Systematics, Evolution and Biogeography of Viviparous Sea snakes of the Indo-Pacific

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Doctor of Philosophy

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Abstract

Viviparous sea snakes are an exceptionally diverse radiation of secondarily marine reptiles that inhabit the shallow tropical and subtropical waters of the Indian and Pacific Oceans with the peak diversity in the Indo-Australian Archipelago (IAA). Although sea snake biology, natural history and diversity are relatively well known, they have a highly unstable taxonomy, and poorly understood evolutionary and biogeographic histories. This thesis examined the systematics, species limits, historical biogeography and diversification of Indo-Pacific viviparous sea snakes using molecular phylogenetics and a combination of external and internal morphological characters.

In the second chapter of this thesis, I describe a highly distinctive new species of viviparous sea snake from shallow estuarine waters of the Gulf of Carpentaria, northern Australia. Molecular analyses placed the new species, named ‘rough scaled’ sea snake, *Hydrophis donaldi*, as a deeply divergent lineage within the *Hydrophis* subgroup.

A multi-locus analysis and a morphological examination of the dangerously venomous and widely distributed ‘beaked’ sea snakes, *Hydrophis schistosus*, in the third chapter showed that they actually consist of two separate species in Asia and Australia that are not each other’s closest relatives. This finding suggested that the beaked sea snakes represent an extreme case of convergent phenotypic evolution in response to similar dietary specialisations, providing important implications for snakebite management.

In the third chapter of this thesis I investigated how past and present barriers to dispersal caused by historical geoclimatic events in the IWP have influenced fine-scale population genetic structure and speciation in the widely distributed ‘spine-bellied’ sea snake, *Hydrophis curtus*. Analyses of mitochondrial and nuclear sequences and microsatellite variation sampled across the IWP strongly indicated population subdivision in *H. curtus* with a deep species level genetic break across the Indian Ocean and West Pacific. These findings further demonstrated that the Indo-Pacific biogeographic barrier in the Plio-Pleistocene may have a significant role in generation of biodiversity in the IAA.

Phylogenetic analyses and biogeographic reconstructions of Indo-West Pacific (IWP) viviparous sea snakes in chapter four indicate that despite their origins in Australasia, sea snakes underwent an explosive *in-situ* radiation during the last 2.5 to 0.3 million years after colonizing Southeast Asia with subsequent dispersals to Australasia and the Indian Ocean. The high speciation rates in the core *Hydrophis* group and allopatric population divergence between the Indian and Pacific Oceans indicate an association with the Pleistocene sea level changes. Together these findings provide important insights to the origins and maintenance of high biodiversity in this marine biodiversity hotspot.

Findings on species boundaries, endemism and population structure in this thesis will directly benefit sea snake conservation and marine reserve management in the IWP. However, a need for more basic systematic studies on sea snakes is strongly implied by the discovery of cryptic lineages and the new species. The inability of temporal diversification patterns to explain the rapid speciation of *Hydrophis* suggests that non-geographic speciation might be a major driving force in sea snake speciation. Hence other avenues of research (e.g niche relationships, adaptation genomics) may provide possible explanations to the high species diversity.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Kanishka D. B. Ukuwela

November, 2013

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CHAPTER 1: General Introduction

1.1. Investigating biological diversity and diversification processes

Systematics is the study of biological diversity, both in the past and present, and the relationships among evolutionary lineages through time (Schuh, 2000). It is a fundamental science in biology because it provides the foundation for all studies of organisms, by showing how organisms are related to each other. Relationships among species and their evolutionary histories are traced through phylogenies. Historically, phylogenies were reconstructed using morphological characters following the assumption that closely reated speceis are similar in morphology due to their shared ancestry. However, due to homoplasy this was not always reliable; after the advent of modern molecular biological techniques, DNA sequnces became an increasingly important source of data for reconstructing phylogenies. Although DNA sequences can also exhibit problems (e.g. saturation and homoplasy), they provide vastly more independent characters for phylogeny reconstructon. Currently, molecular phylogenies based on DNA data are not only used to infer relationships but also to trace morphological and ecological character evolution (Pagel, 1999), estimate speciation and extinction rates (Nee *et al.*, 1994; Ricklefs, 2007), infer geographical modes of speciation (Barraclough & Nee, 2001) and reconstruct biogeographic histories (Lomolino *et al.*, 2006). Furthermore, molecular phylogenies as well as population genetics are frequently used to set conservation priorities by defining evolutionary significant units (ESU) based on genetic uniqueness (Moritz, 1994).

One of the greatest advantages of molecular methods in systematics is the ability to discover morphologically cryptic species. Since speciation is not always accompanied by obvious phenotypic change, morphological traits do not always accurately delimit species boundaries. As a consequnce, recognition of cryptic species has increased exponentially after the development of PCR and DNA sequencing techniques (Bickford *et al.*, 2007). This suggests that the Earth's actual biological diversity is likely to be much higher than the current estimates, which are based primarily on species delimited largely on morphological grounds.

This thesis aimes to answer questions on the systematics, evolution and biogeographic history of viviparous sea snakes, a taxonomicaly challenging group of reptiles using modern molecular phylogenetic, population genetic and as well as traditional morphological methods.

1.2. Sea snake species diversity and phylogenetic context

Nearly 90 species of snakes with marine habits are known from the families Acrochordidae, Colubridae, Homalopsidae and Elapidae (Heatwole, 1999; Rasmussen *et al.*, 2011). Although all these species have occasionally been termed sea snakes/marine snakes, only the marine snakes in the family Elapidae qualify as sea snakes (Heatwole, 1999). The fully aquatic monogeneric family Acrochordidae comprise three species, of which only two species have marine or brackish habits (Figure 1 [A]). Colubridae is the largest snake family with nearly 2000 species in seven subfamilies (Pyron *et al.*, 2011), but contains only 11 species (Natricinae: Figure 1 [B]) that have definite marine affinities (Heatwole, 1999; Rasmussen *et al.*, 2011). The fully aquatic Asian and Australasian family Homalopsidae (mud snakes) have about 12 species that live in coastal habitats such as mangrove forests and salt marshes (Figure 1 [C]) (Murphy, 2007).

