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Multilocus phylogeography of the sea snake *Hydrophis curtus* reveals historical vicariance and cryptic lineage diversity

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The Indo-Australian archipelago (IAA) supports the world's highest diversity of marine fish, invertebrates and reptiles. Many of the marine fish and invertebrates show congruent phylogeographic patterns, supporting a view that the region's complex geo-climatic history has played an important role in generating its exceptional biodiversity. Here, we examine population genetic structure of the viviparous sea snake, *Hydrophis curtus*, to assess how past and present barriers to gene flow in the IAA have contributed to genetic and species diversity in a fully marine reptile. Mitochondrial and anonymous nuclear sequences and ten microsatellite loci were used to identify patterns of historical genetic structure and population expansion, reconstruct dated genealogies and assess levels of recent gene flow. These markers revealed strong concordant geographic structure within *H. curtus* with a prominent genetic break between populations broadly distributed in the Indian Ocean and the West Pacific. These populations were estimated to have diverged in the late Pliocene or early Pleistocene, and microsatellite admixture analyses suggested limited recent gene flow between them despite the current lack of barriers to dispersal, indicating possible cryptic species. Subsequent divergence in the mid–late Pleistocene was detected within the West Pacific clade among the populations in the Phuket-Thailand region, South-East Asia and Australia, and two of these populations also showed genetic signals of recent range expansions. Our results show that climatic fluctuations during the Plio-Pleistocene generated high levels of cryptic genetic diversity in *H. curtus*, and add to similar findings for diverse other marine groups in the IAA.

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Introduction

The Indo-Australian Archipelago (IAA), situated between the Indian and Pacific Oceans, supports the highest marine biodiversity in the world and is of exceptional conservation value (Bellwood & Hughes 2001; Hughes *et al.* 2002). In addition to this conspicuous species richness, many IAA taxa show high levels of cryptic lineage diversity that have been linked to the region's recent geological and climatic history (Woodruffe 2003; Williams & Duda 2008; Bellwood *et al.* 2012). Glacial cycles throughout the last ~2.6 million years (my) periodically lowered sea levels by more than 100 m below current levels (Pirazzoli 1996; Pillans *et al.* 1998; Voris 2000; Woodruffe 2003), creating varied opportunities for vicariance and cryptic allopatric

speciation within the IAA. Most dramatically, the exposed Sunda (South-East Asia) and Sahul (Australia and New Guinea) continental shelves (Fig. 1) subjected marine populations spanning the Indian Ocean (IO) and West Pacific (WP) to alternating episodes of isolation and secondary contact during low sea level stands (Voris 2000; Lambeck *et al.* 2002). The 2–3 km deep and 80 km wide Timor trench permanently separates the Sunda and Sahul shelves, also limiting dispersal of shallow marine organisms between the Asian and Australian regions (Ovenden *et al.* 2009).

Molecular evidence of Plio-Pleistocene vicariance has been found in numerous marine taxa spanning the Indo-West Pacific (IWP). Studies of marine invertebrates and fish have shown complex patterns of geographic population genetic structure, but many groups show concordant population structure and/or cryptic species boundaries among the Australian, WP and IO marine basins (Carpenter *et al.* 2011). The predominant phylogeographic pattern in the region is the clear genetic break between the IO and WP seen in marine invertebrates (e.g. Lavery *et al.* 1996; Benzie 1998; Duda & Palumbi 1999; Crandall *et al.* 2008a) and numerous fish groups (e.g. Magsino & Juinio-Meñez 2008; Drew & Barber 2009; Leray *et al.* 2010; Gaither *et al.* 2011). However, contrastingly, certain groups including moray eels (Reece *et al.* 2010), surgeonfishes (Horne *et al.* 2008) and some echinoderms (Lessios *et al.* 2003) show a complete lack of population genetic structure across the Indo-Pacific. Some taxa also show genetic signals of population contractions and expansions that are consistent with demographic changes driven by sea level fluctuations during glacial cycles (e.g. Crandall *et al.* 2008a; Fitzpatrick *et al.* 2011). The role of life-history traits in promoting or constraining lineage divergence remains unclear: many taxa showing strong phylogeographic structure have widely dispersing pelagic larval stages that could promote connectivity among regions and potentially disrupt phylogeographic patterns (Hoskin 1997).

In this study, we investigated population genetic structure in a fully aquatic viviparous sea snake that is distributed throughout the IWP and differs markedly in dispersal potential from most previously studied taxa. Viviparous sea snakes (Hydrophiinae: Hydrophiini) are the only extant fully marine reptiles (Rasmussen *et al.* 2011). They have peak diversity in the IAA (Rasmussen *et al.* 2011; Elfes *et al.* 2013), with the majority of extant lineages having diversified very rapidly within the last ~3.5 million years (Sanders *et al.* 2013a). Unlike many species previously investigated in the IWP, Hydrophiini are viviparous and direct developing (i.e. give birth to live young), resulting in potentially low reproductive outputs and dispersal rates (Heatwole 1999) that may lead to rapid population subdivision (Lukoschek *et al.* 2007, 2008). The viviparous sea snakes thus present promising opportunities to examine historical biogeographic events in the IAA and their role in generating biodiversity. However, there have been very few phylogeographic studies of sea snakes to date, and these have focused primarily above the species level (Sanders *et al.* 2013b) or on species with restricted distributions in the Australasian region (Lukoschek *et al.* 2007, 2008).

Hydrophis curtus (Shaw, 1802), the 'spine-bellied' sea snake, occupies shallow marine habitats from the Arabian Gulf through Asia and Australia to New Caledonia (Lukoschek *et al.* 2010). We sampled this species across ~70% of its range and used mitochondrial and nuclear sequences and microsatellite markers to reconstruct dated genealogies and historical population size changes, and assess levels of recent gene flow. Together, our inferences suggest that climatic fluctuations during the Plio-Pleistocene generated high levels of previously unrecognized cryptic lineage diversity in *H. curtus*.

