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

Simon T. Maddock [1,2,3,4,*], Aaron Childerstone [3], Bryan Grieg Fry [5], David J. Williams [6,7], Axel Barlow [3,8], Wolfgang Wüster [3]

[1] Department of Life Sciences, The Natural History Museum, London, SW7 5BD, UK.
[2] Department of Genetics, Evolution and Environment, University College London, London, WC1E 6BT, UK.
[3] School of Biological Sciences, Environment Centre Wales, Bangor University, Bangor, LL57 2UW, United Kingdom.
[4] Department of Animal Management, Reaseheath, College, Nantwich, Cheshire, CW5 6DF, UK.
[5] Venom Evolution Lab, School of Biological Sciences, University of Queensland, St Lucia QLD, 4072 Australia.
[6] Australian Venom Research Unit, Department of Pharmacology, University of Melbourne, Parkville, Vic, 3010, Australia.
[7] School of Medicine & Health Sciences, University of Papua New Guinea, Boroko, NCD, 121, Papua New Guinea.
[8] Institute for Biochemistry and Biology, University of Potsdam, 14476 Potsdam (Golm), Germany.

[*] corresponding author: s.t.maddock@gmail.com

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

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

66 While most species of *Pseudechis* are restricted to specific regions of Australia or
67 New Guinea, the *P. australis* complex is widespread across most of Australia and parts of
68 southern New Guinea. This vast distribution and accompanying morphological variation
69 have rendered this complex the subject of taxonomic confusion and controversy (Smith,
70 1982). Beside the large, widespread *P. australis* sensu stricto, multiple additional taxa
71 have been described within the complex, but except for *P. butleri*, these additional
72 species, *P. weigeli* (Wells & Wellington, 1987), *P. pailsi* (Hoser, 1998) and *P. rossignolii*
73 (Hoser, 2000), collectively termed "pygmy mulga snakes", were described outside the
74 peer-reviewed scientific literature (e.g. Wells and Wellington, 1987; Hoser 1998). As a
75 result, most have only gained tentative and sporadic recognition by later authors (e.g.
76 Williams et al., 2008; Cogger, 2014). However, several distinctive mitochondrial lineages
77 within the *P. australis* complex appear to correspond to these taxa, suggesting that they
78 may warrant recognition as separate species (Kuch et al., 2005). Nevertheless, there
79 remains considerable uncertainty over the status and nomenclature of these taxa (e.g.,
80 Inagaki et al., 2010), not least due to the confusion created by their largely evidence-free
81 naming in the unreviewed hobbyist or self-published literature (see Kaiser et al., 2013,
82 for comments).

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

93 each locus equally and accounts for gene tree incongruence resulting from incomplete
94 lineage sorting, something that traditional concatenation methods cannot do.
95 Due to the confusion surrounding the status of the pygmy mulga snakes and other
96 members of the genus, this study aims to (i) clarify species boundaries within the *P.*
97 *australis* complex, and (ii) employ a multi-locus approach to test past phylogenetic
98 hypotheses for *Pseudechis*. We use an expanded mitochondrial phylogeny to identify
99 candidate species and test these using recently developed Bayesian approaches (Yang and
100 Rannala, 2010, 2014). The phylogenetic relationships among the identified species are
101 then inferred using multispecies coalescent methods.
102
103 **2. Materials and Methods**
104 2.1. *Tissue collection and extraction*
105 *Pseudechis* specimens from all known species were sampled from across Australia and
106 New Guinea (see table 1 and figure 2), and genomic DNA was extracted from tissue,
107 blood or sloughed skin using a Qiagen DNeasy™ Tissue Kit according to the
108 manufacturer's protocol.
109
110 2.2. *PCR amplification*
111 Sequences of two mitochondrial gene fragments (*nadh4* and *cytb*) and partial exon
112 sequences of three single copy protein coding nuclear (nuDNA) genes (*prlr*, *ubn1* and
113 *nt3*) were generated (see supplementary material 1 for primer details). PCRs were carried
114 out in 11ul volumes with the following thermocycling conditions: denature at 94 °C for 2
115 minutes (min); then 35 (*ubn1, nt3* and *prlr*) or 40 (*nadh4* and *cytb*) cycles of [94 °C for
116 30 seconds (s); annealing at 48 °C (*cytb, nt3*), 50 °C (*ubn1* and *prlr*), or 54 °C (*nadh4*) for
117 30 seconds; 72 °C amplification for 45 s]; and a final extension of 72 °C for 5 min.
118 Mitochondrial DNA was Sanger sequenced in the forward direction only and nuDNA in
119 both directions by Macrogen Inc., South Korea.
120
121 2.3 *Sequence data preparation*
122 PHASE v. 2.1.1 (Stephens et al., 2001) was used to reconstruct individual alleles
123 (haplotypes) from diploid nuclear sequences. Input files for PHASE were produced using