The large majority of marine snakes are found in two independent marine lineages of the family Elapidae (Heatwole, 1999; Rasmussen *et al.*, 2011). These two lineages include eight species of oviparous sea kraits that feed in coral reefs but come on to land to digest their prey and lay eggs (genus *Laticuada*: Figure 1 [D]) and at least 60 species of viviparous sea snakes (Figure 2). Molecular phylogenies show that these five lineages of marine-associated snakes have independantly colonized marine habitats (Sanders & Lee, 2008; Vidal *et al.*, 2009). The viviparous sea snakes not only represent the more recent marine snake radiation within Elapidae (Sanders *et al.*, 2008; Lukoschek *et al.*, 2012), but are also by far the most species rich and marine adapted (Dunson, 1975; Heatwole, 1999; Rasmussen *et al.*, 2011).

Figure 1. Some representative marine snakes: [A] *Acrochordus granulatus*, [B] *Nerodia clarkii* (photo: Kenneth Wray), [C] *Cerberus rynchops*, [D] *Laticuada colubrina* (photo: Aaron Lobo)

1.2.1 Study taxon: the viviparous sea snakes (Hydrophiinae)

This thesis focuses on the viviparous sea snakes or the ‘true’ sea snakes, an adaptive radiation that is phylogenetically nested within the Australasian terrestrial snakes of the Hydrophiinae subfamily in the family Elapidae (Mcdowell & Cogger, 1967; Slowinski *et al.*, 1997; Keogh, 1998; Sanders & Lee, 2008). Molecular evidence suggests that viviparous Australian terrestrial elapid snakes of the genus *Hemisapis* Fitzinger, 1861 are the closest terrestrial living relatives of the viviparous sea snakes (Keogh *et al.*, 1998; Sanders *et al.*, 2008) and these two groups shared a common ancestor about 10.6-6.5 million years ago (mya) (Sanders *et al.*, 2008; Lukoschek *et al.*, 2012). Currently 62 ecomorphologically diverse species of viviparous sea snakes are known from coastal marine habitats in the Indian and Pacific oceans (Elfes *et al.*, 2013).

Viviparous sea snakes show many adaptations to marine life such as paddle-shaped tails for locomotion, sublingual glands to excrete excess salt, skin with low permeability to water, nostrils with valves that exclude water when diving, relatively higher lung capacity and significantly high amounts of cutaneous respiration that helps in oxygen uptake and nitrogen excretion when underwater (Dunson, 1975; Heatwole, 1999). Although they are commonly called viviparous sea snakes, they are actually ovoviviparous and give birth to live young at sea. This likely represents an important pre-adaptation to marine habits. In contrast, the sea kraits depend on land for oviposition. Venom might also have contributed to the ecological and evolutionary success of sea snakes: most species are dangerously venomous (excluding five species that feed exclusively on fish eggs: (Li *et al.*, 2005; Dotsenko, 2011; Sanders *et al.*, 2012), and like all other elapids they have neurotoxic venom and a front fanged (proteroglyphous) venom delivery system (McCarthy, 1985). Viviparous sea snakes feed mostly on fish and eels and they display a remarkable diversity of ecomorphological adaptations for dietary specialization (Voris, 1966; Voris & Voris, 1983). Although they have well developed eyes, they find prey mainly by olfaction with the assistance of the tongue and the Jacobson’s organ (Heatwole, 1999).

Viviparous sea snakes are found only in warm shallow tropical and subtropical seas of the Indian and Pacific Oceans. It is believed that the viviparous sea snakes could not get to the Atlantic Ocean prior to the formation of the Isthmus of Panama ~5 mya and since they avoid cold waters, they could not enter the Atlantic Ocean from other temperate seas (i.e. Southern tip of Africa and Southwestern coast of South America). However, the recent finding of two specimens of the most widespread sea snake *Hydrophis platurus* from the Namibian coast (Branch, 1998) indicate that sea snakes may be extending their range into the Atlantic Ocean.

Most viviparous sea snakes inhabit shallow regions less than 100m in depth, and are found in diverse habitats, including coral reefs, estuaries, lagoons, in-shore river systems and sea grass beds. Two species are also known to occur in fresh water ecosystems (Rasmussen *et al.*, 2001; Rasmussen *et al.*, 2011). However, throughout much of their range viviparous sea snakes are threatened due to destructive fishery practices and coastal habitat degradation (Livingstone, 2009). Thus urgent conservation measures are needed to ensure the future survival of the sea snakes throughout their range. Nevertheless, more data is needed to assess the conservation status of many sea snakes before conservation measures can be implemented. Furthermore, the taxonomy of many species of viviparous sea snakes is not fully resolved and present phylogenetic study also indicate the presence many cryptic species/lineages. Since they pose a significant health risk to humans especially the fishermen handling nets, taxonomic resolution is important since accurate identification is essential for sea snake bite treatment. Moreover, venom variation has a strong phylogenetic component and is of vital importance in antivenom preparation (Chippaux *et al.*, 1991). Many conventional taxonomic methods which use morphology have provided limited success to resolve these uncertainties due to morophological convergence. However, molecular phylogenetics may offer more reliable insights to resolve most of these taxonomic and systematic complications.

This thesis uses molecular phylogenetics and as well as morphological methods to address these issuses. The following section reviews the historical development of the understanding of the sea snake systematics, phylogenetics and evolution as a precursor to the specific research questions addressed in this thesis.

1.3. Phylogeny and morphological systematics of viviparous sea snakes

1.3.1. Current taxonomy

Viviparous sea snakes are an explosively speciating group that has undergone extensive convergent diversification in skull shape and head-body proportions associated with dietary specializations (Voris & Voris, 1983; Sanders *et al.*, 2013a). As a consequence taxonomy and systematics of viviparous sea snakes were poorly resolved and they have variously been classified in 10 to 16 often monotypic or paraphyletic genera (Smith, 1926; McDowell, 1972; Voris, 1977; Rasmussen, 1997; Kharin, 2004a; Kharin, 2004b; Kharin, 2005; Kharin, 2012). The lack of a phylogenetic framework for these species has hindered attempts to assess their systematics, evolutionary relationships and patterns of diversification.

