Title: Recent rapid speciation and ecomorph divergence in Indo-Australian sea snakes

Running title: Ecomorph evolution and speciation in sea snakes

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Abstract

The viviparous sea snakes (Hydrophiinae) are a young radiation comprising at least 62 species that display spectacular morphological diversity and high levels of local sympatry. To shed light on the mechanisms underlying sea snake diversification, we investigated recent speciation and eco-morphological differentiation in a clade of four nominal species with overlapping ranges in Southeast Asia and Australia. Analyses of morphology and stomach contents identified the presence of two distinct ecomorphs: a 'macrocephalic' ecomorph that reaches >2m in length, has a large head, and feeds on crevice-dwelling eels and gobies; and a 'microcephalic' ecomorph that rarely exceeds 1m in length, has a small head and narrow fore-body, and hunts snake eels in burrows. Individual assignment based on newly developed microsatellites separated 52 co-distributed specimens into four significantly differentiated clusters corresponding to morphological species designations, indicating limited recent gene flow and progress towards speciation. A coalescent species tree (based on mitochondrial and nuclear sequences) and isolation-migration model (mitochondrial and microsatellite markers) suggest between one and three transitions between ecomorphs within the last ~1.2 million to ~840,000 years. In particular, the large-headed 'eastern' population of *H. cyanocinctus* and small-headed *H. melanocephalus* appear to have diverged very recently and rapidly, resulting in major phenotypic differences and restriction of gene flow in sympatry. These results highlight the viviparous sea snakes as a promising system for speciation studies in the marine environment.

Key words: marine speciation, ecomorph evolution, sea snake, *Hydrophis*, Southeast Asia, Australia

Introduction

Ecological speciation occurs when barriers to gene flow arise as a direct correlate of adaptation to divergent resource environments (Funk 1998; Schluter 2000). Evidence of this process has been found in a range of natural systems: reproductive isolation has been attributed to divergent selection on nuptial coloration in cichlid fish (e.g. Seehausen et al. 2008), host choice in phytophagous insects (Feder et al. 1994; Nosil et al. 2002), feeding morphology in Galapagos finches (Grant 1986; 1993) and stickleback fishes (Schluter 1994; Rundle et al. 2000), and mimetic wing patterns in butterflies (Jiggins 2008). These studies and laboratory experiments using *Drosophila* and yeast (Rice & Hostert 1993; Dettman et al. 2007) have shown that reproductive barriers can evolve remarkably quickly in response to divergent selection, with speciation intervals in the range of tens of generations to hundreds of thousands of years (Hendry et al. 2007).

Such rapid bursts of ecologically driven speciation are frequently linked to adaptive radiation, where a single lineage rapidly diversifies into an array of ecomorphologically differentiated and often co-existing species (Schluter 2000). However, a preponderance of ecological speciation has been found in only a few model adaptive radiations (Schluter 2001), such as lacustrine fishes (e.g. Schliewen et al. 1994; Østbye et al. 2006). Moreover, recent studies have emphasised that speciation during adaptive radiation is often non-ecological (Rundell and Price 2009; Losos & Mahler 2010): the archipelago model of adaptive radiation primarily implicates allopatry, which can be either accompanied or followed by ecological differentiation facilitating co-existence (e.g. Grant & Grant 2008). Identifying and distinguishing the relative

70 roles of ecological and non-ecological speciation drivers is especially challenging for radiations
71 with poorly constrained biogeographic histories, such as is typical in the marine environment.
72 However, powerful evidence of ecological speciation can be found if a particular ecomorph
73 independently and repeatedly evolves reproductive isolation in response to similar selection
74 pressures (Funk 1998). Selection is implicated in these cases because a replicated response to
75 similar environments is unlikely to be due to neutral processes such as genetic drift and
76 geographical founder effects.

77 The focus of this study is a unique adaptive radiation of marine snakes. The 62 species of
78 viviparous sea snakes (Hydrophiinae) share a terrestrial ancestor only ~6-13 million years ago,
79 yet exhibit spectacular morphological diversity and high levels of local sympatry in shallow
80 marine ecosystems throughout the Indo-West Pacific (Sanders et al. 2008; Lukoschek et al. 2011;
81 Rasmussen et al. 2011a; Sanders et al. 2012). Sea snake assemblages typically comprise one or
82 two dietary generalists and up to seven specialists including fish egg eaters and predators on
83 catfishes, frogfishes, gobies or crevice-sheltering reef fish (McCosker 1975; Glodek & Voris
84 1982; Voris & Voris 1983). Particularly conspicuous are 'microcephalic' forms adapted to hunt
85 eels in burrows, having very small heads and narrow fore-bodies that rarely exceed half to one-
86 quarter of the girth of the hind body (Voris 1977; Voris & Voris 1983). Remarkably,
87 microcephaly has evolved at least eight (but potentially as many as 14) times in the *Hydrophis*
88 group, a clade that has undergone exceptionally rapid diversification in the last ~3.5 million years
89 and accounts for ~80% of (extant) sea snake species richness (Voris 1977; Lukoschek & Keogh
90 2006; Sanders et al. 2010; Sanders et al. 2012). The microcephalic ecomorph is not represented in
91 any other sea snake lineage (*Aipysurus, Emydocephalus, Ephalophis, Hydrelaps* and

92 *Parahydrophis*) and none of these heavily exploits burrowing eel prey (Voris & Voris 1983).
93 Rapid evolution of head size variation is therefore a likely contributing factor in the explosive
94 speciation in *Hydrophis* group sea snakes. Parallel ecomorph evolution is a common feature of
95 rapid adaptive radiations and has often confounded morphology-based phylogenetic inferences.
96 *Hydrophis* group species have variously been classified in 10 to 16 often paraphyletic or
97 monotypic genera reflecting their complex patterns of phenotypic evolution (Smith 1926;
98 McDowell 1972; Voris 1977; Rasmussen 1997; Rasmussen 2002; Kharin 2004).
99 In this paper, we investigate recent eco-morphological diversification and speciation in
100 four closely-related *Hydrophis* species with overlapping ranges in Southeast Asia and Australia
101 (Fig. 1). *Hydrophis cyanocinctus* reaches >2m in total length, is heavy-bodied with a large head
102 and similar girths at neck and hind-body ('macrocephalic'), and preys on crevice-sheltering eels
103 and gobies, whereas *H. coggeri, H. melanocephalus* and *H. parviceps* all rarely exceed 1.2m in
104 total length and are typical microcephalic species that feed near-exclusively on snake eels in
105 burrows. Initial mitochondrial sampling of these species revealed shallow relationships and lack
106 of reciprocal monophyly between macro- and microcephalic forms and among putative species
107 (this study), suggesting very recent speciation and/or ongoing gene-flow. Body size is thought to
108 be a primary cue for mate recognition in viviparous sea snakes (Shine 2005) so that ecomorph
109 transitions associated with diet might also promote reproductive isolation in sympatry via
110 assortative mating (e.g. Podos 2001). *Hydrophis melanocephalus* is fully sympatric with *H.*
111 *cyanocinctus* in the north-eastern part of the latter species' range in Vietnam, China, Taiwan and
112 Japan. *Hydrophis coggeri* was until recently considered an allopatric population of *H.*
113 *melanocephalus* and overlaps with *H. cyanocinctus* in the south: Borneo, Sulawesi and northern

Australia, extending to New Caledonia and Fiji. The third microcephalic species in the present study, *H. parviceps*, is known from only five specimens collected in South Vietnam (Rasmussen et al. 2012), where it is sympatric with both *H. melanocephalus* and *H. cyanocinctus*. A broad phylogenetic sampling of *Hydrophis* group sea snakes robustly recovered a clade of *H. cyanocinctus*, *H. coggeri* and *H. parviceps* (Sanders et al. 2012); here we show that *H. melanocephalus* is nested inside the latter grouping, confirming that all four species in the present study form a clade.