Materials and methods

Tissue sampling and DNA extraction

Tissue samples from liver and muscle tissue preserved in 90% Ethanol/Iso-propanol were obtained from *H. curtus*

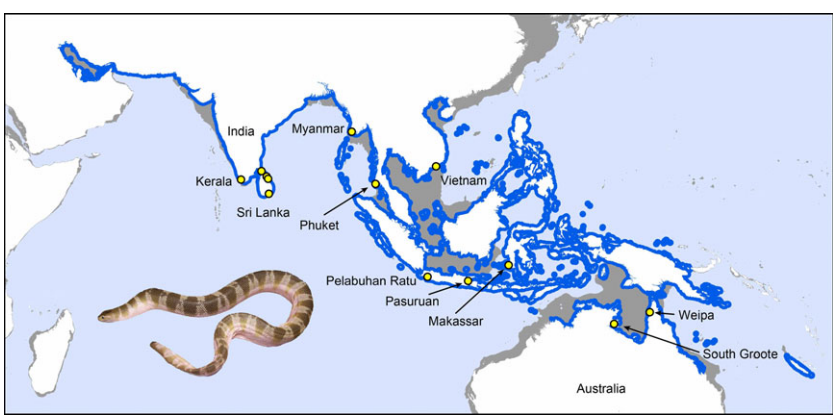

Fig. 1 Current distribution of *Hydrophis curtus* (dark blue) and the sampling locations in this study (yellow circles). The grey areas denote the 120 m isobath which indicate the extent of land when sea levels were ~120 m below present levels during Pleistocene glacial maxima. Distribution data for *H. curtus* are from IUCN red list and the bathymetric data are from http://www.gebco.net/.

specimens collected mostly as fisheries by-catch and provisionally identified using published descriptions and diagnoses (Smith 1926; Rasmussen 2001). Forty-three specimens were collected by the authors during sampling trips in Australia, Indonesia (Pasuruan-East Java, Pelebuhan Ratu-West Java, Makassar-Sulawesi) and Sri Lanka; and nine additional samples from Australia, India, Vietnam, Phuket-Thailand (PT) and Myanmar were acquired from museum collections and collaborators (Fig. 1). Collection localities for most bycatch specimens are of approximate provenance, but the Phuket specimens were landed at the harbour and may have been fished from further north on the Andaman coast or south in the Malacca Straits. Whole genomic DNA was extracted from liver/muscle tissues using standard proteinase K protocols (Puregene™ DNA Isolation Tissue Kit, Gentra Systems). Details of specimen collection localities and museum voucher numbers are provided in the Appendix S1.

Mitochondrial and nuclear DNA sequencing

Three mitochondrial markers and two anonymous nuclear markers were used to identify patterns of genetic structure and reconstruct dated genealogies. These markers have been successfully used in previous phylogenetic (Lukoschek & Keogh 2006; Sanders *et al.* 2013a) and phylogeographic studies of sea snakes (Lukoschek *et al.* 2007) and other snakes (Burbrink *et al.* 2000). The three mitochondrial markers were *Cytochrome b* (*Cytb*) gene (1095 bp) (Burbrink *et al.* 2000), *NADH dehydrogenase subunit 4* (*ND4*) and the adjacent *tRNA* region (840 bp) (Arevalo *et al.* 1994), and *16S small subunit ribosomal RNA* (*16S rRNA*) region (531 bp) (Kocher *et al.* 1989). The two anonymous nuclear markers were *G1894* (395 bp) and *G1888* (393 bp) (Bertozzi *et al.* 2012; Ukuwela *et al.* 2012). All DNA sequence markers were amplified using standard PCR protocols with HotMaster Taq reagents (Applied Biosystems, Foster City, CA, USA). The PCR amplification employed 34 cycles with annealing temperatures of 52 °C for mitochondrial markers and 59 °C for the two anonymous nuclear markers. Sequencing of the PCR products was outsourced to the Australian Genome Research Facility (AGRF) in Adelaide. Consensus sequences from forward and reverse reads were aligned using the GENEIOUS PRO 5.4 software (Drummond *et al.* 2009) and then manually edited and refined by eye. Aligned sequences of the protein coding genes were translated into amino acid sequences to check for premature stop codons that might indicate amplification of pseudogenes and determine the correct reading frame. The program PHASE v2.1.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003) was used to assign single nucleotide polymorphisms (SNPs) derived from the anonymous nuclear markers to a single allelic copy. The sequences generated in this study are deposited in the GenBank under the accession numbers KJ653824-KJ654006 (see Appendix S1).

Microsatellite genotyping

We used ten microsatellite markers developed for *Hydrophis elegans* and shown to cross-amplify successfully in *H. curtus* (Lukoschek & Avise 2011) to examine population subdivision and recent gene flow. These loci were amplified using Multiplex ready technology (MRT) (Hayden *et al.* 2008). Amplifications of each locus were done independently following thermal cycler settings specified for the MRT method (Hayden *et al.* 2008). All forward and reverse primers were tagged with MRT tag sequences to their five prime ends. Amplifications were performed in 12 μL volumes using 3.36 μL of genomic DNA (DNA concentration ~6.5 μg/mL), 3.0 μL of nuclease-free water, 2.4 μL of MRT buffer, 0.09 μL of the fluorescent tag (Fam, Vic, Pet, Ned), 0.09 μL of the reverse tag (tag R), 0.06 μL of Immolase taq polymerase (Bioline Reagents Pty. Ltd., Alexandria, NSW, Australia) and 3.0 μL of the 0.4 μM locus-specific primer pair. After successful amplification, the PCR products for each individual were pooled, and the pooled products were cleaned using vacuum filtration and sent for fragment analysis to AGRF in Adelaide. Allele sizes were scored using the software GENEMAPPER version 3.7 (Applied Biosystems).