The viviparous sea snakes are currently placed in the family Elapidae, subfamily

Hydrophiinae along with 8 species of sea kraits and ~100 species of terrestrial Australasian elapids. Molecular data indicate that the sea kraits are the sister lineage to the clade that includes the Australasian terrestrial elapids and viviparous sea snakes, with the viviparous sea snakes nested within the Australasian terrestrial elapid clade (Slowinski *et al.*, 1997; Keogh *et al.*, 1998; Sanders & Lee, 2008). Recent molecular phylogenetic analyses suggests that the viviparous sea snakes consist of two major clades, the *Aipysurus* and *Hydrophis* groups (Lukoschek & Keogh, 2006; Sanders *et al.*, 2013a). The *Aipysurus* group comprise the genera *Aipysurus* (Figure 2 [A]) and *Emydocephalus*; the more diverse *Hydrophis* group consists of four primary lineages: three monotypic semi-aquatic genera *Ephalophis* (Figure 2 [B]), *Parahydrophis and Hydrelaps*, *Microcephalophis* (Figure 2 [C]) and core *Hydrophis* (Figure 2 [D]) (Sanders *et al.*, 2013a).

Figure 2. Some representative viviparous sea snakes: [A] *Aipysurus laevis* (photo: James Fatherree) [B] *Ephalophis greyae* (photo: Ray Lloyd), [C] *Microcephalophis gracilis*, [D] *Hydrophis spiralis*

1.3.2. Taxonomic history

Early studies based on morphological comparisons placed viviparous sea snakes and the oviparous sea kraits together, in their own family Hydrophiidae: as a result all sea snakes were thought to have a single origin (Boulenger, 1896; Wall, 1909). Subsequent morphological analyses further indicated two divisions in Hydrophiidae: Laticaudinae (sea kraits) and Hydrophiinae *sensu stricto* (viviparous sea snakes) (Smith, 1926). Based on geographic distributions of these two groups, it was speculated that the laticaudines had an Australian origin and hydrophiines had an Indo-Malayan origin (Smith, 1926). Later examination of temporal musculature implied that the viviparous sea snakes originated from the Australasian terrestrial elapid radiation and the sea kraits were an independent marine lineage that derived from the *Calliophis-Micrurus-Maticora* group of terrestrial elapids (Mcdowell & Cogger, 1967). Further studies on musculature and osteology strongly suggested the placement of Hydrophiidae (*sensu* Smith 1926) as a subfamily of Elapidae (McDowell, 1969; McCarthy, 1985) and the division of viviparous sea snakes into three groups; *Hydrelaps*, *Aipysurus* and *Hydrophis* (McDowell, 1972).

The first sea snake phylogeny based on morphology, identified three groups of sea snakes within Elapidae: *Laticauda*, *Aipysurus-Emydocephalus* and *Hydrophis* (Voris, 1977), a finding that was later supported by parietal and maxillary bone characters (Rasmussen, 1997). Based on these findings, it was believed that these three groups could have independent origins among the elapids, or they could have a single origin with early divergences soon after marine invasion (Voris, 1977). However, morphological examinations indicated that characters based on aquatic adaptations such as flattened tail and tail osteology were not always helpful in resolving evolutionary relationships of sea snakes due to convergence (Rasmussen, 1997). A subsequent morphological phylogenetic analysis (Rasmussen, 2002) recovered results similar to the previous phylogenetic study, but placed the *Aipysurus-Emydocephalus* clade with a separate group of Australasian terrestrial elapids. This led to the speculation that marine life evolved three times separately among elapids (Rasmussen, 2002). However, later molecular phylogenetic analysis (see below) challenged this idea.

Biomolecular analyses further advanced and increased the understanding of sea snake systematics and origins. A serological study strongly indicated the grouping of viviparous sea snake genera in to the two groups: *Acalyptophis, Astrotia, Pelamis, Lapemis* and *Hydrophis*; and *Aipysurus* and *Emydocephalus* (Minton & Da Costa, 1975). The immuno-electrophoretic patterns in this study also showed that the sea kraits are distantly related to the viviparous sea snakes. Immunological analyses (Mao *et al.*, 1977; Cadle & Gorman, 1981) and a peptide fingerprint assay of Hemoglobins (Mao *et al.*, 1978) provided biomolecular evidence for the three groups of sea snakes identified from the phylogenies based on morphology (Voris, 1977). Further, the close relationship between the *Aipysurus* and *Hydrophis* groups, the relationship between viviparous sea snakes and Australian terrestrial elapids and the young age of the *Hydrophis* lineage has been strongly implied through these Immunological (Cadle & Gorman, 1981) and serum albumin (Mao *et al.*, 1983) analyses.

1.3.3. Molecular phylogeny: recent rapid radiation

Molecular phylogenetic studies that examined elapid systematics using amino acid sequences of venom proteins (Slowinski *et al.*, 1997), DNA sequence data (Keogh, 1998; Keogh *et al.*, 1998; Sanders & Lee, 2008; Sanders *et al.*, 2008; Kelly *et al.*, 2009) and combined molecular and morphological data (Scanlon & Lee, 2004) independently verified that the viviparous sea snakes were closely related to Australasian terrestrial elapids, while the sea kraits were basal to the clade that contain both Australasian terrestrial elapids and viviparous sea snakes. These findings further validated the previously held beliefs that sea kraits and viviparous sea snakes are distantly related groups that independently colonized marine habitats. However, these studies also indicated the monophyly of the viviparous sea snakes (*Aiypysurus* and *Hydrophis* groups) challenging the previous finding of Rasmussen (2002). Further, the divergence dates implied by these molecular phylogenetic analyses were consistent with the discoveries of previous immunological and biochemical studies (Minton and da Costa, 1975; Mao *et al.*, 1978; Mao *et al.*, 1983). Time-calibrated molecular phylogenetic analyses using DNA sequence data estimated the split between sea kraits and the

Australasian terrestrial elapid-viviparous sea snake clade to be between 23-13.1 my (Sanders & Lee, 2008; Kelly *et al.*, 2009). Subsequent molecular phylogenetic analysis suggested that the viviparous sea snakes were closely related to the Australian terrestrial viviparous elapid snakes of the genus *Hemiaspis* (Keogh *et al.*, 1998; Sanders *et al.*, 2008) or basal to the clade that contained *Hemiaspis* and other Australian viviparous elapids (Sanders *et al.*, 2008; Lukoschek *et al.*, 2012). The split between the two lineages was estimated to be 8.3 my old (6.5-10.6 my 95% Highest Posterior Density) while the age of the viviparous sea snake crown group was estimated to be ~6.2 my (4.5-7.9 95% HPD) (Sanders *et al.*, 2008). This important finding suggested that viviparous sea snakes have rapidly diversified during a very short time period, corroborating the previous conclusion based on immunological distances (Cadle and Gormans 1981).