We analysed phenotypic and genetic variation in each of these species using morphology, microsatellite markers, and mitochondrial and nuclear sequences, and integrated new and published diet records. These data were used to: i) Assess correspondence between taxonomic, genetic and phenotypic groupings, ii) Infer the number and direction of evolutionary changes between macro- and microcephalic ecomorphs, and iii) Test whether reproductive segregation occurs among ecomorphs and/or among recognised species. Together these inferences were used to assess a possible role for ecological specialisation in promoting speciation in this complex of sea snakes.

Methods

Sampling

Sea snakes were obtained by the authors during collecting trips to Indonesia, Vietnam, Thailand, Sri Lanka and Australia between 1998 and 2010. Most specimens were obtained opportunistically from fisheries by-catch. Vouchers were fixed in formalin and deposited in museum collections. DNA tissues (liver and muscle biopsies) were sampled for 58 individuals

spanning most of each species' geographic range. Standard protocols were used to extract genomic DNA (Puregene™ DNA Isolation Tissue Kit, Gentra Systems). Mitochondrial sequence fragments of *H. melanocephalus* from Japan and *H. cyanocinctus* from Thailand were obtained from GenBank. Specimen localities, voucher numbers and GenBank accessions for samples used in molecular analyses are given in Table S1. Table S2 shows numbers of specimens included in genetic, morphological and diet analyses for each species and locality.

Morphological analyses

Morphological data were collected for 122 museum and field-collected specimens representing the four species (42 *Hydrophis melanocephalus*, 45 *H. coggeri*, 30 *H. cyanocinctus*, and five *H. parviceps*). We examined four ecologically significant traits involving body size and proportions, in addition to nine taxonomically important scalation and colour pattern characters used to delimit the four species (Smith 1926; Rasmussen et al. 2011b). Morphometric characters (recorded to the nearest 1.0mm using string and a ruler) were: body length measured from snout to vent (SVL), tail length from vent to tip of the tail, girth at the neck, and girth at 0.75 SVL. Scale counts were the number of scale rows at the neck and at midbody (measured using the number of ventrals following Voris (1977)), the number of ventral scales following Smith (1926), number of supralabials, and number of sublabials. Colour pattern characters were number of bands on the body and number of bands on the tail. After excluding sub-adults, gravid females and specimens with stomach and gut contents, a bivariate plot was used to assess variation in relative girth (girth at 0.75 SVL : girth at neck) and SVL among species and ecomorphs. Adults were identified by large, non-flaccid testes in males and thickened oviducts and/or visible

158 vitellogenic follicles in females. Interspecific differences in relative girth were tested statistically
159 in Excel using single-factor ANOVA analyses on log-transformed ratios of girth at 0.75 SVL
160 versus girth at neck for *H. cyanocinctus*, *H. coggeri* and *H. melanocephalus* (*H. parviceps* was
161 excluded due to low sample size). Multiple comparisons were controlled for using
162 a Bonferroni-corrected alpha (of 0.05 divided by 3).
163
164 **Diet data**
165 New and published diet data were collated and summarised for adult specimens of the
166 four species. Specimens collected during fieldtrips were dissected to examine stomach contents,
167 where possible these were identified to family level by relevant experts in our institutions.
168 Additional diet data were obtained from the literature (Voris 1972; McCosker 1975; Glodek &
169 Voris 1982; Voris & Voris 1983 and references therein; Fry et al. 2001; Lobo 2006).
170 Interspecific diet differences were tested for *H. cyanocinctus*, *H. coggeri* and *H. melanocephalus*
171 in Excel using a chi-square test for a 3 x 3 contingency table of counts for the three diet
172 categories recorded for these species: gobies, crevice eels (moray and conger eels) and burrowing
173 eels (snake and worm eels) (see diet results below). Single prey items were recorded with the
174 exception of one *H. cyanocinctus* specimen that contained two gobies. Multiple comparisons
175 were controlled for using a Bonferroni-corrected alpha (of 0.05 divided by 3).
176
177 **Microsatellite analysis**
178 Twelve microsatellite loci were developed for this study using perfect repeats from next
179 generation shotgun data (Sanders & Gardner 2012). Genotype profiles were generated for the

four nominal species using Multiplex-Ready Technology, with capillary electrophoresis outsourced to the Australian Genomic Research Facility in Adelaide, Australia. Allele sizes were determined against a Genescan 500 Liz size standard using the Applied Biosystems programs GeneMapper 4.0 and PeakScanner 1.0. Each locus was tested for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium using GenePop 4.0 (Rousset 2008). MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) was used to identify null alleles, large allele dropout and stuttering errors.

Several genetic distance measures are available for microsatellite data (Goldstein et al., 1995). At inter-specific levels, stepwise-like mutations are expected to contribute significantly to microsatellite variation so that allele-size based measures of differentiation (such as R_{ST}) might perform better than allele-identity based measures (such as F_{ST}), which fail to increase linearly with time since divergence (Goldstein & Pollock 1997; Hardy et al. 2003). We used two approaches to investigate whether stepwise mutations are likely to have contributed to inter-specific differentiation in our data. First, we used SPAGEDI 1.3 (Hardy & Vekemans 2002) to generate 20,000 allele size permutations and perform a one-tailed test to assess whether observed R_{ST} values between all possible species pairs were significantly higher than permuted R_{ST} values. We then used the analysis of molecular variance (AMOVA) framework in Arlequin 3.5 (Excoffier & Lischer 2010) to investigate whether measures including (R_{ST}) or excluding (F_{ST}) allele-size variation explain a larger proportion of microsatellite variance among species.

Microsatellite population structure and individual assignment

To investigate whether microsatellite population structure corresponds to nominal species and/or divergent phenotypes, we used the individual-based Bayesian clustering approach implemented in STRUCTURE 2.3 (http:// pritch.bsd.uchicago.edu). This method probabilistically assigns individuals to ancestral populations based on their genotypes by minimising deviation from Hardy–Weinberg equilibrium and linkage equilibrium (Pritchard et al. 2000). Admixture (Q) is estimated for each individual from each of K ancestral population clusters, where K is specified in advance (see below). All runs were done using the admixture model (allowing individuals to have ancestry in multiple populations), with independent allele frequencies and no *a priori* population classifications. Default parameter settings were used with a burnin step of 1,000,000 followed by 1,000,000 Markov Chain Monte Carlo (MCMC) iterations. Ten runs per different K were performed for K = 1 to K =5; these were averaged using CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) as very small differences in log likelihood were observed at each value of K. We used STRUCTURE Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) to estimate the most likely number of clusters based on i) the K value with the peak posterior probability distribution, and ii) likelihood ratio tests performed on the log-likelihood of the data for each value of K ($Pr(X|K)$) (Pritchard et al. 2000). STRUCTURE results were plotted using Distruct 1.1 (Rosenberg 2004).