124 the online resource seqPHASE (Flot, 2010). For each locus, PHASE was executed three
125 times at a random starting seed for 1000 iterations, a 10 thinning interval, and 100 burn-
126 in. Each run was examined against the other two to test for mean frequency concordance,
127 and the most similar to zero selected for further analysis. Sites with heterozygous
128 probabilities of ≥0.7 were considered to be correctly called by PHASE, a standard
129 employed in many phylogenetic studies.
130 The optimal partitioning strategy and evolutionary substitution models (for
131 Bayesian inference (BI) were selected based on results using PartitionFinder (Lanfear et
132 al., 2012) (supplementary material 2), from a total set of six possible partitions for
133 mtDNA analyses comprising (codon positions 1, 2 and 3 from *nadh4* and *cytb*); and from
134 four possible partitions for each locus for complete dataset analyses. The optimal
135 partitioning strategy and evolutionary substitution models were identical across all
136 optimality criteria (AIC, AICc and BIC).
137
138 2.4. *Mitochondrial phylogeny*
139 *Acanthophis rugosus and Oxyuranus scutellatus* were used as outgroups in the mtDNA
140 analyses based on their relationships with *Pseudechis* (sister genus and a more distant
141 outgroup respectively) (Sanders et al., 2008). All sequences were aligned using
142 CodonCode Aligner v. 3.7.1 and checked for any unexpected indels or stop codons using
143 MEGA5 (Tamura et al., 2011).
144 Maximum likelihood (ML) and BI methods were employed to infer phylogenetic
145 relationships of the mitochondrial haplotypes. The dataset was first partitioned into four
146 pertinent partitions based on suggestions from PartitionFinder (*nadh4* codon position (cp)
147 1; *nadh4* cp2 and *cytb* cp1; *nadh4* cp3 and *cytb* cp2; and *cytb* cp3). For ML, Randomized
148 Axelerated Maximum Likelihood (RAxML) v.7.2.2 (Pfeiffer and Stamatakis, 2010) was
149 used to estimate the ML tree under the GTR-gamma model and assess clade support
150 using 500 bootstrap replicates using the GTR-CAT model. RAxML was run through the
151 CIPRES Science Gateway v.3.1 (Miller et al., 2010). Data generated in this study was
152 also compared with the *nadh4* sequence data from Kuch et al. (2005) and obtained
153 through GenBank (see supplementary material 5 for further information).

154 MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) was implemented for BI and
155 was run for 10^7 generations, with two independent parallel runs each with one cold and
156 three heated chains each. Trees were sampled every 1000 generations. Convergence and
157 sufficient parameter sampling was assessed using Tracer v.1.5 (available from
158 http://beast.bio.ed.ac.uk/Tracer) by examining effective sample size of each parameter.
159
160 2.5. *Species delimitation*
161 To visualise patterns of haplotype sharing between candidate species, we
162 constructed median-joining networks using the software Network v.4.6.11 (Fluxus-
163 engineering.com).
164 The reversible-jump Markov Chain Monte Carlo (rjMCMC) method of Yang &
165 Rannala (2010), implemented through the software BPP v.3 (Rannala and Yang, 2003;
166 Yang and Rannala, 2014), was used to test whether the candidate species suggested by
167 the mitochondrial gene tree are supported by the nuclear gene sequences. This method
168 tests alternative speciation hypotheses while accommodating uncertainty in the species
169 tree. Posterior probabilities are determined for different speciation models by collapsing
170 (no speciation) or expanding (speciation) nodes of the species tree using rjMCMC
171 sampling.
172 In any Bayesian analysis, the results may be strongly influenced by the priors.
173 The key priors for Bayesian species delimitation are the ancestral population size (θ) and
174 root age (τ_0). Both can affect the posterior probabilities for speciation models (Yang &
175 Rannala, 2010). Since we lacked adequate prior knowledge of these parameters, we
176 assessed the impact of different combinations of priors on the posterior speciation
177 probabilities. We considered four different combinations of priors, both being assigned a
178 gamma $G(\alpha, \beta)$ distribution, consisting of four combinations of small or large ancestral
179 population sizes and shallow or deep divergences. Large and small ancestral population
180 size estimates were modelled as $\theta \sim G(1,10)$ and $\theta \sim G(2,2000)$, respectively, and deep
181 and shallow divergences as $\tau_0 \sim G(1,10)$ and $\tau_0 \sim G(2,2000)$ respectively (following the
182 scheme of Leaché & Fujita, 2010). Priors assuming large ancestral population sizes and
183 shallow divergences are more conservative in terms of numbers of species estimated
184 (Yang & Rannala, 2010).