Molecular phylogenetic analyses that examined the relationships among viviparous sea snakes confirmed the reciprocal monophyly of the *Aipysurus* and *Hydrophis* groups *sensu* Smith (1926) (Lukoschek & Keogh, 2006; Sanders *et al.*, 2013a). Further, the latter study provided evidence for four distinct lineages within the *Hydrophis* group: two semi aquatic lineages (genera *Ephalophis-Parahydrophis* and *Hydrelaps*), *Microcephalophis,* and a 'core *Hydrophis* lineage' (genera *Acalyptophis, Astrotia, Disteira, Enhydrina, Hydrophis, Kerilia, Lapemis, Pelamis* and *Thalassophina)* (Sanders *et al.*, 2013a). The results further showed that the genus *Hydrophis* which includes the majority of viviparous sea snake species is broadly paraphyletic with respect to many other genera (*Acalyptophis, Astrotia, Distiera, Enhydrina, Kerilia, Lapemis, Pelamis, Thalassophina*) (Lukoschek & Keogh, 2006; Sanders *et al.*, 2013a). This finding is again consistent with previous immunological (Cadle & Gorman, 1981) and morphological (Rasmussen, 1994) evidence. Thus the placement of these mostly monotypic genera in the genus *Hydrophis* Latreille 1802 was proposed (Sanders *et al.*, 2013a).

Many hypotheses have been proposed to explain the rapid speciation of viviparous sea snakes. It has been assumed that the colonization of a novel habitat might have accelerated speciation as a result of the sudden availability of unoccupied ecological niches (Lillywhite *et* *al.*, 2008). However, this hypothesis has been challenged since it has been shown that the initial invasion of marine habitats was not accompanied by rapid diversification rates, but rather accelerated diversification occurred ~3-5 million years after colonization (Sanders *et al.*, 2010; Sanders *et al.*, 2013a). Recently it was proposed that rapid evolution of head and fore-body size variation within the *Hydrophis* group is a likely contributing factor in the explosive speciation in viviparous sea snakes (Sanders *et al.*, 2013b). Although, paleoclimatic and past geological events are also known to accelerate speciation (Hewitt, 2003; Janis, 2003), their role in the speciation of viviparous sea snakes is yet to be assessed. Despite these various hypotheses, the exact mechanisms responsible for rapid speciation in viviparous sea snakes still remain incompletely known.

1.4. Biogeographic history of viviparous sea snakes and Indo-Pacific marine biogeography

Given their phylogenetic position nested inside the terrestrial elapids endemic to Australasia, the ancestors of present day viviparous sea snakes most likely originated in the tropical or sub-tropical Australian seas and subsequently colonized the Southeast Asian, Indian and Pacific Oceans. Despite their origins in Australia, their greatest diversity is found in the Southeast Asian region of the Indo-Australian Archipelago (IAA) (Figure 3) in the Indo-West Pacific (Rasmussen *et al.*, 2011; Elfes *et al.*, 2013). As a result, the high diversity of viviparous sea snakes in Southeast Asia rather than Australasia raises an interesting question regarding their biogeographic origins and geographic patterns of diversification.

Coincidentally, the IAA is a marine biodiversity hotspot (Hughes *et al.*, 2002), home to the highest diversity of reef fishes and corals in the world (Hoeksema, 2007; Allen, 2008). Three competing hypotheses have been proposed to explain the high marine biodiversity in the region. It has been suggested that this region represents (1) a centre of origin/speciation, where new species are generated and are subsequently exported to peripheral areas (Ekman, 1953); (2) a centre of accumulation of diversity due to the dispersal of novel taxa into the region after speciation in isolated peripheral locations (Ladd, 1960); or (3) a region of overlap for marine biodiversity that originated in the Pacific and Indian Oceans (Woodland, 1983). A plethora of studies have provided evidence for each of these hypotheses (centre of origin: Veron, 1995; Briggs, 1999; Lind *et al.*, 2007; centre of accumulation: Jokiel & Martinelli, 1992; Drew & Barber, 2009; Eble *et al.*, 2011; centre of overlap: reviewed in Gaither & Rocha, 2013). Together these studies may indicate that a combination of all the above processes have generated the high marine biodiversity in the IAA (Randall, 1998; Bernardi *et al.*, 2004; Barber & Bellwood, 2005). As a result the 'biodiversity feedback model' has been proposed to explain the high marine biodiversity in the IAA and other tropical marine biodiversity hotspots (Bowen *et al.*, 2013). According to this new paradigm, biodiversity hotspots act as both centers of speciation and centers of accumulation and/or overlap.

Indo-Australian Archipelago has a complex geological and climatic history (Voris, 2000; Woodruffe, 2003). Paleoclimatic events such as Quaternary glacial cycles during the last 2.6 million years have had a major effects on sea levels, shorelines and climate of the region's Oceans (Lambeck *et al.*, 2002). These climatic and geological processes have created dynamic and varied opportunities for dispersal and vicariance for the marine biota (Lister & Rawson, 2003). Pleistocene glacial cycles throughout the last ~2.6 million years periodically lowered sea levels by more than 100m below current levels (Pillans *et al.*, 1998; Voris, 2000). Most dramatically, the exposed Sunda (Thai-Malay peninsula and Greater Sunda islands) and Sahul (Australia and New Guinea) continental shelves (Figure 3) subjected marine organisms spanning the Indian Ocean and West Pacific to alternating episodes of isolation and secondary contact. These cyclic climatic and geological processes are known to recurrently influence population genetic structure and speciation (Hewitt, 2000; Hewitt, 2003). Although, the affects of these historical climatic and geological processes on viviparous sea snakes are yet to be assessed, direct development and lack of a freely dispersing larval stage in viviparous sea snakes is expected to retain strong signals of historical population structure compared to most other marine groups (fishes and invertebrates) (Hoskin, 1997). Thus viviparous sea snakes present promising opportunities to examine the role of these past geo-climatic events on speciation and generation of biodiversity.