Analyses of mtDNA sequence data

Mitochondrial genealogies were reconstructed using Bayesian inference and maximum likelihood (ML) methods. The three mtDNA markers were concatenated and analysed together because they are inherited as a single locus. Partitioning schemes and best-fit substitution models for each partition were assessed for the concatenated data set using the Bayesian information criterion (BIC) implemented in PARTITIONFINDER v1.0.1 (Lanfear *et al.* 2012). Three partitions were selected: (i) first codon positions of *Cytb and ND4*, *16SrRNA* and *tRNA*; (ii) second codon positions of *Cytb* and *ND4*; and (iii) third codon positions of *Cytb* and *ND4*. The best-fit substitution models were HKY+i+G, HKY+G and HKY+G for the first, second and third partitions, respectively.

Bayesian estimation of mitochondrial genealogies and divergence times was implemented in BEAST v1.7.4 (Drummond & Rambaut 2007). As there are no viviparous sea snake fossils that can be used to calibrate the tree, we used a pairwise mtDNA divergence rate of 2.7% per my, calculated using the root age prior mean of 6.2 my and the maximum-corrected sequence divergence within Hydrophiini for the concatenated mitochondrial alignment (Sanders *et al.* 2013a). The analysis was run for 50 million generations, sampling every 10 000 generations, with an Lognormal uncorrelated relaxed clock model of branch rate

variation and a Bayesian Skyline tree shape prior. Model parameters and clock models were linked across partitions. The analysis was repeated four times with different random number seeds to test the consistency of the outcome of the analyses. Convergence was assessed by examining effective sample sizes (ESS values >200) and likelihood plots through time in TRACER v1.5 (Rambaut & Drummond 2007). The BEAST maximum credibility trees were summarized in TREEANNOTATOR v1.7.4 (distributed with BEAST package) with the first 25% of trees discarded from each run as burn-in.

Partitioned maximum likelihood analyses were implemented in RAXML v7.2.6 (Stamatakis 2006). *Hydrophis (Astrotia) stokesii* was used as an out-group to root the ML tree based on the close but reciprocally monophyletic relationship between this species and *H. curtus* (Sanders *et al.* 2013a). The analysis used the GTR+G substitution model and the same partitions used in the Bayesian analyses with 200 independent ML searches. Branch support was estimated using 1000 bootstrap pseudoreplicates.

Levels of mtDNA sequence divergence were calculated between mitochondrial lineages and sampling localities using corrected (HKY) pairwise distances for the *Cytb* gene in GENEIOUS PRO 5.6 (Drummond *et al.* 2009). Hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) was conducted for the mitochondrial *Cytb* gene to examine the proportion of molecular variance explained by differences between regions, between sampling locations within regions and within locations. We only used the *Cytb* gene in this analysis because this gene was sequenced for the most individuals (and all mitochondrial markers are linked and thus should show the same demographic history). The analysis was done in ARLEQUIN version 3.5.1 (Excoffier & Lischer 2010) using mitochondrial haplotype frequencies and variance structure defined according to the four geographically delimited clades recovered by the mitochondrial genealogy: (i) Indian Ocean (Sri Lanka, India, Myanmar), (ii) Phuket-Thailand, (iii) South-East Asia (West Java, East Java, Sulawesi, Vietnam) and (iv) Australia (South Groote, Weipa).

Analyses of nuclear DNA sequence data

Nuclear allele networks were generated for the anonymous nuclear loci *G1894* and *G1888* using the median-joining method (Bandelt *et al.* 1999) implemented in NETWORK v.4.6 (fluxus-engineering.com). The analysis employed an equal weighting for each nucleotie substitution with the default zero epsilon parameter value.

Analyses of microsatellite data

The ten microsatellite markers were initially tested for significant deviations from Hardy–Weinberg equilibrium and linkage equilibrium within populations (defined based on Structure analysis) using exact tests (Guo & Thompson 1992) implemented in GENEPOP Web Version 4.2 (Raymond & Rousset 1995; Rousset 2008). Significance levels were estimated using the Markov chain algorithm of Guo & Thompson (1992) with 10 000 runs and 1000 dememorization steps.

Population differentiation and fixation indices. To estimate population differentiation, allelic differentiation was calculated between population pairs for combined microsatellite loci using Fisher's exact probability test in GENEPOP Web version 4.2 with 10 000 MC runs (Guo & Thompson 1992) and 1000 dememorization steps. To estimate microsatellite differentiation between sampling locations and the four clusters recovered by Structure (see below), pairwise fixation indices (F_{ST} values) were calculated with 1000 permutation tests of significance for all loci combined in ARLEQUIN version 3.5.1 (Excoffier & Lischer 2010).

Bayesian population genetic assignment. Population structure was assessed for the ten microsatellite loci combined using Bayesian cluster analysis executed in the software Structure 2.3.4 (Pritchard *et al.* 2000). Analyses were run using an admixture model (allowing mixed ancestry in multiple clusters) with correlated allele frequencies among populations. To infer the most probable number of ancestral clusters (K), analyses were run with $K = 1$ to $K = 10$ with ten runs for each K using 500 000 MCMC iterations after a burn-in period of 200 000 iterations. The optimum number of K was assessed using log-likelihood values visualized in Structure Harvester Web version 0.6.93 (Earl & vonHoldt 2012) and likelihood ratio tests performed on the mean log-likelihood values of each K. This K value was used in the final analysis, and the analysis was run by increasing the MCMC iterations to 700 000 after a burn-in period of 300 000 iterations with three replicates; convergence of parameters and likelihood values among the separate runs were estimated by examining α and likelihood values.

Analysis of molecular variance. Finally, hierarchical AMOVA (Excoffier *et al.* 1992) was performed in ARLEQUIN version 3.5.1 (Excoffier & Lischer 2010) using all ten microsatellite loci to compare proportions of molecular variance between regions, between sampling locations within regions and within locations (defined as for the mitochondrial analyses, above).