185 We used the clades found in the mitochondrial phylogeny as candidate species for
186 species delimitation. We tested the following candidate species using this procedure: the
187 universally recognised taxa *P. porphyriacus*, *P. colletti*, *P. guttatus*, *P. papuanus*, *P.*
188 *butleri*, and *P. australis* sensu stricto, and four contentious taxa from the *P. australis*
189 complex: the New Guinea pygmy mulga snake, *P. rossignolii* (Hoser, 2000), the
190 Kimberley form described as *Cannia weigeli* Wells & Wellington (1987), the SW
191 Queensland form described as *Pailsus pailsei* Hoser (1998) and a Northern Territory
192 form of unclear nomenclatural status, here referred to as NT dwarf form (see Kaiser et al.,
193 2013). BPP3 was run twice for each combination of tau and theta priors, using 100,000
194 generations, a burnin of 20,000 generations and a sampling frequency of 2.
195
196 2.6 *Multilocus coalescent species tree*
197 A species level phylogeny was produced using all sampled loci based on the
198 results of the mtDNA analysis and the Bayesian species delimitation analysis using
199 *BEAST (Heled and Drummond, 2010). The MCMC chain ran for 3 x 10^8 generations
200 with sampling every 30,000 generations. The first 10% of reads were discarded as burn-
201 in. The dataset was partitioned by locus and following preliminary runs that detected
202 significant variation in evolutionary rate for all genes except *prlr* relaxed molecular
203 clocks were used for all partitions apart from *prlr* where a strict clock was used.
204 Evolutionary rates of the nuclear loci were estimated relative to the mitochondrial locus.
205 A Yule-process prior was used for the species tree in conjunction with a piecewise linear
206 and constant-root population-size model. Convergence and adequate sampling (ESS ≥
207 200) of all parameters was verified using Tracer.
208
209 **3. Results and Discussion**
210 3.1. *Gene variability and mitochondrial phylogenetic relationships*
211 Within *Pseudechis*, *nadh4* (698bp) had 232 variable sites of which 159 were parsimony
212 informative; *cytb* (709bp) had 214 variable sites of which 148 were parsimony
213 informative; *prlr* (538bp) had 16 variable sites of which 11 were parsimony informative;
214 *ubn1* (486bp) had 7 variable sites of which 3 were parsimony informative; and *nt3*
215 (584bp) had 19 variable sites of which 13 were parsimony informative. The low

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

220 The mitochondrial dataset revealed the same major clades presented in Wüster et
221 al. (2005) and the relationships within the *P. australis* complex presented by Kuch et al.
222 (2005) (Fig. 1; supplementary material 5). In particular, the four mtDNA clades within
223 the *P. australis* complex identified by Kuch et al. are represented in our mitochondrial
224 gene tree, and provide the basis for the designation of candidate species for delimitation.
225

226 3.2 Species delimitation

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

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

247 population size. The contentious taxon *P. pailsi*, from western Queensland, received
248 similar support as *P. colletti* and *P. guttatus*. We regard all of these taxa as separate
249 species based on the evidence presented here. The status of the Kimberley (*P. weigeli*)
250 and NT forms was less certain: analyses with priors based on assumptions of large
251 ancestral populations provided little support for separate species status for the two taxa,
252 whereas analyses based on assumptions of small ancestral populations provided stronger
253 support. Nevertheless, in all analyses, separate species status for both *P. weigeli* and the
254 NT dwarf form was the option receiving the highest support. While we acknowledge the
255 need of more data to fully resolve the status of *P. weigeli* and the NT pygmy mulga as
256 separate species, we consider both to be distinct for coalescent species tree inference.
257
258 3.3 Multilocus phylogeny
259 The nuclear genes used here have provided novel evidence on the phylogeny of
260 *Pseudechis*. The multilocus species tree (Fig. 1) is largely congruent with the
261 mitochondrial phylogeny. The three major subclades of *Pseudechis* (Mengden et al.,
262 1986; Wüster et al., 2005) and the relationships among them remain strongly supported.
263 Within the *P. australis* complex, our data were unable to resolve relationships between
264 the four main groups: *P. australis* sensu stricto, *P. butleri*, *P. rossigolii*, and the
265 Australian pygmy mulga clade.
266 There was strong support for the sister species relationship of the Australian *P.*
267 *colletti* with *P. guttatus*, with the Papuan *P. papuanus* forming their closest relatives.
268 Within the strongly supported clade of species with 17 scale rows, our data revealed four
269 major subclades: *P. australis* sensu stricto, *P. butleri*, *P rossignolii* and an Australian
270 pygmy mulga clade containing *P. pailsi*, *P. weigeli* and the NT dwarf form (species A:
271 Fig. 1). However, we were unable to resolve the relationships between these four
272 subclades in the *P. australis* complex.
273 The nuDNA revealed a large amount of variation within *P. australis* sensu stricto
274 (Fig. 2), which was not revealed by mtDNA alone (Kuch et al., 2005; Wüster et al., 2005;
275 Fig. 1). This could be attributed to aridification changes (which occurred in the Pliocene
276 from arid to mesic to arid) (see Byrne et al., 2008) or glacial cycling causing populations
277 to retract to different refugia therefore separating populations. This would contradict the

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

291 In conclusion, our multilocus phylogeny largely confirmed previous
292 mitochondrial (Kuch et al., 2005; Wüster et al., 2005) and morphological phylogenies
293 (Mengden et al., 1986) of *Pseudechis*, supporting the existence of three major clades
294 within the genus. Our new nuclear data confirm the validity of separate species across
295 several lineages that have previously been revealed by mtDNA phylogeographic
296 analyses, but whose status had remained contentious due to a lack of other corroborating
297 evidence. Based on the current body of evidence, our results suggest that 10 species of
298 *Pseudechis* should be recognized pending further analysis.
299

300 **Acknowledgements**

301 For providing and facilitating the acquisition of tissue samples we thank P. Couper, P.
302 Doughty, C. Hay, R. Mastenbroek, P. Mirtschin, S. Richards, W. Schneyer, S. Trim and
303 VenomTech, J. Wilmer, and P. Rowley and Liverpool School of Tropical Medicine. We
304 also thank W. Grail for advice during lab work. This study was financed in part by Grant
305 APSF 07/1 from the Australia & Pacific Science Foundation, and Royal Society
306 International Outgoing Short Visit grant 2006/R2 to WW.