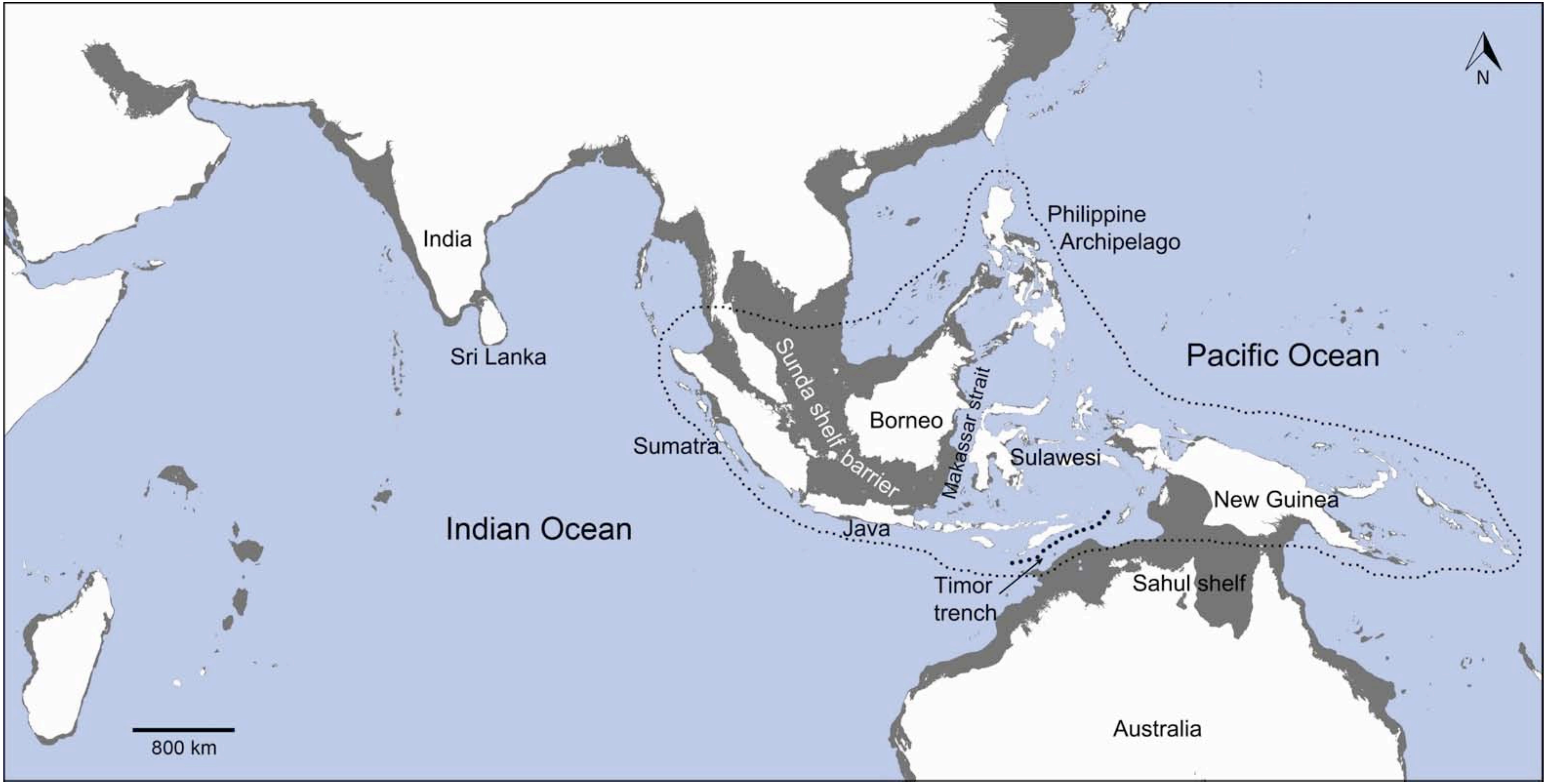

Figure 3. The Indo-Pacific marine biogeographic region with the Indo-Australian Archipelago demarcated by the thin dotted line. The grey areas denote the 120m isobath which indicate the extent of land when sea levels were ~120 m below present levels during the Pleistocene glacial maxima. Bathymetric data were taken from General Bathymetric Chart of the Oceans (GEBCO: http://www.gebco.net/).

Currently, the species boundaries, systematics and biogeographic history of viviparous sea snakes are very incompletely resolved. Phylogenetic relationships and phylogeographic affinities of many Indian Ocean taxa in particular remain undetermined. Further, hypotheses that may explain the origins and diversification of viviparous sea snake diversity in the IAA remains unanswered. Very little is known about the role of historical geo-climatic events and past and present barriers to dispersal on population genetic structure and speciation of the Indo-Pacific viviparous sea snakes. In this thesis, I examine the systematics, biogeographic history, temporal patterns of diversification and gene flow in viviparous sea snakes to shed light on the above issues using morphological, molecular phylogenetic and population genetic approaches.

1.5. Aims of the thesis

The overall aims of this thesis were to examine the systematics, diversity, biogeographic history and temporal patterns of diversification of viviparous sea snakes in the Indo-Pacific, with a particular focus on the poorly known Indian Ocean sea snake fauna. To address these objectives the research was carried out with four specific aims:

1. Use mitochondrial and nuclear sequences, external morphology and skeletal anatomy to formally describe a highly distinctive but previously undiscovered species of Australian viviparous sea snake, and investigate its placement within the broader sea snake phylogeny.
2. Examine species boundaries in medically important beaked sea snakes of the genus *Enhydrina* which spans Asia to Australia, using seven mitochondrial and nuclear loci in addition to diagnostic morphological characters and CT-scanning of skeletal anatomy.
3. Assess how past and present geographic barriers to gene flow in the Indo-Pacific have influenced population genetic structure and speciation in the spine-bellied sea snake, *Hydrophis curtus*, using population genetic analyses of microsatellite markers and

mitochondrial and nuclear sequences.

