen			*	*	*
P. weigeli	WAM R171499	Prince Regent Nature Reserve, Kimberley, WA	*		*	*	*
P. weigeli	WW3209	Litchfield, NT	*	*	*	*	*
P. weigeli	WW3212	Kununurra, WA	*	*	*	*	*

Table 2. Posterior probabilities for species status for candidate species of *Pseudechis* and different numbers of species under different tau and theta prior combinations. Only scores from the first run of each analysis are shown. BPP scores are given to two decimal places and alternatives receiving BPP ≤ 0.01 in all combinations are not shown. Complete scores for both runs of each of the BPP analysis are shown in Appendix 1.

	thetaprior = 2 2000 tauprior = 2 2000	**thetaprior = 1 10 tauprior = 1 10**	**thetaprior = 1 10 tauprior = 2 2000**	**thetaprior = 2 2000 tauprior = 1 10**
	Small ancestral population, shallow divergences	**Large ancestral population, deep divergences**	**Large ancestral population, shallow divergences**	**Small ancestral population, deep divergences**
australis	1	1	1	1
butleri	1	1	1	1
pailsi	0.99	0.84	0.95	0.98
*pailsi+*NT dwarf form	0.01	0.15	0.05	0.02
weigeli	0.94	0.73	0.61	0.92
*weigeli+*NT dwarf form	0.05	0.26	0.39	0.08
NT dwarf form	0.93	0.59	0.56	0.9
rossignolii	1	1	1	1
guttatus	0.98	0.9	0.93	0.98
guttatus+colletti	0.02	0.09	0.07	0.02
colletti	0.98	0.91	0.93	0.98
papuanus	1	1	1	1
porphyriacus	1	1	1	1
BPP for number of species				
P[7]		0.00	0.00	
P[8]	0.00	0.04	0.03	0.00
P[9]	0.08	0.43	0.45	0.12
P[10]	0.91	0.53	0.52	0.88



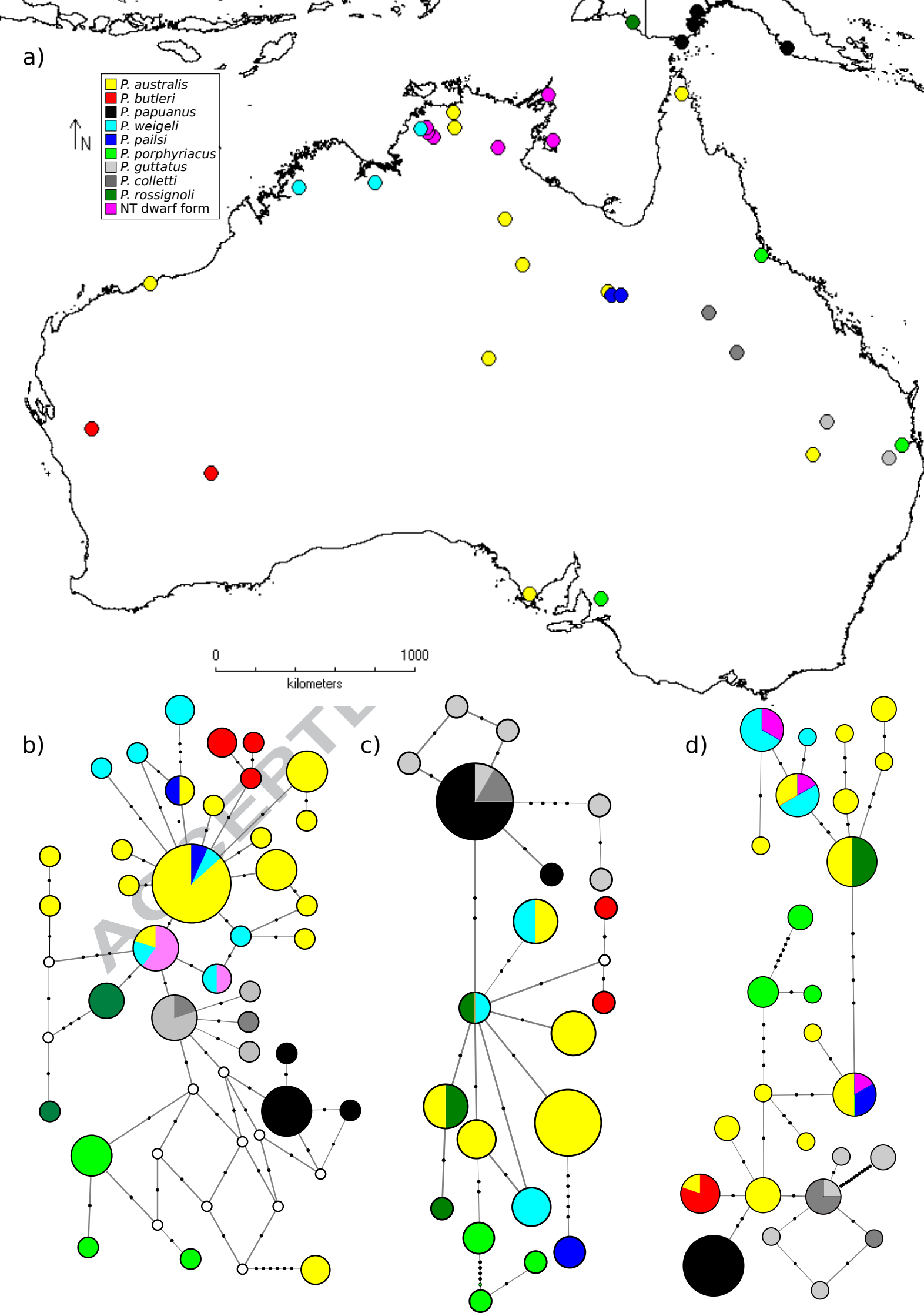

a)
P. australis
P. butleri
P. papuanus
P. weigeli
P. pailsi
P. porphyriacus
P. guttatus
P. colletti
P. rossignoli
NT dwarf form
N
0
1000
kilometers
b)
c)
d)

Fig. 1 Phylogeny of *Pseudechis*. (a) Phylogenetic tree of the relationships inferred from the mitochondrial dataset. Bayesian inference tree with maximum likelihood support values placed on (BI posterior clade probabilities / ML bootstrap percentages). Where there is a disagreement in topology between BI and ML analyses a '*' indicates this. The separate ML and BI trees are presented in supplementary material 4. (b) Bayesian species tree of the relationships within *Pseudechis* inferred using the multispecies coalescent in *BEAST. Support on branches is BI posterior clade probabilities. State abbreviations are as follows: WA = Western Australia, NT = Northern Territory, Qld = Queensland, SA = South Australia (Australia); WP = Western Province, CP = Central Province (Papua New Guinea).

Fig. 2 (a) Sampling map of *Pseudechis* used in this study. Colours used refer to individual species supported in the bpp analyses and are indicated in the key and are the same used in the nuclear haplotype networks; (b) haplotype network for the *nt3* locus; (c) haplotype network for the *ubn1* locus; (d) haplotype network for the *prlr* network. White circles indicate median vectors; black circles indicate mutational steps.

HIGHLIGHTS

- Species delimitation analyses support the recognition of 10 species of blacksnake (*Pseudechis*).
- One undescribed species of pygmy mulga snake from the Northern Territory of Australia was identified.
- Phylogenetic relationships support three major clades within the blacksnakes.