Neutrality tests and historical demography

To assess the demographic history of *H. curtus* populations, we examined mismatch distributions and calculated Tajima's D (Tajima 1989), Fu's Fs (Fu 1997), Ramos-

Onsins and Rozas' R_2 statistic and nucleotide diversity (π) for the SE Asian and Australian mitochondrial clades using the mitochondrial *Cytb* gene. Because the PT population had a small sample size and the IO population was represented by a single *Cytb* haplotype, we did not estimate demographic parameters for these two clades. Calculation of Tajima's D, Fu's Fs, Ramos-Onsins and Rozas' R_2 statistic and nucleotide diversity (π) were done in DNASP version 5.0 (Librado & Rozas 2009). Ramos-Onsins and Rozas' R_2 statistic was calculated with coalescent simulations to test for significant deviations from a constant population. Mismatch distributions, sum of squares deviation (SSD) and the Harpending's raggedness index (RI) were estimated for the observed data and compared to the test statistics from data simulated (100 bootstrap replicates) under a sudden demographic expansion model in ARLEQUIN version 3.5.1 (Excoffier & Lischer 2010). Tajima's D and Fu's Fs values were calculated to detect significant departures from equilibrium conditions, indicating recent population expansion. Because these analyses do not separate the effect of population expansion from positive selection, Tajima's D was calculated within each clade for synonymous sites and for non-synonymous sites, separately. If population expansion has occurred, then, Tajima's D calculated for synonymous sites should be significantly negative (following Burbrink *et al.* 2008).

Isolation by distance

To test whether the observed pattern of genetic structure can be explained by isolation by distance (IBD), tests of correlations between genetic distances and geographic distance matrices were implemented in the R Statistical analysis software (R Development Core Team 2008). Genetic distances were estimated as a measure of corrected (HKY) sequence divergence between populations for the mitochondrial *Cytb* gene and as a measure of population differentiation (Fixation index (F_{ST})) for the ten microsatellite loci. The geographic distances were measured as the coastal distances between pairs of locations using the software Google Earth version 5.1, because *H. curtus* is largely restricted to shallow habitats and most likely disperses along coastlines (Lukoschek *et al.* 2010). Sampling localities within Sri Lanka and Australia were grouped as single (separate) populations due to the short distances between sampling sites at these localities. Geographic distances were log-transformed before the analysis. Data were initially tested using residuals vs. fitted values plot to check whether they satisfied the assumptions of a linear regression model. As both data sets appeared to violate a linear model, Spearman's rank correlation test was used to test the relationship between genetic distance and geographic distance.

Results

Analysis of mtDNA sequence data

Bayesian and Maximum Likelihood analysis yielded very similar topologies and strongly recovered a deep basal divergence between monophyletic groups (clades) corresponding to Indian Ocean (IO) vs. mostly West Pacific (WP) localities (BPP > 0.9, BS > 70) (Fig. 2). The IO clade was separated from the WP clade by mean pairwise-corrected *Cytb* genetic distances of 9.3–9.5%. The IO clade consisted of specimens from Myanmar, Sri Lanka and India, but these regions were not reciprocally monophyletic. The WP clade consisted of three sub-clades with unresolved inter-relationships and relatively shallow mean genetic divergences (0.401–0.648%): (i) a Phuket-Thailand (PT) clade of five individuals collected from Phuket, Thailand; (ii) a SE Asian clade of 25 specimens from the south coast of West Java, East Java, south Sulawesi and Vietnam; and (iii) an Australian clade of 13 specimens collected from two locations in northern and north-eastern Australia. Bayesian divergence time estimates suggest that the IO and WP clades diverged about 2.8 million years ago (mya) (0.85–4.83 mya, 95% HPD interval), whereas divergences within the WP clade occurred much more recently, approximately 0.15–0.7 mya (95% HPD interval).

AMOVA for the *Cytb* gene showed that a significant ($P = 0.013$) proportion (95.9%) of the genetic variation was explained by variation among regions (i.e. IO, PT, SE Asia and Australia) (Table 1), consistent with the geographic population subdivision observed in the mitochondrial genealogy and microsatellite cluster analysis (see Structure analysis).

Analyses of nuclear DNA sequence data

Hydrophis curtus in the IO and WP did not share any alleles at either nuclear locus, indicating strong population subdivision between these two Oceanic regions.

Six alleles were present in the *G1894* anonymous nuclear locus (Fig. 3A). Indian Ocean specimens (all from Sri Lanka) were represented by two unique alleles that were not shared with the other individuals in PT, SE Asia and Australia (the sample from Myanmar failed to amplify for this locus). Individuals from Australia, PT and Vietnam were represented by a single allele that was also shared with individuals from other WP localities. Individuals from Sulawesi were represented by four alleles, while samples from East Java and West Java were represented by two alleles.

Eleven nuclear alleles were present for the *G1888* anonymous nuclear locus (Fig. 3B). Individuals from the IO (Sri Lanka and Myanmar) did not share any alleles with specimens from other sampling locations. Individuals from