4. Investigate the biogeographic history and geographical and temporal patterns of diversification in Indo-West Pacific viviparous sea snakes by reconstructing the phylogenetic relationships and geographic range evolution across the Indian Ocean, West Pacific and Australian marine biogeographic regions.

1.6. Thesis Structure

The main body of this thesis consists of four research chapters that have been either published, accepted for publication or prepared to be submitted for publication in international, peer-reviewed academic journals. These research chapters are followed by a discussion chapter summarising the overall findings of this thesis. The research chapters are provided in the relevant journal format preceded by a title page and statements of authorship. The first two chapters of this thesis focus primarily on the systematics and phylogenetic relationships, and the third and fourth chapters examine the biogeographic history, temporal diversification patterns and phylogeography of viviparous sea snakes. Supplementary information is presented at the end of each chapter. The final discussion chapter summarises the overall findings, examine the broader implications and highlights promising future directions of this research.

CHAPTER 2: *Hydrophis donaldi* (Elapidae, Hydrophiinae), a highly distinctive new species of sea snake from northern Australia

STATEMENT OF AUTHORSHIP

Title of Paper: *Hydrophis donaldi* (Elapidae, Hydrophiinae), a highly distinctive new species of sea snake from northern Australia

Publication status: Published

Publication Details: Ukuwela, K.D.B., Sanders, K.L. & Fry, B.G. (2012) *Hydrophis donaldi* (Elapidae, Hydrophiinae), a highly distinctive new species of sea snake from northern Australia. *Zootaxa*, 3201: 45-57.

Author Contributions

Kanishka D. B. Ukuwela

Generated and analysed data, co-authored manuscript and acted as the corresponding author.

I hereby certify that the statement of contribution is accurate.

Signature………………………………….. Date…………………………………..

Kate L. Sanders

Assisted with generating data and contributed to writing the manuscript.

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Bryan G. Fry

Collected specimens and contributed to writing the manuscript.

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Signature………………………………….. Date…13th November, 2013…………….

Ukuwela, K.D.B., Sanders, K.L. & Fry, B.G. (2012) *Hydrophis donaldi* (Elapidae, Hydrophiinae), a highly distinctive new species of sea snake from northern Australia.
Zootaxa, v. 3201(21st February), pp. 45-57

NOTE:
This publication is included on pages 18-30 in the print copy
of the thesis held in the University of Adelaide Library.

CHAPTER 3: Molecular evidence that the deadliest sea snake *Enhydrina schistosa* (Elapidae: Hydrophiinae) consists of two convergent species

STATEMENT OF AUTHORSHIP

Title of Paper: Molecular evidence that the deadliest sea snake *Enhydrina schistosa* (Elapidae: Hydrophiinae) consists of two convergent species

Publication status: Published

Publication Details: Ukuwela, K.D.B., de Silva, A., Mumpuni, Fry, B.G., Lee, M.SY. & Sanders, K.L. (2013) Molecular evidence that the deadliest sea snake *Enhydrina schistosa* (Elapidae: Hydrophiinae) consists of two convergent species. *Molecular Phylogenetics & Evolution,* 66 (1): 262-269.

Author Contributions

Kanishka D. B. Ukuwela

Collected specimens, co-developed the research concept, generated and analysed data and co-authored the manuscript.

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Ukuwela, K.D.B., de Silva, A., Mumpuni, Fry, B.G., Lee, M.S.Y. & Sanders, K.L. (2013) Molecular evidence that the deadliest sea snake *Enhydrina schistosa* (Elapidae: Hydrophiinae) consists of two convergent species.
Molecular Phylogenetics and Evolution, v. 66(1), pp. 262-269

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CHAPTER 4: Multi-locus phylogeography of the spine-bellied sea snake (*Hydrophis curtus*, Elapidae) reveals historical vicariance and cryptic speciation

STATEMENT OF AUTHORSHIP

Title of Paper: Multi-locus phylogeography of the spine-bellied sea snake (*Hydrophis curtus*, Elapidae) reveals historical vicariance and cryptic speciation

Publication status: Accepted for Publication

Publication Details: Ukuwela, K.D.B., de Silva, A., Mumpuni, Fry, B.G., & Sanders, K.L. (2014) Multi-locus phylogeography of the spine-bellied sea snake (*Hydrophis curtus*, Elapidae) reveals historical vicariance and cryptic speciation. *Zoologica Scripta* (accepted).

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Signature………………………………….. Date…………………………………..

Multi-locus phylogeography of the sea snake *Hydrophis curtus* reveals historical vicariance and cryptic speciation

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Abstract

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 populations in the Phuket-Thailand region, Southeast 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.

Keywords

Coral triangle, Indo-Australian archipelago, Indo-West Pacific, marine biodiversity, Pleistocene, Sunda shelf barrier

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 (Bellwood *et al.* 2012; Williams & Duda Jr 2008; Woodruffe 2003). Glacial cycles throughout the last ~2.6 million years (my) periodically lowered sea levels by more than 100m below current levels (Pillans *et al.* 1998; Pirazzoli 1996; Voris 2000; Woodruffe 2003), creating varied opportunities for vicariance and cryptic allopatric speciation within the IAA. Most dramatically, the exposed Sunda (Southeast 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 (Lambeck *et al.* 2002; Voris 2000). The 2-3km deep and 80km 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. Benzie 1998; Crandall *et al.* 2008a; Duda & Palumbi 1999; Lavery *et al.* 1996) and numerous fish groups (e.g. Drew & Barber 2009; Gaither *et al.* 2011; Leray *et al.* 2010; Magsino & Juinio-Meñez 2008). However, contrastingly, certain groups including moray eels (Reece *et al.* 2010), reef fish (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 (Elfes *et al.* 2013; Rasmussen *et al.* 2011), 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.* 2008; Lukoschek *et al.* 2007). 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.* 2008; Lukoschek *et al.* 2007).

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* specimens collected mostly as fisheries by-catch and provisionally identified using published descriptions and diagnoses (Rasmussen 2001; Smith 1926). Forty-three specimens were collected by the authors during sampling trips in Australia, Indonesia (Pasuruan-East Java, Pelebuhanratu-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 harbor 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 supplementary 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 52ºC for mitochondrial markers and 59ºC 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 & Donnelly 2003; Stephens *et al.* 2001) 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 (see Appendix S1).