Arlequin 3.5 (Excoffier & Lischer 2010) was used to calculate R_{ST} (Slatkin 1995) and F_{ST} (Weir & Cockerham 1984) values between taxonomic and geographic groups, with tests for significant differentiation performed via 1000 random permutations of the data.

Mitochondrial and nuclear sequencing

~1100 base pairs of the mitochondrial cytochrome b (cytb) gene was amplified for 52 individuals using forward primer L14910 (5'- GAC CTG TGA TMT GAA AAA CCA YCG TTG T -3′) and reverse primer H16064 (5'- CTT TGG TTT ACA AGA ACA ATG CTT TA -3') (Burbrink et al. 2000). To provide additional independent loci for species tree inference, two nuclear markers were sequenced for 21 individuals (5 eastern and 3 western *Hydrophis cyanocinctus*, 5 *H. melanocephalus*, 6 *H. coggeri* and 2 *H. parviceps*), with the same individuals sampled across nuclear and mitochondrial markers. Nuclear loci were G1888 (402bp) and G1894 (429bp); these non-coding anonymous markers were selected from shotgun sequencing (see Bertozzi et al. 2012) and were amplified using forward primer G1888 (5'-CAG GGC CTT GCC TTG TGC CA-3') and reverse primer G1889 (5'-ACC TCT GCG CAC TAT GAC TCT TGA-3'), and forward G1894 (5'- ACC CTT TCA GTC ACA GGT CTG CT-3') and reverse G1895 (5'- GAG CGA AAC AGG GAG TTA TCC AAG C-3'). For all markers, PCR was carried out in 25µL volumes using HotMaster reagents (Perkin Elmer/Applied Biosystems) and double-stranded sequencing was outsourced to the Australian Genome Research Facility Ltd (AGRF) in Adelaide, Australia. Sequences were checked for ambiguities, and alignments were assembled from consensus sequences of forward and reverse reads using Geneious Pro v5.1.7 (Drummond et al. 2010). Pairwise distances among mitochondrial clades were calculated for sequence data using the Species Delimitation plugin for Geneious (Masters et al. 2011).

Phylogeny and divergence times

243 The MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) plugin for Geneious was used to
244 reconstruct a Bayesian mitochondrial tree using the optimum data partitioning scheme
245 determined using Bayes factors and best-fit models of nucleotide evolution identified using the
246 Akaike Information Criterion (AIC) in MrModeltest v. 2.3 (Nylander 2004) and PAUP* 4.0
247 (Swofford 2002): codon positions 1+2 (GTRig) and codon position 3 (GTRig). Values for model
248 parameters were unlinked across partitions. MCMC analyses were run using default settings and
249 different starting seeds and four chains each. The final analysis was run for 6,000,000 generations
250 and sampled every 1000 generations. The first 30% of sampled trees were excluded as burn-in.
251 Convergence was assessed by examining effective sample sizes (ESS values) and likelihood plots
252 through time in TRACER (Rambaut & Drummond 2007), and by comparing the posterior
253 probabilities from different runs. *Kerilia jerdoni* was used as an outgroup because there is robust
254 morphological and molecular evidence that this species is closely related to but outside the
255 *Hydrophis cyanocinctus* complex (Voris 1977; Rasmussen 1997; Sanders et al. 2012).
256 Although mitochondrial markers provide large numbers of polymorphic sites for
257 resolving population and species histories, they are also susceptible to introgression and/or
258 stochastic retention of ancestral polymorphisms, which can confound these inferences. For this
259 reason, we used *BEAST (Heled and Drummond 2010) in the BEAST 1.7 .1 package
260 (Drummond et al. 2012) to reconstruct a species tree from the mitochondrial and two nuclear loci
261 while accounting for coalescent stochasticity among simultaneously sampled gene trees.
262 *BEAST requires *a priori* assignment of individuals to putative species, i.e. sufficiently
263 divergent groups of individuals (Heled and Drummond, 2010). Western and eastern *H.*
264 *cyanocinctus* were assigned as separate putative species due to high levels of divergence at all

three loci. Haplotypes shared by eastern *H. cyanocinctus* and *H. melanocephalus* were excluded from the analysis due to likely introgression (see Cytonuclear Discordance). We used a strict clock and applied the substitution rate of 1.65% divergence per lineage per million years estimated for cytochrome b based on a relaxed clock calibrated using age estimates for eight squamate fossils (Sanders and Lee 2008; Sanders et al. 2008). A Yule branching process was used for the species tree prior and default settings were used for all remaining priors, including a Piecewise linear and constant population size model (Heled and Drummond, 2010). We ran the analysis five times with different random starting seeds for 400,000,000 generations and sampled every 5000 generations; convergence of Markov chains was assessed as for the mitochondrial analysis (above). The first 50% of sampled trees were excluded as burn-in and the remaining 40,000 trees were used to generate a maximum credibility species tree for each run in Tree Annotator 1.6.1 (Rambaut and Drummond, 2007).

Finally, we attempted to resolve the polytomy among eastern *H. cyanocinctus*, *H. coggeri* and *H. melanocephalus* by comparing divergence times between *H. melanocephalus* versus *H. cyanocinctus* and *H. melanocephalus* versus *H. coggeri* using a coalescent isolation-with-migration (IM) model implemented in the program IMa (Hey & Nielsen, 2004; Hey & Nielsen, 2007). Whereas IM does not assume reproductive isolation, extensive admixture of distantly related species can confound phylogenetic estimates based on gene/species trees and comparisons of microsatellite distance/diversity. The full demographic IM model was fitted to the13 microsatellite and mitochondrial loci simultaneously, using a Markov chain Monte Carlo (MCMC) approach and applying the Hasegawa-Kishino-Yano (HKY) model for the mitochondrial sequence data and the Stepwise Mutation Model (SMM) for each microsatellite