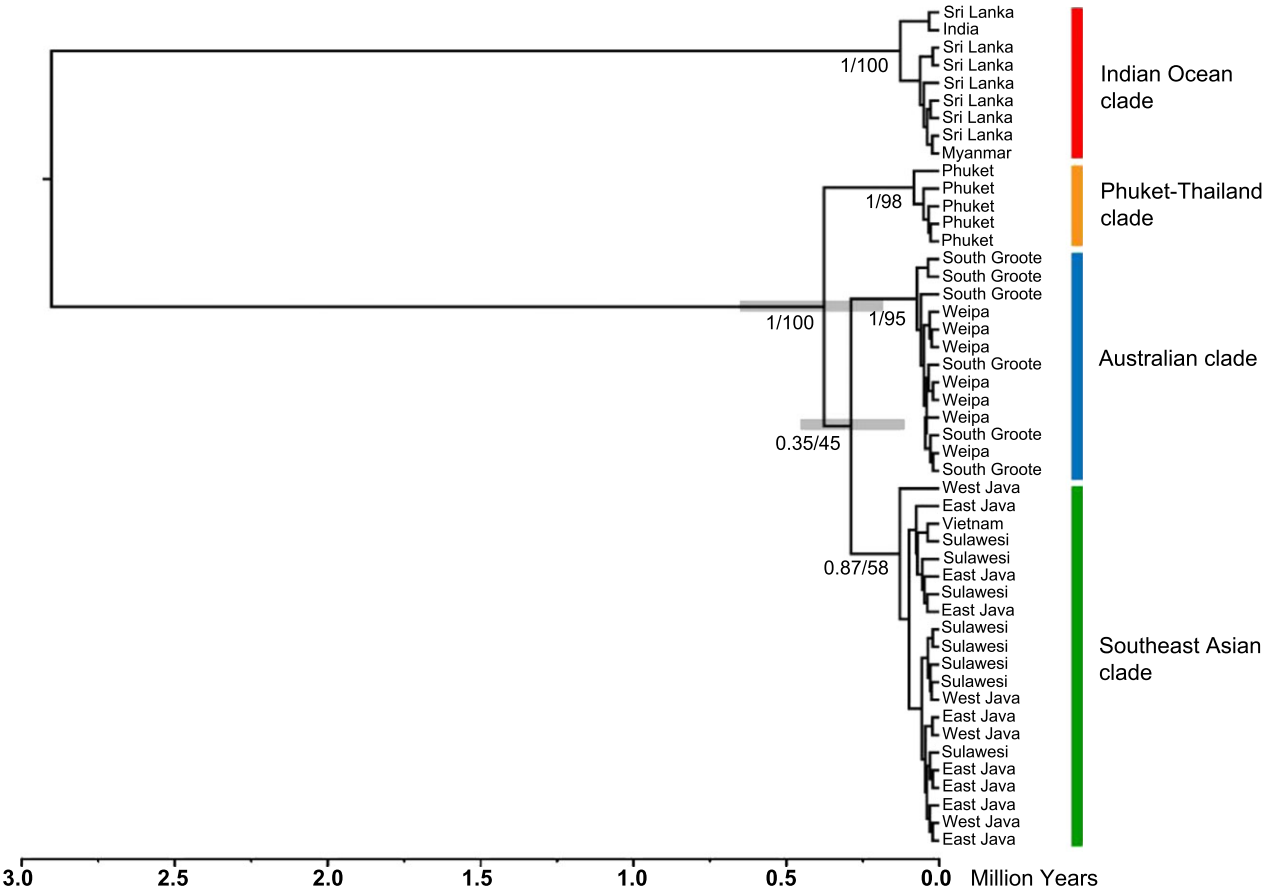

Fig. 2 Time calibrated maximum clade credibility ultrametric tree of concatenated mitochondrial DNA of *Hydrophis curtus*. The time scale is in millions of years before present. The grey horizontal bars indicate 95% highest posterior distribution (HPD) intervals of node ages, and node support is indicated at each major node (Posterior probability/Bootstrap support).

Table 1 Hierarchical AMOVA analysis for *Hydrophis curtus* from nine sampling locations

	Mitochondrial DNA (*Cytb*)			Microsatellite loci		
Source of variation	Variation	Percentage of variation (%)	*P*-value	Variation	Percentage of variation (%)	*P*-value
Among regions	11.382	95.90	0.012	0.684	19.60	0.003
Among locations within regions	2.638	0.11	0.341	0.026	0.74	0.402
Within locations	15.135	3.99	0.000	2.780	79.66	0.000

Sri Lanka were represented by three alleles of which one was shared with the sample from Myanmar. The most common allele for the *G1888* locus was shared among individuals from East Java, West Java, Australia and Vietnam. Individuals from PT were represented by three unique alleles that were not shared with other samples from any other locations. Samples from East Java and Australia were represented by two different alleles, West Java by three alleles, while samples from Sulawesi and Vietnam were represented by a single allele.

Analyses of microsatellite data

The total number of alleles per locus screened for 51 individuals ranged from 7 (loci He792 and He953) to 18 (locus He962) with a mean of 11.8. Exact tests indicated that all ten microsatellite markers were in Hardy–Weinberg

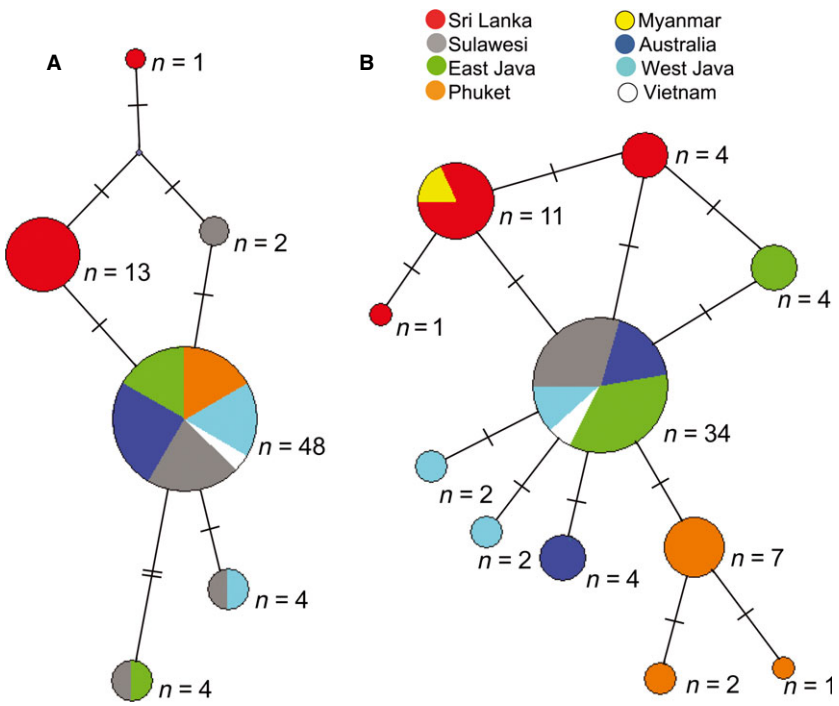

Fig. 3 Median-joining allele networks of (A) *G1894* and (B) *G1888* anonymous nuclear loci. Each allele is represented by a circle, and the size of each circle is proportional to the number of alleles (*n*). Colours correspond to the sampling location, and each small black cross line indicates a single positional change between two alleles.

equilibrium ($P > 0.05$) and linkage equilibrium ($P > 0.05$) within populations.