307
308

References

1. Barbanera, F., Zuffi, M.A.L., Guerrini, M., Gentilli, A., Tofanelli, S., Fasola, M., Dini, F., 2009. Molecular phylogeography of the asp viper *Vipera aspis* (Linneaus, 1758) in Italy: Evidence for introgressive hybridisation and mitochondrial DNA capture. Mol. Phylogenet. Evol. 52, 103-114.

2. Barraclough, T. G., & Nee, S. (2001). Phylogenetics and speciation. Trends Ecol. Evol. 16, 391-399.

3. Beheregaray, L.B., 2008. Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. Mol. Ecol. 17, 3754–3774.

4. Byrne, M., Yeates, D.K., Joseph, L., Kearney, M., Bowler, J., Williams, M.A.J., Cooper, S., Donnellan, S.C., Keogh, J.S., Leys, R., Melville, J., Murphy, D.J., Porch, N., Wyrwool, K-H., 2008. Birth of a biome: insights into the assembly and maintenance of the Australian arid zone biota. Mol. Ecol. 17, 4398-4417.

5. Cogger, H.G., 2014. Reptiles and amphibians of Australia, 7th edition. New Holland, Sydney.

6. Flot, J.F., 2010. SeqPHASE: a web tool for interconverting PHASE input/output files and FASTA sequence alignments. Mol. Ecol. Resour. 10, 162-166.

7. Greer, A.E., 1997. The Biology and Evolution of Australian Snakes. Surrey Beatty and Sons Pty Limited, Chipping Norton, NSW.

8. Heled, J., Drummond, A.J., 2010. Bayesian inference of species trees from multilocus data. Mol. Biol. Evol. 27, 570–80.

338 9. Hoser, R., 1998. A new snake from Queensland, Australia (Serpentes: Elapidae).
339 Monitor. 10, 5-9.

340 10. Inagaki, H., Yamauchi, Y., Toriba, M., Kubo, T., 2010. Regional divergence of
341 phospholipase A_2-like protein cDNAs between New Guinean and Australian
342 *Pseudechis australis*. Toxicon 56, 637-639.
343
344 11. Kaiser, H., Crother, B.I., Kelly, C.M.R., Luiselli, L., O'Shea, M., Ota, H., Passos,
345 P., Schleip, W.D., Wüster, W. 2013. Best practices: In the 21st century, taxonomic
346 decisions in herpetology are acceptable only when supported by a body of
347 evidence and published via peer-review. Herpetol. Rev. 44, 8–23.
348
349 12. Keogh, J.S., 1999. Evolutionary implications of hemipenial morphology in the
350 terrestrial Australian elapid snakes. J. Linn. Soc. London, Zool. 125, 239–278.
351
352 13. Kuch, U., Keogh, J.S., Weigel, J., Smith, L.A., Mebs, D., 2005. Phylogeography
353 of Australia's king brown snake (*Pseudechis australis*) reveals Pliocene
354 divergence and Pleistocene dispersal of a top predator. Naturwissenschaften, 92,
355 121–127.

356 14. Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S., 2012. PartitionFinder:
357 combined selection of partitioning schemes and substitution models for
358 phylogenetic analyses. Mol. Biol. Evol. 29, 1695–1701.

359 15. Leaché, A.D., Fujita, M.K., 2010. Bayesian species delimitation in West African
360 forest geckos (*Hemidactylus fasciatus*). Proc. Biol. Sci. 277, 3071-3077.

361 16. Mengden, G.A., Shine, R., Moritz, C., 1986. Phylogenetic relationships within the
362 Australian venomous snakes of the genus *Pseudechis*. Herpetologica 42, 215–229.

363 17. Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science
364 Gateway for inference of large phylogenetic trees, in: Proceedings of the Gateway
365 Computing Environments Workshop (GCE). New Orleans, pp. 1–8.