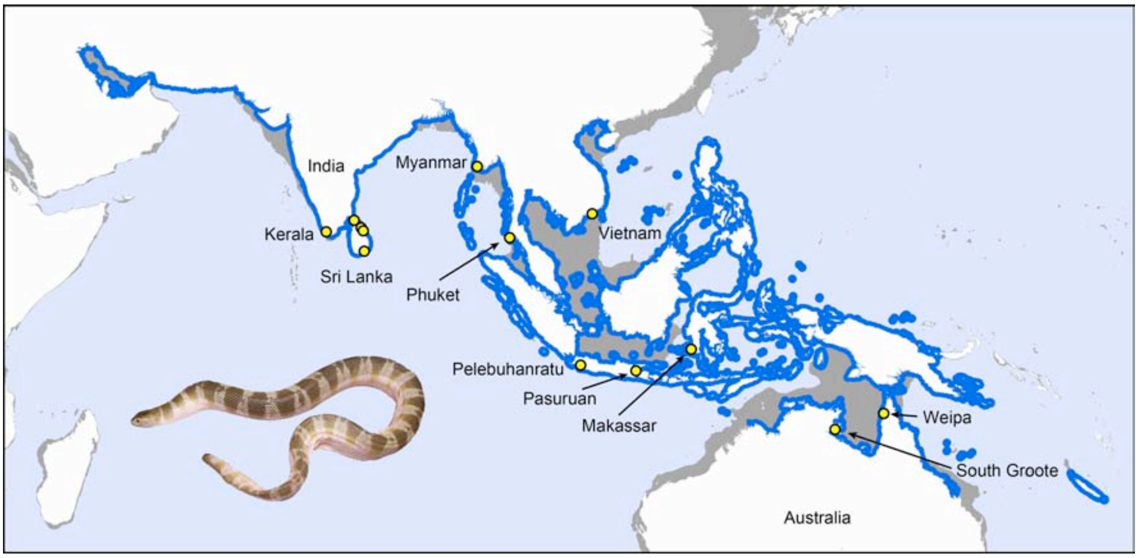

Fig. 1 Current distribution of *Hydrophis curtus* (dark blue) and the sampling locations in this study (yellow circles). The grey areas denote the 120m isobath which indicate the extent of

land when sea levels were ~120 m below present levels during Pleistocene glacial maxima. Distribution data for *H. curtus* are from IUCN redlist and the bathymetric data are from http://www.gebco.net/.

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 5 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, 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, Foster city, CA, USA).

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 since they are inherited as a single locus. Partitioning schemes and best-fit substitution models for each partition were assessed for the concatenated dataset using the Bayesian information criterion (BIC) implemented in Partitionfinder v1.0.1 (Lanfear *et al.*

2012). Three partitions were selected: 1) first codon positions of *Cytb and ND4*, *16SrRNA* and *tRNA*; 2) second codon positions of *Cytb* and *ND4*; and 3) third codon positions of *Cytb* and *ND4*. The best fit subsitituion 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). Since 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 10000 generations, with an uncorrelated lognormal 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 summarised 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 outgroup 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 5.4 (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 thus should show the same demographic history). The analysis was done in Arlequin ver 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: 1) Indian Ocean (Sri Lanka, India, Myanmar), 2) Phuket-Thailand, 3) Southeast Asia (West Java, East Java, Sulawesi, Vietnam) and 4) 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 nucleotide 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 and Thompson, (1992) with 10000 runs and 1000 dememorization steps.

Population differentiation and Fixation indices (F_{ST}): 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 10000 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 ver 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 500000 MCMC iterations after a burn-in period of 200000 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 700000 after a burn-in period of 300000 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 ver 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 ver 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 ver 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. Since 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 geographical 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 geographical distances were measured as the coastal distances between pairs of locations using the software Google Earth version 5.1, since *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. Geographical distances were log transformed before the analysis. Data were initially tested using residuals versus fitted values plot to check if they satisfied the assumptions of a linear regression model. Since 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) versus mostly West Pacific (WP) localities (Posterior probability >0.9, Bootstrap Support >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%): 1) a Phuket-Thailand (PT) clade of five individuals collected from Phuket on the Andaman (IO) coast of Thailand; 2) a SE Asian clade of 25 specimens from the south coast of West Java, East Java, south Sulawesi and Vietnam; and 3) an Australian clade of 13 specimens collected from two locations in Northern and Northeastern 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), whereas divergences within the WP clade occurred much more recently, approximately 0.15-0.7 mya.

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).