Population differentiation and fixation indices. The Fisher's exact probability tests for combined microsatellite loci indicated highly significant ($P < 0.05$) population differentiation between all population pairs. The overall F_{ST} value for all loci between IO and WP (combined PT, SE Asia, Australia) was 0.174 and significant ($P < 0.05$). Overall F_{ST} values between sampling regions were high and significant ($P < 0.05$) ranging from 0.117 (PT–SE Asia) to 0.297 (IO–Australia) (Table 2). Overall F_{ST} values between sampling locations (excluding Myanmar and Vietnam due to the low sample sizes) ranged from 0.001 (East Java–West Java) to 0.289 (Sri Lanka–South Groote, Australia) (Table 2).

Bayesian population genetic assignment. Initial Bayesian cluster analyses in Structure recovered the highest mean

Table 2 Overall F_{ST} values for ten microsatellite loci in *Hydrophis curtus* among the sampling regions and locations

		Sampling region			
Sampling region	Sample size	Indian Ocean	Phuket	SE Asia	Australia
Indian Ocean	9	–			
Phuket	5	**0.243**			
SE Asia	25	**0.191**	**0.117**		
Australia	13	**0.297**	**0.207**	**0.194**	–

		Sampling location						
Sampling location	Sample size	SL	PT	WJ	EJ	SU	SG	WE
Sri Lanka (SL)	8	-	**0.245**	**0.204**	**0.176**	**0.189**	**0.289**	**0.273**
Phuket (PT)	5		-	**0.121**	**0.118**	**0.158**	**0.227**	**0.190**
West Java (WJ)	6			-	0.001	0.005	**0.225**	**0.192**
East Java (EJ)	9				-	0.011	**0.210**	**0.201**
Sulawesi (SU)	9					-	**0.212**	**0.210**
South Groote, Australia (SG)	5						-	0.082
Weipa, Australia (WE)	8							-

Bold text indicates significant ($P < 0.05$) F_{ST} values.

log-likelihood score (LnP(K)) of −1611.24 for four clusters ($K = 4$). Likelihood ratio tests also confirmed '$K = 4$' as the best value for K at a significance level of 0.05. The three runs with '$K = 4$' successfully converged resulting in the same geographically correlated clusters and similar log-likelihood values: (i) an IO cluster (Sri Lanka and Myanmar); (ii) a PT cluster (Phuket, Thailand); (iii) a SE Asian cluster (East Java, West Java, Sulawesi and Vietnam); and (iv) an Australian cluster (South Groote and Weipa) (Fig. 4). The IO, PT and Australian clusters showed limited mixed ancestry (qK values 1.6–12%) with other clusters, whereas all individuals in the SE Asian cluster shared at least 20–25% of ancestry with the PT cluster. Analysis with '$K = 5$' recovered the IO and Australian clusters but did not yield geographically meaningful divisions for the PT and SE Asian samples. The four clusters recovered by Structure closely agree with the groups delimited by mtDNA genealogy.

Analysis of molecular variance. AMOVA for all ten microsatellite loci revealed highly significant population subdivision among regions (IO, PT, SE Asia, Australia) ($P = 0.003$) and within sampling locations ($P = 0.000$). However, only 19.60% of the molecular variation was explained by among region variation (Table 1), whereas 79.66% of the variation was explained by within sampling location variation.

Neutrality tests and historical demography

Mismatch analysis of pairwise distances for the Australian and SE Asian clades each showed unimodal distributions (Fig. 5) suggesting a recent or sudden population expansion (Slatkin & Hudson 1991; Rogers & Harpending 1992). Comparisons of sum of squares deviation and raggedness index indicated that the hypothesis of sudden/recent population expansion could not be rejected ($P > 0.05$) for these two clades (Table 3). Tajima's D and Fu's Fs values for the whole gene were also negative and significant ($P < 0.05$) for these two clades. However, Tajima's D values for the synonymous sites were significantly negative ($P < 0.05$)

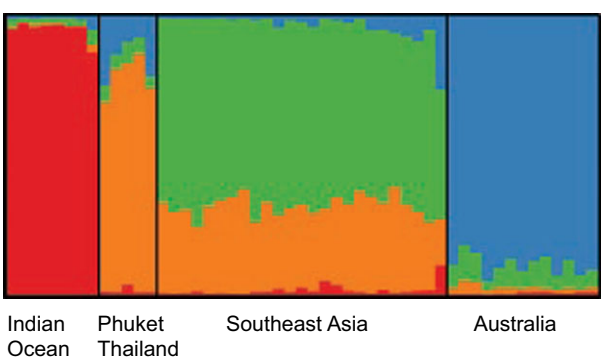

Fig. 4 Bayesian population assignment test of 51 *Hydrophis curtus* individuals based on 10 microsatellite loci. The four clusters that partition the data are displayed with different colours. Each vertical line represents one individual and its assignment likelihood (*Y*-axis from 0 to 1.0) into the four clusters is shown by the colour.