366 18. Pfeiffer, W., and Stamatakis, A. 2010. Hybrid MPI/Pthreads Parallelization of the
367 RAxML Phylogenetics Code. In: Ninth IEEE International Workshop on High
368 Performance Computational Biology (HiCOMB 2010) Atlanta.
369
370 19. Rato, C., Carranza, S., Perera, A., Carretero, M.A., Harris, D.J., 2010. Conflicting
371 patterns of nucleotide diversity between mtDNA and nDNA in the Moorish
372 gecko, *Tarentola mauritanica*. Mol. Phylogenet. Evol. 56, 962-971.
373
374 20. Rato, C., Carranza, S., Harris, D.J., 2011. When selection deceives
375 phylogeographic interpretation: The case of the Mediterranean house gecko,
376 *Hemidactylus turcicus* (Linnaeus, 1758). Mol. Phylogenet. Evol. 58, 365-373.
377
378 21. Ronquist, F. and Huelsenbeck J.P. 2003. MrBayes 3: Bayesian Phylogenetic
379 Inference under mixed models. Bioinformatics, 19, 1572-1574.
380
381 22. Sanders, K.L., Lee, M.S.Y., Leys, R., Foster, R., Keogh, J.S., 2008. Molecular
382 phylogeny and divergence dates for Australasian elapids and sea snakes
383 (Hydrophiinae): evidence from seven genes for rapid evolutionary radiations. J.
384 Evol. Biol. 21, 682-695.
385
386 23. Smith, L.A., 1982. Variation in *Pseudechis australis* (Serpentes: Elapidae) in
387 Western Australia and description of a new species of *Pseudechis*. Rec. West.
388 Aust. Mus. 10, 35-45.
389
390 24. Stephens, M., Smith, N., Donnelly, P., 2001. A new statistical method for
391 haplotype reconstruction from population data. Am. J. Hum. Genet. 68, 978-989.
392
393 25. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011.
394 MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood,
395 Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28,
396 2731-2739.

397 26. Wells, R.W., Wellington, C.R., 1987. A new species of proteroglyphous snake
398 (Serpentes: Oxyuranidae) from Australia. Australian Herpetologist. 503, 1-8.

399 27. Williams, D.J., O'Shea, M., Daguerre, R.L., Pook, C.E., Wüster, W., Hayden,
400 C.J., McVay, J.D., Paiva, O., Matainaho, T., Winkel, K.D., Austin, C.C., 2008.
401 Origin of the eastern brownsnake, *Pseudonaja textilis* (Duméril, Bibron and
402 Duméril) (Serpentes: Elapidae: Hydrophiinae) in New Guinea: evidence of
403 multiple dispersals from Australia, and comments on the status of *Pseudonaja*
404 *textilis pughi* Hoser 2003. Zootaxa 1703, 47-61.
405
406 28. Wüster, W., Dumbrell, A.J., Hay, C., Pook, C.E., Williams, D.J., Fry, B.G., 2005.
407 Snakes across the Strait: trans-Torresian phylogeographic relationships in three
408 genera of Australasian snakes (Serpentes: Elapidae: *Acanthophis*, *Oxyuranus,* and
409 *Pseudechis*). Mol. Phylo. Evol. 34, 1-14.
410
411 29. Yang, Z. and Rannala, B., 2010. Bayesian species delimitation using multilocus
412 sequence data. Proc. Natl. Acad. Sci. USA, 107, 9264-9269
413
414 30. Yang, Z. and Rannala, B., 2014. Unguided species delimitation using DNA
415 sequence data from multiple loci. Mol. Biol. Evol. 31:3125–3135

416
417
418
419
420

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

Species	Accession number	Locality	Locus				
			NADH4	CYTB	PRLR	UBN1	NT3
NT dwarf form	NT R28277	Wigram Island, English Company Islands, NT	KX981683				
NT dwarf form	NT R29812	Oolloo Road, Daly River, NT	KX981667	KX981640	KX981721		KX981783
NT dwarf form	NT R35195	Umbakumba, Groote Eylandt, NT	KX981682				
NT dwarf form	NT R35582	Daly River, NT	KX981668	KX981641	KX981722		KX981784
NT dwarf form	NT R36768	Wongalara, NT	KX981666	KX981639	KX981720		KX981782
NT dwarf form	WW1234	Adelaide River, NT	KX981654	KX981623	KX981732		KX981762
P. australis	NT R35196	Kapalga, Kakadu NP, NT	KX981665	KX981638	KX981719	KX981689	KX981781
P. australis	QM A007284	Heathlands Resource Reserve, Cape York Peninsula, QLD		KX981637	KX981717	KX981687	KX981779
P. australis	WW1139	Alice Springs, NT	KX981648	KX981617	KX981729	KX981709	KX981757
P. australis	WW1149	Port Hedland, WA	KX981647	KX981615		KX981706	KX981759
P. australis	WW1157	Mt. Isa, QLD	KX981678	KX981616		KX981727	KX981760
P. australis	WW1235	Barkly Tableland, NT	KX981653	KX981622			KX981763
P. australis	WW1237	Mt. Isa, QLD	KX981650	KX981619			KX981765
P. australis	WW1238	Mt. Isa, QLD	KX981651	KX981620	KX981730		KX981766
P. australis	WW1260	Kakadu Hwy, NT	KX981649	KX981618			KX981767
P. australis	WW1512	Frewena Station, NT	KX981655	KX981627			KX981769
P. australis	WW3207	Alice Springs, NT	KX981658	KX981630	KX981746	KX981707	KX981772
P. australis	WW855	St George, QLD	KX981672		KX981737	KX981705	KX981755
P. australis	WW856	Eyre Peninsula, SA	KX981676		KX981747	KX981697	KX981756
P. butleri	WAM R156863	Murchison, WA	KX981669		KX981725	KX981692	KX981787