287 locus (with alleles first converted into numbers of repeats). Nuclear sequences were excluded due
288 to a lack of inter-specific variation (see Results). After a burn-in period of 1 million steps, we ran
289 the program in M-mode for 10 million steps (with the default sampling of every 100 steps), so
290 that effective sample sizes (ESS) were at least 45 for each parameter. Prior distributions for
291 demographic parameters were set based on posterior distributions from several preliminary runs.
292 The analyses were then run at least five times each using different random number seeds to check
293 for convergence of the Markov chain. Log-likelihood ratio (2LLR) tests were performed on the
294 16 nested models implemented in IMa (with population, migration and divergence time
295 parameter estimates variously to set to zero, fixed as equal to other parameters, or free to vary).
296 We did not convert the divergence time parameter to an absolute time estimate because a reliable
297 mutation rate is not currently available for our microsatellite markers. An earlier divergence
298 between *H. melanocephalus* and *H. coggeri* compared to *H. cyanocinctus* and *H. melanocephalus*
299 would support a closer relationship between the latter two species. Pairwise comparisons could
300 not be made for western *H. cyanocinctus* (microsatellite markers not available) or *H. parviceps*
301 (molecular sample size of two individuals).
302
303 **Species delimitation**
304 Due to species-level paraphyly or polyphyly at all three sequence loci (see Results), we
305 attempted to delimit species boundaries using the Bayesian approach implemented in the program
306 BP&P version 2.1 (Rannala and Yang, 2003; Yang and Rannala, 2010). This coalescent based
307 method accommodates the species tree as well as lineage sorting effects but assumes no recent
308 gene flow. Reversible-jump MCMC is used to estimate the posterior distribution of the set of

trees that can be generated by collapsing nodes in a guide tree. We used two alternative guide trees: 1) based on the maximum credibility tree estimated by *BEAST (Fig. 5) with western *H. cyanocinctus* as the sister lineage to a clade of eastern *H. cyanocinctus* and the three microcephalic species; and 2) based on current taxonomy but with western and eastern *H. cyanocinctus* as separate (sister) species, and *H. melanocephalus, H. coggeri* and *H. parviceps* as successive outgroups. The mitochondrial and two nuclear sequence loci were used with the gamma prior G (2, 1000) for the population size parameters (θs) with mean 2/2000 = 0.001. The age of the root in the species tree (tau 0) was assigned the gamma prior G (2, 1000), while the other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala, 2010: equation 2). The heredity parameter was used to assign inheritance scalars of 0.25 and 1.0 to the mitochondrial and nuclear loci, respectively; and the locusrate parameter was used to allow different rates among loci generated from the Dirichlet distribution with α = 2. To confirm consistency between runs, the analysis was run four times for each guide tree using both speciation delimitation algorithms and different random number seeds. Each MCMC was run for 200,000 generations with a burn-in of 50,000.

Results

Morphological analyses and diet

Fig. 2 shows a bivariate plot of relative girth versus snout to vent length (SVL) in the four species. The three microcephalic species exhibited broadly overlapping distributions. *Hydrophis coggeri* and *H. melanocephalus* showed very similar means for both traits, with SVL rarely

exceeding 1m and hind-body girths typically twice to almost three times the girth of the neck; the three *H. parviceps* specimens showed a mean SVL of ~1m and mean hind-body girth of approximately three times the girth of the neck. Notably, adult *H. coggeri* and *H. melanocephalus* exhibit non-overlapping SVL distributions where they occur in sympatry in Sulawesi (SVL 660-710 and 800-990, respectively; not shown). *Hydrophis cyanocinctus* specimens formed a separate cluster from all three microcephalic species, with SVL measurements ranging from almost 1m to over 2m (mean 1.45m) and hind-body girths of 1.2 to 1.8 times the girth of the neck (mean 1.5). Relative girths differed significantly between *H. cyanocinctus* and both *H. coggeri* and *H. melanocephalus* after Bonferroni correction ($df = 1$, $p < 0.0001$), but not between *H. coggeri* and *H. melanocephalus* ($df = 1$, $p > 0.016$).

Hydrophis cyanocinctus was also differentiated from the three microcephalic species by higher counts on the head and body for all scale characters, including marginally overlapping distributions of scale rows at neck and mid-body, and supra- and sub-labial scale counts. The microcephalic species were distinguished from each other by fewer scale rows at the neck in *H. parviceps* (19-21 versus >23 in *H. coggeri* and *H. melanocephalus*), the number of bands on the body (28-35 in *H. coggeri*, 33-72 in *H. melanocephalus* and 61-73 in *H. parviceps*) and the number of bands on the tail (2-5 in *H. coggeri*, 3-7 in *H. melanocephalus* and 7-11 in *H. parviceps*). Means and ranges for all scalation and colour pattern characters are given in Appendix 1.

Diet items ascertained for 75 individuals showed clear patterns, with only the microcephalic species having a high proportion of snake eels (Ophichthidae). All snake eels identifiable to genus level were *Leiuranus* and *Myrichthys* species; these are nocturnal, inhabit

353 mucus-lined burrows in sandy substrates, and many mimic the banded colour-patterns of sea
354 snakes and sea kraits to deter predators (hence 'snake' eel) (McCosker et al. 1998). Of the 12 diet
355 records available for *H. cyanocinctus*, 66% had fed on crevice-associated eels in the families
356 Muraenidae (moray eels) and Congridae (conger eels) and 34% on gobies (Gobiodidae and
357 Gobiidae). Of 56 diet records for *H. coggeri*, 94% of individuals had fed on burrowing snake eels
358 (Ophichthidae), 4% on burrowing worm eels (Moringuidae) and 2% on congrid eels. Records
359 from 5 *H. melanocephalus* suggest this species also feeds primarily on snake eels (4 individuals),
360 and occasionally congrids (1 individual). Foraging observations of both *H. coggeri* (McCosker
361 1975; Heatwole et al. 1978; Guinea 1981) and *H. melanocephalus* (Takahashi 1981) report
362 diurnal individuals successively probing burrows on the sea floor until eels are captured.
363 *Hydrophis parviceps* is known from 5 specimens only, but 2 of these contained stomach contents
364 also identified as snake eels. Diet composition differed significantly between *H. cyanocinctus* and
365 both *H. coggeri* and *H. melanocephalus* ($df = 1$, $p < 0.0001$), but not between *H. coggeri* and *H.*
366 *melanocephalus* ($df = 1$, $p > 0.016$).
367
368 **Microsatellite analysis**
369 Genotype profiles were generated for a total of 52 individuals with two missing loci in
370 five individuals and one missing locus in three individuals. A total of 69 alleles were identified
371 with the number of alleles per locus ranging between 2 and 10 with an average of 5.7. MICRO-
372 CHECKER tests showed the final set of 12 loci to be free from large allele dropout and stuttering
373 errors both when populations were examined together and separately. No significant linkage
374 disequilibrium or deviation from HWE was detected among the 12 loci using GenePop ($p <$