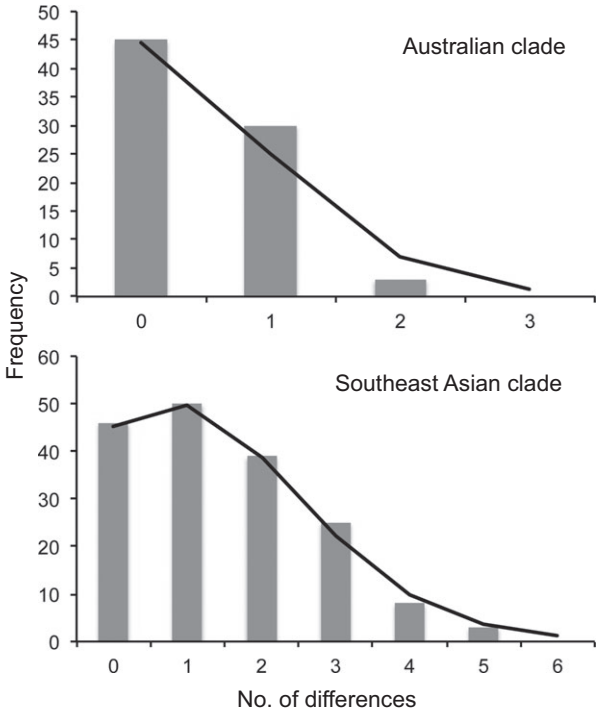

Fig. 5 Mismatch distributions for the *Cytb* gene in each mitochondrial clade. The grey bars depict the observed pairwise distributions, and black lines show the distribution simulated under a model of sudden/recent population expansion.

Table 3 Historical demographic analyses of geographically delimited mitochondrial clades

	Clade	
Test	Australian	SE Asian
N (number of samples)	13	19
π	0.461	1.462
Fu's Fs	**−0.537**	**−4.931**
Fu's Fs 95% CI	−2.042 to 3.359	−4.953 to 4.548
Tajima's D (all)	**−1.599**	**−2.231**
Tajima's D (all) 95% CI	−1.599 to 2.045	−2.414 to 1.791
Tajima's D (synonymous)	–	**−2.126**
Tajima's D (non-synonymous)	−1.429	−1.511
R_2 statistic	**0.140**	**0.072**
P-value (SSD)	0.570	0.990
P-value (RI)	0.620	0.990

Bold text indicates significant ($P < 0.05$) values.

only for the SE Asian clade. It was not significant ($P > 0.05$) for both clades for the non-synonymous sites. Significantly negative values of Tajima's D and Fu's Fs indicate an excess of low-frequency polymorphisms compared to that expected for null neutral hypothesis in an equilibrium population. Significantly negative values for Tajima's D for the synonymous sites rather than the non-synonymous sites in the SE Asian clade robustly indicate population expansion. Ramos-Onsins and Rozas' R_2 statistic significantly deviated from a constant population size in both SE Asian ($R_2 = 0.072$, $P = 0.000$) and Australian ($R_2 = 0.140$, $P = 0.036$) populations.

Isolation by distance

Spearman's rank correlation tests revealed a significant positive correlation between the genetic and geographic distances for both *Cytb* ($\rho = 0.534$, $P = 0.003$) and the microsatellite markers ($\rho = 0.678$, $P = 0.006$). Spearman's rank correlation tests within the WP (excluding samples from IO) found a significant correlation between genetic and geographic distances for microsatellites ($\rho = 0.818$, $P = 0.006$), but not for *Cytb* ($\rho = 0.508$, $P = 0.053$).

Discussion

Our molecular analyses revealed strong geographic subdivision within *Hydrophis curtus* with a prominent genetic break between populations distributed primarily in the Indian Ocean (IO) and West Pacific (WP). These two groups showed reciprocally monophyletic mitochondrial relationships and fixed nuclear sequence differences. Microsatellite population assignment (using Structure) and F_{ST} values (0.174) further suggested little recent gene flow between the two oceanic regions despite the present lack of geographic barriers. Within the WP group, genetic subdivision was present with distinct populations in the Phuket-Thailand region, South-East Asia and Australia. These three regions were represented by reciprocally monophyletic mitochondrial clades that had unresolved interrelationships, and concordant microsatellite clusters that showed limited admixture and relatively high and significant pairwise F_{ST} values (0.117–0.207). This cryptic lineage diversity and our divergence time estimates are discussed below with reference to the geo-climatic history of the region and findings for other Indo-Australian Archipelago (IAA) taxa.

The Indo-West Pacific break: vicariance and cryptic speciation?

BEAST divergence time estimates (Fig. 2) suggest that the major split between *H. curtus* in the IO and the WP took place in the Plio-Pleistocene approximately 2.8 mya (95% HPD interval: 0.85–4.83). This date is consistent with the ~9% sequence difference and a pairwise substitution rate of 3.3% per million years estimated for the *Cytb* gene in Hydrophiinae (Sanders *et al.* 2013a), and closely corresponds to the onset of sea level fluctuations in the region ~2.6 mya (Voris 2000; Lambeck *et al.* 2002). Hence, it is possible that the Sunda shelf/Indo-Pacifc biogeographic barrier that formed during low sea level stands caused vicariance of *H. curtus* populations spanning the IO and WP. Under this scenario, we would expect samples from the western Sunda shelf to show closest affinity to the IO samples. However, snakes from Phuket-Thailand and the south coast of West Java were robustly placed with WP samples in mitochondrial genealogies (Fig. 2), nuclear networks (Fig. 3) and microsatellite population assignment tests (Fig. 4). Interestingly, this pattern is consistent with recent phylogeographic studies of two other aquatic snakes: the salt water tolerant amphibious *Cerberus rynchops* (Alfaro *et al.* 2004) and the viviparous sea snake *Hydrophis cyanocinctus* (Sanders *et al.* 2013a). It is possible that the IO *H. curtus* genotypes on the western side of the Sunda shelf (PT and West Java) were replaced by WP genotypes following the disappearance of the biogeographic barrier with rising sea levels. Molecular evidence suggests that the coastal regions around the southern Thai-Malay peninsula and western coasts of the islands of Sumatra and Java act as a zone of secondary contact for previously isolated IO and WP marine biota (Marie *et al.* 2007; Hobbs *et al.* 2009; Gaither *et al.* 2011). Denser population sampling on the Andaman coast is needed to quantify recent gene flow between IO and WP *H. curtus* populations; however, in the present study, no admixed individuals were sampled in the microsatellite analysis, and *H. curtus* from these regions did not share any mitochondrial haplotypes or alleles at nuclear sequence markers. The distribution of WP individuals in West Java might alternatively be explained by the presence of cold surface-water patches off the southern coast of Java and north-western coast of Australia that resulted from upwellings during the last glacial maximum (LGM) (Martinez *et al.* 1999; Takahashi & Okada 2000). This phenomenon may have restricted the dispersal of temperature-sensitive animals so that as a thermal conformer, *H. curtus* may have been excluded from the waters of southern Java during the LGM and only recently colonized from neighbouring WP populations.