P. butleri	WW1148	Leonora, WA	KX981680	KX981626	KX981739		KX981758
P. colletti	QM A001703	Audreystone, QLD	KX981663	KX981636	KX981716		
P. colletti	QM A007566	Ballater Stn, 123 km SE Richmond, QLD	KX981664				
P. colletti	WW853	QLD	KX981673		KX981749	KX981704	KX981753
P. guttatus	QM A000609	Warwick, QLD	KX981662		KX981715	KX981686	KX981778
P. guttatus	QM A000613	Wallumbilla, QLD	KX981661		KX981714	KX981685	KX981777
P. guttatus	WW854	QLD	KX981675		KX981748	KX981699	KX981754
P. pailsi	QM A000587	Dajarra Road, Mt. Isa, QLD		KX981634	KX981712	KX981684	KX981775
P. pailsi	WW1158	Captive specimen	KX981677	KX981624	KX981731	KX981710	KX981761
P. pailsi	WW1236	Mt. Isa, QLD	KX981679	KX981625	KX981738	KX981711	KX981764
P. papuanus	WW1367	Papua, Indonesia	KX981644	KX981610	KX981740	KX981700	
P. papuanus	WW1368	Papua, Indonesia	KX981645	KX981611	KX981741	KX981698	KX981768
P. papuanus	WW1502	Iamega Village, WP, PNG	KX981657	KX981629	KX981742		
P. papuanus	WW1735	Sabuia, CP, PNG	KX981646	KX981612	KX981743	KX981701	KX981770
P. papuanus	WW1736	Saibai Island, QLD	KX981656	KX981628	KX981744	KX981702	KX981771
P. papuanus	WW844	Bamustu, WP, PNG	KX981643	KX981609	KX981745	KX981703	KX981751
P. porphyriacus	QM A001270	Twin Falls, Paluma, QLD			KX981718	KX981688	KX981780
P. porphyriacus	QM A004039	Karrabin-Rosewood Road, Thagoona, QLD	KX981681	KX981635	KX981713		KX981776
P. porphyriacus	WW852	Borossa Valley, SA	KX981642	KX981605	KX981733	KX981694	KX981752
P. rossignolii	WW275	Merauke, Indonesia	KX981652	KX981621	KX981736	KX981708	KX981750
P. rossignolii	WW4238	Captive specimen			KX981723	KX981690	KX981785
P. rossignolii	WW4239	Captive specimen			KX981724	KX981691	KX981786
P. weigeli	WAM R171499	Prince Regent Nature Reserve, Kimberley, WA	KX981670		KX981726	KX981693	KX981788
P. weigeli	WW3209	Litchfield, NT	KX981659	KX981631	KX981734	KX981695	KX981773
P. weigeli	WW3212	Kununurra, WA	KX981660	KX981632	KX981735	KX981696	KX981774

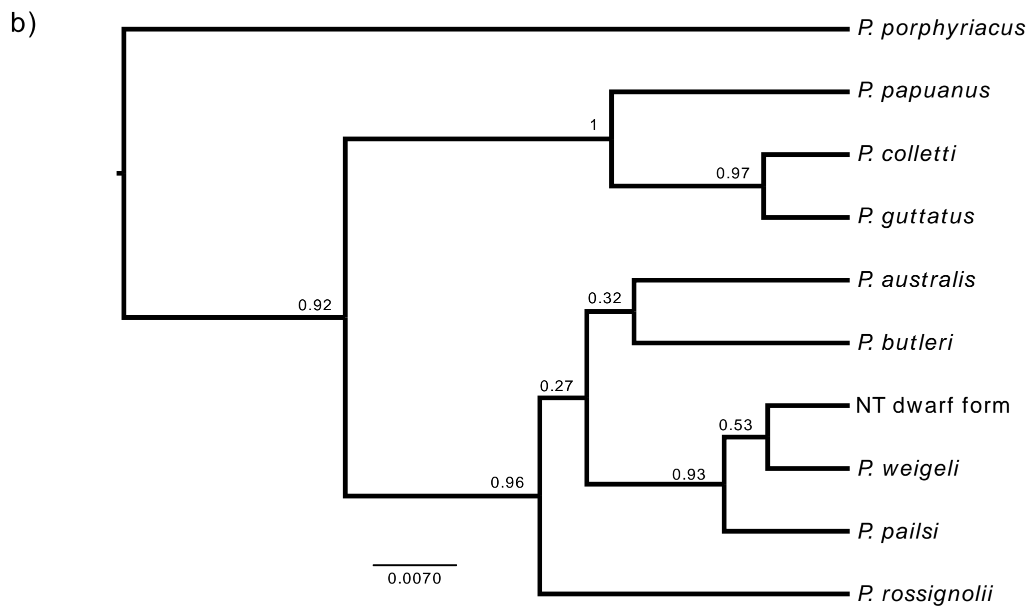
425
426

427 **Table 2.** Posterior probabilities for species status for candidate species of *Pseudechis* and different numbers of species under different tau and
428 theta prior combinations. Only scores from the first run of each analysis are shown. BPP scores are given to two decimal places and alternatives
429 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.
430