375 0.05), although MICRO-CHECKER suggested null alleles might be present at SSM12 in
376 *Hydrophis coggeri* (frequency = 0.193), and at SSM27 in *H. cyanocinctus* (frequency = 0.324)
377 (Sanders and Gardner 2012).
378 All populations of each nominal species were sampled for microsatellites with the
379 exception of Indian Ocean (western) *Hydrophis cyanocinctus* specimens, which were not
380 available at the time of laboratory analysis. Hence, the microsatellite results below refer only to
381 eastern (Southeast Asian and Australian) *H. cyanocinctus.* The allele size permutation test (Hardy
382 et al. 2003) indicated that allele sizes contribute to among population differentiation in at least 4
383 of the 12 microsatellite loci: observed R_{ST} values were significantly higher than permuted R_{ST}
384 values for *H. melanocephalus* versus *H. coggeri* in SS8 (p=0.03); *H. cyanocinctus* versus *H.*
385 *melanocephalus* in SS12 (p=0.05); *H. melanocephalus* versus *H. coggeri* in SS14 (p=0.05), and
386 each of *H. melanocephalus* and *H. coggeri* versus *H. parviceps* in SS25 (p=0.04 and 0.04,
387 respectively). The AMOVA with genetic variation partitioned according to the three species
388 groups (*H. cyanocinctus*, *H. coggeri* and *H. melanocephalus*) described 13.3% of variation
389 among groups based on R_{ST} measures (p = 0.01) compared to 8.6% of variation based on F_{ST}
390 measures (p = 0.03). Together these results suggest that distance statistics that account for allele
391 size variation are most appropriate for our data.
392
393 **Microsatellite population structure and individual assignment**
394 Multiple STRUCTURE runs with a given value of K led to virtually identical results.
395 Using the full dataset, STRUCTURE Harvester revealed a peak posterior probability of four (K =
396 4), and a minimum of three (K = 3, $\Delta K = 292.5264$), ancestral population clusters. At K=4,

clusters corresponded to the four nominal species irrespective of their geographic origin: *H. cyanocinctus* from Vietnam clustered with conspecifics from Java and Australia, and were separated from *H. parviceps* from Vietnam, *H. coggeri* from Australia and Sulawesi, and *H. melanocephalus* from Sulawesi and Vietnam (Fig. 3). In this analysis, only one individual showed >25% ancestry from more than one population: the specimen from Sulawesi was identified as *H. melanocephalus* on the basis of morphology and had shared ancestry between the *H. melanocephalus* cluster (Q = ~0.5) and the *H. coggeri* and *H. cyanocinctus* clusters (Q = ~0.25 each). This individual was excluded from subsequent population genetic distance calculations. At K=3, all *H. cyanocinctus* plus *H. parviceps* were distinguished from *H. coggeri* and *H. melanocephalus*. Higher K values (K=5-6) failed to extract additional meaningful geographic or taxonomic clusters. Individual assignment thus provides evidence of limited recent introgression among the four geographically overlapping species.

For the 12 microsatellite loci combined, among-species pairwise R_{ST} and F_{ST} values were relatively high and significant at $p < 0.05$ based on 1000 permutations (Table 1). The lowest inter-specific values were found between *H. cyanocinctus* and *H. melanocephalus* ($R_{ST} = 0.114$; $F_{ST} = 0.181$), with $R_{ST} = 0.317$ and $F_{ST} = 0.211$ between *H. melanocephalus* and *H. coggeri*, and $R_{ST} = 0.333$ and $F_{ST} = 0.297$ between *H. cyanocinctus* and *H. coggeri*. Within species distances were R_{ST} 0.061 and F_{ST} 0.041 between *H. cyanocinctus* from Southeast Asia and Australia, and R_{ST} 0.089 and F_{ST} 0.059 between *H. melanocephalus* from Vietnam and Sulawesi.

Phylogeny and divergence times

418 The final mitochondrial alignment consisted of 1107 sites for 54 individuals representing
419 24 haplotypes. The Bayesian majority-rule consensus tree (Fig. 4) did not retrieve monophyly of
420 individuals classified as *Hydrophis cyanocinctus* and *H. melanocephalus*. The basal ingroup
421 divergence is between western *H. cyanocinctus* and a well supported clade (posterior 0.98)
422 containing all other sampled individuals. The latter group comprises 3 main clades: 1) a clade of
423 *H. melanocephalus* from Sulawesi (posterior 0.99); 2) all *H. coggeri* from Australia and Sulawesi
424 (posterior 0.99); 3) a grouping of eastern *H. cyanocinctus* (from Australia and SE Asia), *H.*
425 *melanocephalus (*from Sulawesi, Vietnam and Japan) and *H. parviceps* (posterior 0.96). Within
426 clade 3, the two sampled *H. parviceps* form sister lineages, although more samples are required
427 for a robust test of monophyly. Neither eastern *H. cyanocinctus* nor the "clade 3" *H.*
428 *melanocephalus* are monophyletic. A single haplotype is shared by four *H. melanocephalus* from
429 Vietnam and three *H. cyanocinctus* from Java. The mean corrected (HKY) pairwise divergence
430 between clades 1 and 2 versus 3 is 1.5%; mean within-clade divergence is 0.7% in clade 1, 0.3%
431 in clade 2, and 0.5% in clade 3. A considerably higher divergence of 3.6% is found between the 2
432 major ingroup clades (western *H. cyanocinctus* versus the clade consisting of eastern *H.*
433 *cyanocinctus* plus the 3 microcephalic species).
434 The nuclear loci G1894 and G1888 contained 5 and 4 polymorphic sites, respectively. At
435 G1894, eastern *H. cyanocinctus*, *H. coggeri* and *H. melanocephalus* shared two haplotypes,
436 neither of which was found in any other species; *H. parviceps* was represented by a single unique
437 haplotype with two fixed substitutions, and western *H. cyanocinctus* was represented by three
438 unique haplotypes with one fixed substitution. At G1888, two eastern *H. cyanocinctus*, one
439 western *H. cyanocinctus* and one *H. parviceps* showed unique haplotypes with single fixed

substitutions, and two other haplotypes were shared by eastern *H. cyanocinctus*, *H. coggeri* and *H. melanocephalus*.

*BEAST analyses of the combined mitochondrial and nuclear sequence data yielded ESS values above 500 for all parameters and species trees that were topologically identical among replicate runs. The maximum credibility species tree (Fig. 5) strongly recovered (pp 1.0) western *H. cyanocinctus* as sister to a well supported (pp 1. 0) clade of all other ingroup taxa, i.e. eastern *H. cyanocinctus* plus the three microcephalic species. Eastern *H. cyanocinctus* and *H. melanocephalus* were moderately well supported as sister lineages (pp 0.83) and formed a polytomy (pp 0.52) with *H. coggeri* and *H. parviceps*. Relationships among western *H. cyanocinctus*, *H. melanocephalus* and *H. coggeri* are evidently driven by the mitochondrial locus (due to low nuclear variation); however, the lack of shared haplotypes and presence of fixed differences separating western *H. cyanocinctus* and *H. parviceps* from the remaining taxa provide independent support for the non-monophyly of both macrocephalic (*H. cyanocinctus*) and microcephalic (*H. coggeri, H. parviceps*, *H. melanocephalus*) ecomorphs. Mean divergence time estimates were 840,000 years ago (95% HPD [highest posterior density] 0.4-1.3million) for the root node (western *H. cyanocinctus* versus all remaining taxa), 220,000 years (95% HPD 120,000-340,000) for the basal divergence of the clade containing eastern *H. cyanocinctus* and the three microcephalic species, and 80,000 years (95% HPD 100,000-150,000) for the divergence between eastern *H. cyanocinctus* and its sister taxon *H. melanocephalus*. These species tree dates are somewhat younger than divergence times based on the mitochondrial rate (3.3% pairwise per million years for cytochrome b), which would imply a root divergence ~1.2 million years ago, and a basal divergence between eastern *H. cyanocinctus* and the three