Phylogeographic structure within the West Pacific

Mitochondrial (Fig. 2) and microsatellite data (Fig. 3) revealed further population substructure within the WP. Microsatellite data indicated more frequent gene flow between SE Asian and PT populations compared to the Australian population. This likely reflects the close proximity and current connection of PT to other SE Asian sampling localities via shallow water habitat. In contrast,

Australian and SE Asian populations are separated by the Timor trench, which is ~3 km deep, 80 km wide and carries the Indonesian throughflow current (Fig. 1). The great expanse of deep sea (>200 m) between the SE Asian and northern Australian waters probably also poses a barrier for dispersal of *H. curtus* between these regions. The close timing of divergence among the three WP clades (0.15–0.7 mya, 95% HPD interval) (Fig. 2) suggests their separation could have been initiated by the same or closely spaced sea level fluctuations. Although other mechanisms, such as tectonic activity and changes in sea surface circulation may also have influenced isolation and divergence of WP *H. curtus* populations, their effects are yet to be determined.

Low microsatellite F_{ST} values (0.001–0.011) and lack of geographic structure among mitochondrial haplotypes suggest relatively high levels of connectivity within SE Asia. This is consistent with findings for some species of marine fish (Leray *et al.* 2010; Gaither *et al.* 2011) and invertebrates (Crandall *et al.* 2008a,b) that show no population genetic structure within SE Asia and the WP. The Makassar Strait is a deep-sea trench that runs between Borneo and Sulawesi and delimits Wallace's line, the boundary that separates the terrestrial biogeographic regions of SE Asia and Australasia. Some marine organisms that possess widely dispersing larval stages show deep phylogeographic breaks across the strong currents of the Makassar Strait contributing to the idea of a 'marine Wallace's line' (Barber *et al.* 2000; Lourie & Vincent 2004). However, in contrast to the much wider Timor trench (80 km), the Makassar Strait does not appear to pose a substantial challenge to dispersal in direct-developing marine snakes.

Historical demographic analyses reveal recent population or range expansion of *H. curtus* in the Australian and SE Asian lineages (Fig. 5). Previous studies of another species of viviparous sea snake also found evidence of recent population expansions in the Gulf of Carpentaria (GOC) in Australia (Lukoschek *et al.* 2007, 2008). These findings are consistent with the drying of the GOC during Pleistocene low sea level stands (Torgersen *et al.* 1985): *H. curtus* populations that inhabit the GOC today could be descendants of populations that colonized the area about 10 000 years ago when the sea levels reached their current levels. The similarly reduced extent of shallow seas in SE Asia during Pleistocene low sea level periods (see Fig. 1) may also have subjected *H. curtus* populations to range contractions/population reductions and subsequent postglacial expansions.

Effect of isolation by distance on population genetic structure

Our results from both *Cytb* and microsatellite data supported a pattern of IBD in *H. curtus*. However, the lack of significant correlation between genetic distance and geographic distance among locations in the WP for *Cytb* data indicates that the deep genetic divergence across the Sunda shelf/Indo-Pacific biogeographic barrier accounts for the signal of overall IBD. Therefore, it is most likely that rather than IBD, Plio-Pleistocene vicariance across the Sunda shelf and Timor trench biogeographic barriers explain population subdivision of *H. curtus* in the IWP.

Taxonomic implications

Hydrophis curtus was previously placed in the genus *Lapemis* with one other species, *L. hardwickii*. *Lapemis curtus* was recognized from the Indian Ocean (Arabian Gulf to Myanmar), while *L. hardwickii* was recognized in SE Asia and Australasia (Mergui Archipelago-Myanmar to South China seas and northern Australia) (Smith 1926). These species were diagnosed according to differences in their parietal and ventral scales (Smith 1926). However, Gritis & Voris (1990) examined nearly 1400 specimens and found that these characters varied continuously across the species' collective range and as a result referred both species to *L. curtus*. Given that our molecular genetic analyses strongly support the presence of two largely reproductively isolated species in the IO and WP, a re-evaluation of additional morphological traits that may separate these lineages is needed. Thus, we refrain from delimiting species solely on current evidence; further information is needed to determine the genetic structure at possible contact zones and the mechanisms (biological or environmental) that maintain species boundaries in these zones.

Conclusions

Our molecular results reveal a phylogeographic history of *H. curtus* that is highly concordant with other marine taxa spanning the Indo-West Pacific. Further, the deep species-level divergence and limited recent gene flow between IO and WP populations provide evidence of possible cryptic speciation across the Sunda shelf/Indo-Pacific biogeographic barrier. Overall, these results support an important role for Plio-Pleistocene vicariance events in generating population genetic and species diversity in marine snakes in the Indo-West Pacific.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Specimen collection localities, museum voucher numbers and GenBank accession numbers of the sequences used in the study.