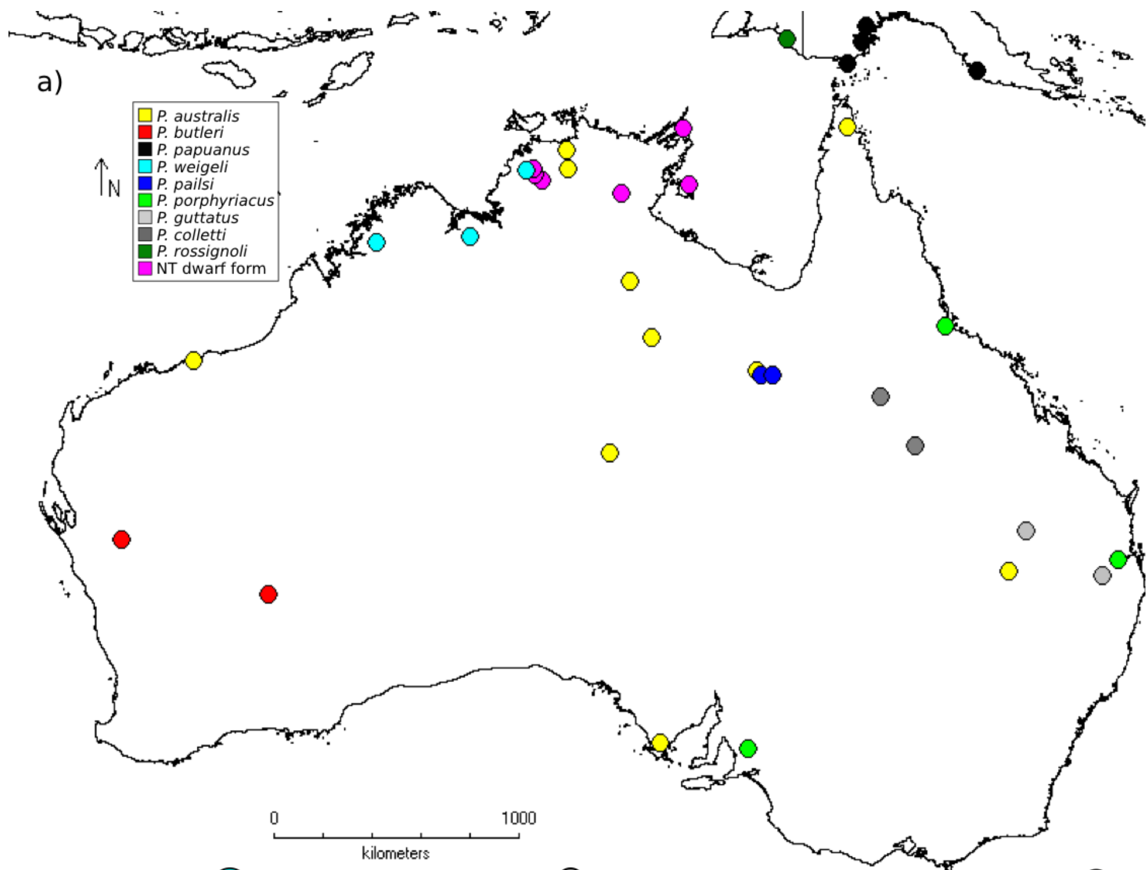
	thetaprior = 2 2000 tauprior = 2 2000	**thetaprior = 1 10 tauprior = 1 10**	**thetaprior = 1 10 tauprior = 2 2000**	**thetaprior = 2 2000 tauprior = 1 10**
	Small ancestral population, shallow divergences	**Large ancestral population, deep divergences**	**Large ancestral population, shallow divergences**	**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