462 microcephalic species ~450,000 years ago. Species divergence times based on multilocus
463 coalescent approaches are expected to be younger than gene-tree estimates given that gene tree
464 divergences will pre-date speciation (Edwards & Beerli 2000).
465 IMa analyses yielded ESS values above 100, unimodal posterior distributions for
466 divergence times and all other demographic parameters, and very concordant results from
467 replicate runs, suggesting good mixing and convergence of the Markov chains. Posterior
468 distributions of the divergence time parameter (μt) indicated an earlier divergence between
469 *Hydrophis melanocephalus* and *H. coggeri* compared to *H. melanocephalus* and (eastern) *H.*
470 *cyanocinctus* (Fig. 6); western *H. cyanocinctus* was not sampled for microsatellites (see above).
471 ML estimates of μt were 0.9 [90% HPD 0.6-2.2] for *H. melanocephalus* versus *H. coggeri* and
472 0.3 [90% HPD 0.1-0.9] for *H. melanocephalus* versus *H. cyanocinctus.* Although the lower 90%
473 HPD interval for *H. melanocephalus* versus *H. coggeri* broadly overlapped the upper 90% HPD
474 interval for *H. melanocephalus* versus *H. cyanocinctus*, it fell well outside of the ML estimate for
475 the latter divergence. For both species pairs, likelihood ratio tests of nested demographic models
476 strongly rejected models were the two migration parameters (representing gene flow in both
477 directions) were set to zero and all other model parameters were free to vary ($2LLR > 200$, $p <$
478 0.001). Our results suggested similar rates of migration between the two species pairs, with
479 slightly lower rates from *H. melanocephalus* into *H. coggeri* ($m_1 = 1.09$) than in the opposite
480 direction ($m_2 = 2.41$), and slightly higher migration rates from *H. melanocephalus* into *H.*
481 *cyanocinctus* into ($m_1 = 2.55$) than in the opposite direction ($m_2 = 1.91$); however, in both
482 analyses we were unable to reject alternative models of equal (but non-zero) migration.
483

Species delimitation

BP&P analyses using the *BEAST guide tree (Fig. 5) supported separate species status for western *Hydrophis cyanocinctus*, but did not support the recognition of the other lineages (eastern *H. cyanocinctus*, *H. melanocephalus*, *H. coggeri* and *H. parviceps*) as separate species. Both species delimitation algorithms consistently recovered the most prevalent tree (>73%) as having all internal nodes collapsed, while the basal node (western *H. cyanocinctus* versus the rest) was identified with >99% posterior probability. The next most prevalent tree (>19%) showed no nodes collapsed but recovered low posterior support for all internal nodes (posterior probabilities 12-27%). Analyses using the guide tree closest to current taxonomy (eastern and western *H. cyanocinctus* as separate sister species) recovered the most prevalent tree (>62%) as having all nodes collapsed (so that all lineages formed a single species); no nodes collapsed were collapsed in the next most prevalent tree (>36%) but all were recovered with low support (posterior probabilities <37%).

Discussion

Our results show correspondence between geographically overlapping genomic clusters and morphological species designations, providing evidence of progress towards speciation in the four nominal species. Mitochondrial haplotype sharing between allopatric populations of two species, and coalescent IM and species delimitation analyses, together indicate historical and/or recent introgression (see Cytonuclear discordance below). However, individual assignment using microsatellite data clearly separated the four widespread species into significantly differentiated clusters, irrespective of their sympatric or parapatric distributions at each sampling locality. Only

506 one hybrid individual was identified (with more than >75% ancestry shared between the two
507 microcephalic species in Sulawesi). This evidence of limited recent gene flow between co-
508 distributed species is strongly supported by non-overlapping distributions in morphological traits:
509 in Vietnam and Australia, eastern *H. cyanocinctus* is clearly separated from the three
510 microcephalic species by much larger girth at the neck relative to the hind body; *H.*
511 *melanocephalus* and *H. parviceps* in Vietnam are distinguished by numbers of scale rows at the
512 neck; in Sulawesi, *H. coggeri* and *H. melanocephalus* are separated by number of bands on the
513 body and body length. Western *H. cyanocinctus* were not sampled for microsatellites but their
514 sister relationship to all other sampled populations, large mitochondrial distance, and fixed
515 nuclear differences, suggest that the eastern form might be a fifth and hitherto overlooked species
516 (the type locality is given as India: Smith 1926). Divergence times estimated using a multilocus
517 coalescent tree and pairwise mitochondrial distances indicate that eastern *H. cyanocinctus* and the
518 three microcephalic species last shared a common ancestor only ~220,000 to 450,000 years ago,
519 while western *H. cyanocinctus* diverged from the latter clade 840,000 to 1.2 million years ago.
520
521 **Cytonuclear discordance**
522 Our mitochondrial and nuclear microsatellite datasets yielded highly discordant patterns.
523 Most notably, eastern *Hydrophis cyanocinctus* and *H. melanocephalus* samples each formed a
524 single microsatellite cluster in individual assignment analyses, but comprised multiple
525 polyphyletic mitochondrial lineages. Such discordance among mitochondrial and nuclear data has
526 been reported for numerous closely related and/or rapidly speciating taxa (see Seehausen 2004)
527 and is typically explained by i) historical hybridisation among mtDNA lineages, coupled with

stochastic loss of haplotypes via genetic drift and ii) incomplete lineage sorting (so that ancestral polymorphisms are retained across multiple lineages). Both processes may have contributed to the cytonuclear discordance reported here for sea snakes. However, the IM models that assumed inter-specific gene flow were a significantly better fit to our data than models with migration parameters set to zero, suggesting an important role for historical introgression (if the discordance was solely due to retention of ancestral polymorphisms, we would expect zero gene flow in the speciation history of these taxa). The failure of the method of Rannala and Yang (2003) to delimit the four nominal species in the present study provides further evidence of historical introgression: this Bayesian method recognises groups that have not experienced recent gene flow and assumes that patterns of species para- and polyphyly and discordance among loci is due to lineage sorting alone (Yang & Rannala 2010). Finally, the mitochondrial haplotype shared by four *H. melanocephalus* from Vietnam and three eastern *H. cyanocinctus* is highly derived (placed at the tips of the tree), which suggests that it was most likely introduced from one species to the other via introgression (e.g. Lawrence et al. 2010). Our findings are consistent with a large number of studies showing introgression between co-distributed species in the early stages of speciation (reviewed in Abbott et al. 2013).

Ecomorph origins and evolutionary transitions

Eastern and western *Hydrophis cyanocinctus* both reach >2m in total length with a large head and similar girths at neck and mid-body, and feed on crevice-dwelling eels and gobies. In contrast, *H. melanocephalus, H. coggeri* and *H. parviceps* have small heads and fore-body girths (half to more than one third of the hind-body), reach maximum lengths of up to 1.2m, and all

550 have a specialist diet of burrowing snake eels (Ophichthidae) which they hunt in their burrows.
551 Species and mitochondrial trees resolve western *H. cyanocinctus* (not sampled in the
552 microsatellite analysis) as basal to a clade comprising eastern *H. cyanocinctus* plus the three
553 microcephalic species. Additionally, *H. belcheri*, the sister lineage of all taxa considered here,
554 and other close relatives (*Kerilia jerdoni*, *H. spiralis*, *H. lapemoides, H. viperinus*) are all also
555 macrocephalic (Sanders et al. 2012). These patterns are most consistent with the macrocephalic
556 phenotype represented by *H. cyanocinctus* being ancestral to all three microcephalic species.
557 If a single shift from macro- to microcephalic phenotypes were to explain the observed
558 diversity patterns, we would expect all microcephalic species to cluster together in the
559 phylogenetic analyses. On the contrary, *H. melanocephalus* (microcephalic) and eastern *H.*
560 *cyanocinctus* (macrocephalic) displayed the lowest inter-specific R_{ST} and F_{ST} values, lacked
561 reciprocal monophyly in the mitochondrial tree, and the two species were sister taxa in the
562 multilocus coalescent species tree. These results appear most consistent with separate origins of
563 microcephaly (from an ancestral *H. cyanocinctus* morphotype) in at least *H. coggeri* and *H.*
564 *melanocephalus.* The alternative scenario of microcephaly evolving only once (in the ancestor of
565 the *H. coggeri*, *H. melanocephalus*, *H. parviceps*, and eastern *H. cyanocinctus* clade), with
566 secondary increase in head size and body length occurring in eastern *H. cyanocinctus*, is also
567 plausible but requires re-evolution of several other morphological traits not obviously correlated
568 with head and body size in eastern *H. cyanocinctus* (Smith 1926; Rasmussen et al. 2011b;
569 Rasmussen and Sanders unpublished data).
570 An important caveat of using population genetic data to infer relationships is that
571 extensive admixture can cause species to cluster together even if they are not closest relatives.

572 Thus, our results might alternatively be explained by single origins of microcephaly and
573 macrocephaly with differential gene flow between eastern *H. cyanocinctus* and the three
574 microcephalic species. This scenario cannot be ruled out but is not supported by current species
575 distributions (the range of eastern *H. cyanocinctus* largely encompasses all three microcephalic
576 species) or estimates of historical migration rates and divergence times based on an IM model
577 which does not assume historical reproductive isolation: *H. melanocephalus* shows more recent
578 common ancestry with eastern *H. cyanocinctus* than it does with *H. coggeri* despite similar
579 migration rate estimates for both species pairs. Although our results appear most consistent with
580 repeated shifts from macro- to microcephalic phenotypes, robustly resolving the exact number
581 and pattern of changes will likely require additional genomic and population sampling for these
582 species.

583 Our inferences on the origin and affinities of *H. parviceps* (the third microcephalic
584 species) are limited by a molecular sample size of only two individuals, yet microsatellite
585 differentiation and fixed substitutions in nuclear sequences clearly separated these specimens
586 from all other sampled populations. Although more sampling is needed, only 5 specimens of this
587 species have been collected in 80 years despite considerable efforts surveying sea snakes within
588 its range in southern Vietnam (Rasmussen et al. 2012).

589

590 **Evidence for ecological speciation?**

591 The repeated association between microcephaly and a specialist diet of burrowing snake
592 eels strongly implicates divergent or disruptive selection in driving phenotypic evolution in these
593 species. Sea snakes are superbly 'pre-adapted' to evolve specialisations for exploiting burrowing

eels, having elongate limbless bodies to penetrate burrows and powerful venom with which to subdue large and aggressive prey. The functional prediction is that small heads and narrow fore-bodies allow microcephalic forms to hunt snake eels by entering their narrow burrows. This association is supported by compelling (albeit often anecdotal) evidence. All eight microcephalic species of *Hydrophis* (including five not considered here) for which diet records are available prey near-exclusively on burrowing eels, and this trophic resource is not heavily exploited by any other phenotype in sea snakes (McCosker 1975; Voris & Voris 1983; Fry et al. 2001). Numerous foraging observations of microcephalic species (including both *Hydrophis coggeri* and *H. melanocephalus* studied here) report diurnal individuals successively probing eel burrows on the sea floor until prey is captured (e.g. McCosker 1975; Heatwole et al. 1978; Guinea 1981; Takahashi 1981). Resource competition is thought to be a major driver of ecological divergence, especially if 'open' or underutilized niches are available (e.g. Levene 1953), and these factors are likely to contribute here also. Sea snake assemblages exhibit strong diet partitioning suggestive of past competitive interactions and typically contain single (or occasionally two) burrowing-eel and crevice-eel specialists (Voris & Voris 1983; Fry et al. 2001).

The rapid recent speciation and evolution of dietary specialisations in this group is consistent with ecological speciation driven by selection on trophic morphology. Periods of allopatric divergence, e.g. during the Pleistocene isolation of ocean basins in Southeast Asia (Porter 1989), might also have promoted speciation and ecological differentiation in this system. However, at least a partial role for ecomorph divergence in promoting speciation is indicated by the lower levels of microsatellite genetic structure between geographically disjunct and reciprocally monophyletic mitochondrial clades within species (R_{ST} 0.061 between Southeast

Asian and Australian *Hydrophis cyanocinctus*; R_{ST} 0.089 between *H. melanocephalus* in Vietnam and Sulawesi), compared to higher levels of divergence between ecomorphs in parapatry and sympatry (R_{ST} >0.114-0.333). In particular, the macrocephalic eastern *H. cyanocinctus* and microcephalic *H. melanocephalus* appear to have diverged very recently and rapidly, resulting in major phenotypic differences and restriction of gene flow in sympatry, but lack of reciprocal monophyly for mitochondrial markers.

Disentangling the relative influence of trophic divergence and non-ecological factors in this system will ultimately require an understanding of the build up of pre- and/or post-zygotic isolating mechanisms. Under divergent selection, assortative mating can lead to reproductive isolation if traits linked to feeding specialisation also affect mate choice (e.g. Schliewen et al. 2001). Body size is thought to be a primary cue for mate recognition in viviparous sea snakes (Shine 2005) and macro- and microcephalic ecomorphs display largely non-overlapping distributions in this trait (Fig. 3). Size-assortative mating would also help to explain the partial reproductive isolation of microcephalic species *H. melanocephalus* and *H. coggeri* in Sulawesi, where these species display non-overlapping body size distributions suggesting a possible role for character displacement. Chemoreception is thought to be of secondary importance in mate recognition in sea snakes (Shine 2005) and is similarly linked to diet via prey-tracking. Habitat segregation can also act as a pre-zygotic barrier in the early stages of speciation (e.g. Eroukhmanoff et al. 2011), and might restrict gene flow between macro- and microcephalic ecomorphs if feeding and mating sites coincide (Australian *H. cyanocinctus* and *H. coggeri* are found in muddy-bottomed rocky habitats versus sandy inter-reef habitats, respectively: Guinea & Whiting 2005; Sanders, pers. obs.).