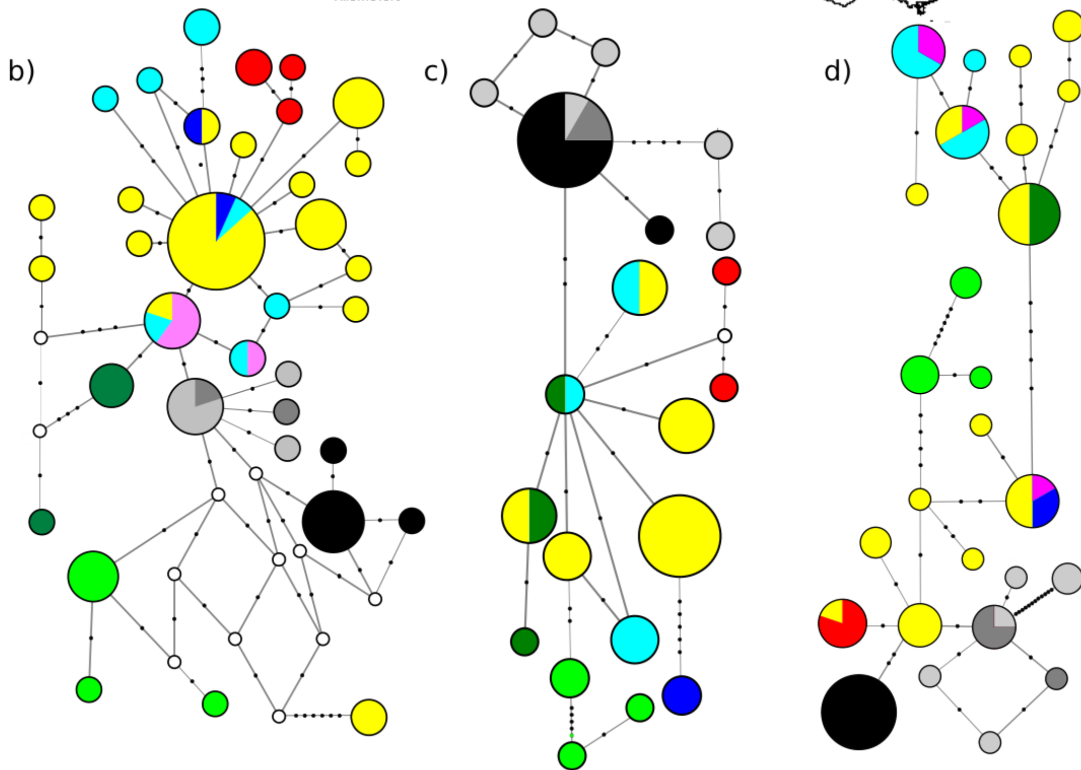
431 **Fig. 1** Phylogeny of *Pseudechis*. (a) Phylogenetic tree of the relationships inferred from
432 the mitochondrial dataset. Bayesian inference tree with maximum likelihood support
433 values placed on (BI posterior clade probabilities / ML bootstrap percentages). Where
434 there is a disagreement in topology between BI and ML analyses a '*' indicates this.
435 The separate ML and BI trees are presented in supplementary material 4. (b) Bayesian
436 species tree of the relationships within *Pseudechis* inferred using the multispecies
437 coalescent in *BEAST. Support on branches is BI posterior clade probabilities. State
438 abbreviations are as follows: WA = Western Australia, NT = Northern Territory, Qld =
439 Queensland, SA = South Australia (Australia); WP = Western Province, CP = Central
440 Province (Papua New Guinea).
441
442 **Fig. 2** (a) Sampling map of *Pseudechis* used in this study. Colours used refer to
443 individual species supported in the bpp analyses and are indicated in the key and are the
444 same used in the nuclear haplotype networks; (b) haplotype network for the *nt3* locus;
445 (c) haplotype network for the *ubn1* locus; (d) haplotype network for the *prlr* network.
446 White circles indicate median vectors; black circles indicate mutational steps.
447
448

WW274 *O. scutellatus*
WW278 *A. rugosus*
WW852 Barossa Valley, SA
WW1306 Cairns, Qld
A004039 Thagoona, Qld
P. porphyriacus
1/100
WW853 Qld
A001703 Audreystone, Qld
A007566 Ballater Stn., Richmond, Qld
P. colletti
1/100
0.88/77
WW854 Qld
A000609 Warwick, Qld
A000613 Wallumbilla, Qld
P. guttatus
0.99/100
1/100
WW844 Bamustu, WP
WW1502 Lamega Vil., WP
WW1736 Saibai Island, Qld
WW1367 West Papua
WW1368 West Papua
WW1735 Sabuia, CP
P. papuanus
1/100
1/100
a)
WW855 St. George, Qld
WW1260 Kakadu Hwy., NT
R35196 Kakadu NP, NT
WW856 Eyre Peninsula, SA
WW1235 Barkly Tableland, NT
WW1512 Frewena Stn., NT
WW1149 Port Hedland, WA
WW1237 Mt. Isa, Qld
WW3207 Alice Springs, NT
WW3769 Fitzroy Crossing, WA
WW1156 Mt. Isa, Qld
WW1139 Alice Springs, NT
WW1238 Mt. Isa, Qld
WW1157 Mt. Isa, Qld
A007284 Cape York Peninsula, Qld
P. australis
1/100
1/100
0.55*
WW1234 Adelaide River, NT
R35582 Daly River, NT
R29812 Daly River, NT
R36768 Wongalara, NT
R35195 Groote Eylandt, NT
R28277 Wigram Island, NT
NT dwarf form
0.84/86
1/100
1/100
WW1158
WW1236 Mt. Isa, Qld
A000587 Mt. Isa, Qld
P. pailsi
1/100
1/95
0.95*
WW3209 Litchfield, NT
WW3212 Kununurra, WA
WAM R171499 Prince Regent N.R., WA
P. weigeli
1/100
0.05
1/100
WW275 Merauke, West Papua
P. rossignolii
WW1148 Leonora, WA
WAM R156863 Murchison, WA
P. butleri
1/100
b)



449
450





451