638

639 **Conclusions**

640 Our results highlight the viviparous sea snakes as a promising system for studies of
641 speciation and adaptive radiation in marine environments. We provide integrative evidence of
642 rapid diversification and at least partial reproductive isolation between large-bodied
643 macrocephalic predators on crevice-dwelling fishes and small-bodied microcephalic specialists
644 on burrowing eels (possibly in only a few hundred thousand years). Ecological shifts are mirrored
645 in a wider phylogenetic context across the *Hydrophis* group of sea snakes, where the
646 microcephalic ecomorph has evolved repeatedly many other times and accounts for more than
647 30% of species richness (at least 15 of 49 described species). Rapid evolution of head size
648 variation is therefore a likely contributing factor in the explosive speciation in this group. Future
649 research should also explore the genetic and ontogenetic basis of phenotype evolution, including
650 the extent to which genomic parallelism underlies rapid diversification, as well as the links
651 between ecomorph divergence and reproductive ecology. However, information on the life
652 history of sea snakes is still very scant and field studies are needed to provide the necessary
653 ecological framework for such inferences.

654

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Table 1. Microsatellite genetic differentiation among species based on R_{ST} (above the diagonal) and F_{ST} (below the diagonal). Bold values were significant at $p < 0.05$ by 1000 permutations of the data.

	H. cyanocinctus (eastern)	*H. coggeri*	*H. melanocephalus*	*H. parviceps*
H. cyanocinctus (eastern)	-	**0.333**	**0.114**	0.132
H. coggeri	**0.297**	-	**0.317**	**0.389**
H. melanocephalus	**0.181**	**0.211**	-	**0.185**

677 Appendix 1. Mean and range of scale counts and colour pattern characters for the four species
678 examined in the present study. Note that sample sizes for characters differ from the overall
679 sample size per locality and sex.

	H. cyanocinctus (eastern)		*H. coggeri*		*H. melanocephalus*		*H. parviceps*	
	Males	Females	Males	Females	Males	Females	Males	Females
Ventrals	334.4 (293-369) n = 14	345.9 (323-367) n = 15	281.5 (271 – 325) n = 19	296.5 (223 – 321) n = 25	304.3 (229 – 350) n = 15	312.2 (248 – 347) n = 26	343.6 (340-348) n = 3	335 (329-341) n = 2
Scale rows neck	30.4 (27-35) n = 14	30.5 (27-36) n = 16	24.4 (23-27) n = 20	25.5 (23-28) n = 25	24.6 (23-26) n = 13	25.2 (23-27) n = 23	19.6 (19-21) n = 3	21 (21) n = 2
Scale rows mid-body	39.5 (36-43) n = 14	41.6 (39-44) n = 16	30.6 (30-37) n = 20	33 (32-37) n = 25	33.8 (29-38) n = 13	35.4 (29-39) n = 23	32 (31-33) n = 3	34 (34) n = 2
Supralabials	8.3 (8-9) n = 7	8.25 (8-9) n = 4	6.25 (5.5-7) n = 4	6.5 (6-7.5) n = 7	6.9 (6.5-8) n = 6	6 n = 1	6.5 (6-7) n=2	7 (7) n=2
Sublabials	9.7 (9-10) n = 7	9.4 (8-10) n = 4	7.25 (6.5-8) n = 4	7.9 (7.5-9.5) n = 7	8 (7.5-9) n = 6	7 n = 1	7 (6-8) n=3	8 (8) n=2
Postoculars	1.85 (1.5-2) n = 7	1.7 (1-2) n = 4	1.9 (1-2) n = 4	1.3 (1-2) n = 7	1.8 (1.5-2) n = 6	1 n = 1	1 (1) n = 3	1 (1) n = 2
Temporals	2 (2) n = 7	2 (2) n = 4	1 (1) n = 4	1.1 (1-2) n = 7	1.2 (1-2) n = 6	1 n = 1	1 (1) n = 3	1 (1) n = 2
Bands on body	50.5 (35-70) n = 13	53.5 (40-68) n = 12	30.2 (28-35) n = 20	30 (25-34) n = 25	50.9 (33-72) n = 13	50.1 (33-65) n = 25	69.3 (68-71) n = 3	67 (61-73) n = 2
Bands on tail	6.2 (5-7) n = 13	6.3 (4-9) n = 12	3.2 (2-5) n = 19	3.8 (2-5) n = 25	4.9 (3-7) n = 16	4.5 (3-5) n = 24	9.3 (8-11) n = 3	7.5 (7-8) n = 2

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Figure 1. Distributions of species in the present study (blue = *H. cyanocinctus*; green = *H. melanocephalus*; orange = *H. parviceps*; red = *H. coggeri*) based on species occurrence data modified from the IUCN Red List (International Union for Conservation of Nature, 2010) and mapped using the Atlas of Living Australia (http://www.ala.org.au/) application. Sampling sites for molecular analyses are indicated using arrows.

Figure 2. Bivariate plot of relative girth versus snout to vent length (SVL) in the four studied *Hydrophis* species. Relative girth is measured as girth at 0.75 SVL : girth at the neck. Blue = *H. cyanocinctus*; green = *H. melanocephalus*; orange = *H. parviceps*; red = *H. coggeri*. Males and females are shown as closed and open symbols, respectively. Species means are marked with plus (+) symbols. Sub-adults, gravid females, and specimens containing stomach and gut contents are excluded.

Figure 3. STRUCTURE plot based on microsatellite data for 50 individuals at K=4. Each individual is represented by a vertical line divided into coloured segments representing their inferred ancestry in four ancestral clusters (K). The y-axis shows the % of each individual's membership in the cluster of corresponding to that colour: Blue = eastern (Southeast Asian and

Australian) *Hydrophis cyanocinctus*; green = *H. melanocephalus*; orange = *H. parviceps*; red = *H. coggeri.*

Figure 4. MrBayes all compatible consensus of 4,000 post burn-in trees for the four *Hydrophis* species sampled in this study (*Kerilia jerdoni* outgroup not shown) based on mitochondrial cytochrome b. Node support values above 75% are shown. The asterisk (*) denotes the haplotype shared by eastern *H. cyanocinctus* and *H. melanocephalus*. Black = western (Indian Ocean) *H. cyanocinctus*; blue = eastern (Southeast Asian and Australian) *H. cyanocinctus*; green = *H. melanocephalus*; orange = *H. parviceps*; red = *H. coggeri*.

Figure 5. *BEAST species tree based on mitochondrial and two nuclear sequences showing transitions between macro- and microcepahlic ecomorphs. Node labels indicate posterior probabilities. Timescale is in millions of years before present. Representative images of body proportions and colour pattern are shown for each species.

Figure 6. Posterior probability distributions of the divergence time parameter for *Hydrophis melanocephalus* versus *H. cyanocinctus* and *H. melanocephalus* versus *H. coggeri*, estimated by fitting an isolation-with-migration (IM) model to 13 microsatellite and mitochondrial loci.