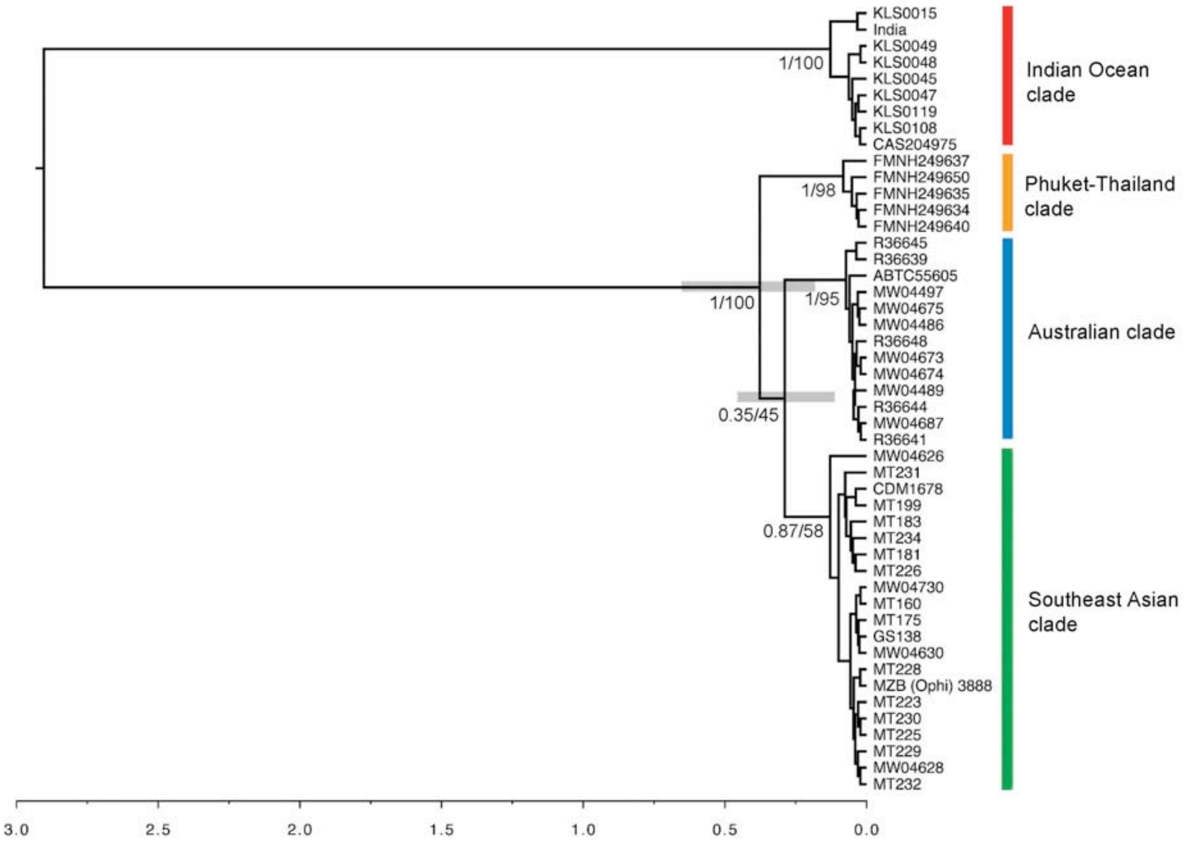

Fig. 2 Time calibrated maximum clade credibility ultrametric tree of concatenated mitochondrial DNA of *Hydrophis curtus*. The time scale is in millions of years before present. The grey horizontal bars indicate 95% highest posterior distributions (HPD) of node ages and node support is indicated at each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

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

Source of variation	Mitochondrial DNA (*Cytb*)			Microsatellite loci		
	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

Analyses of Nuclear DNA sequences 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 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.

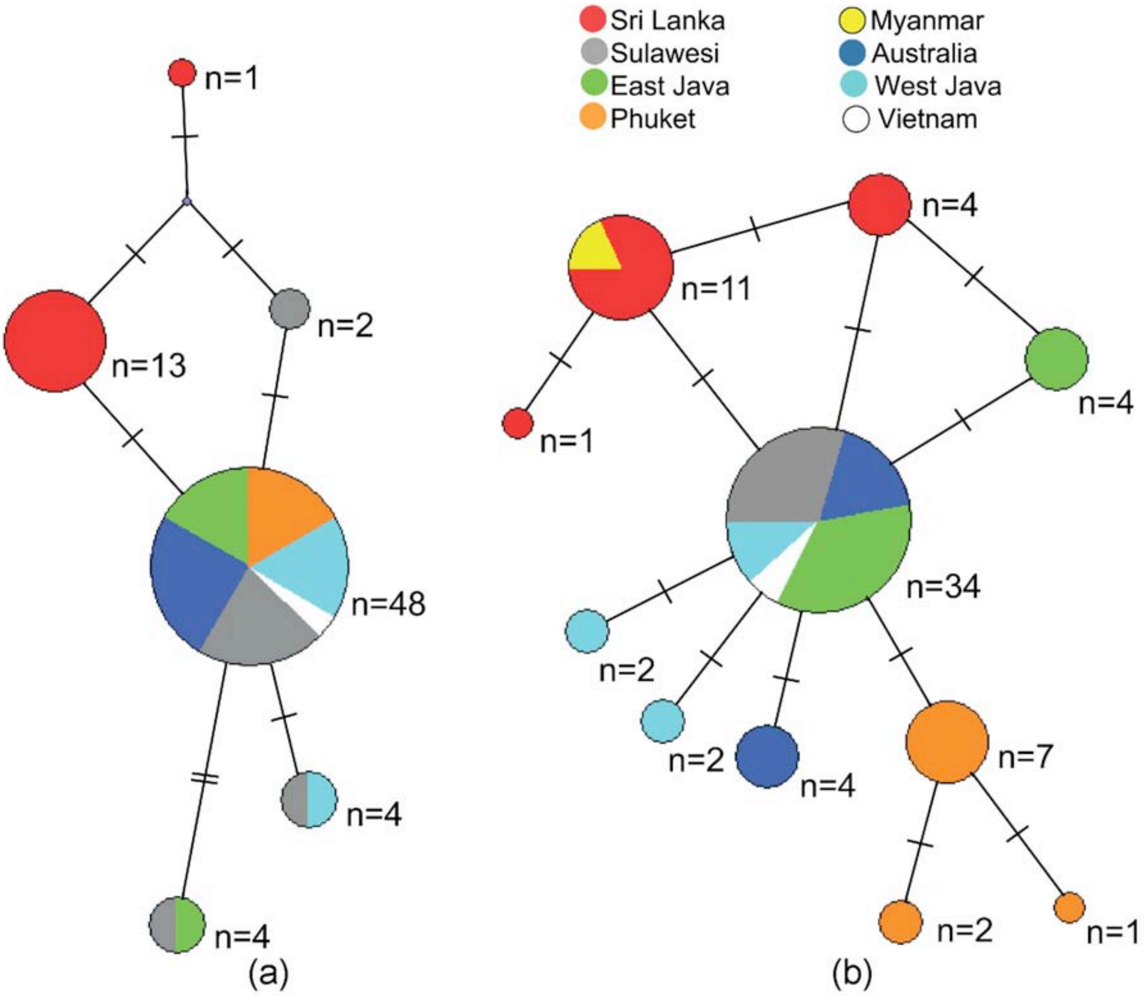

Fig. 3 Median joining allele networks of (A) *G1894* and (B) *G1888* anonymous nuclear loci. Each allele is represented by a circle and the size of each circle is proportional to the number of alleles (n). Colours correspond to the sampling location and each small black cross line indicates a single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

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

Population differentiation and Fixation indices (F_{ST}): 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).

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

Sampling region	Sample size	Sampling region			
		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						
		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

Bayesian Population genetic assignment: Initial Bayesian cluster analyses in Structure recovered the highest mean 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: 1) an IO cluster (Sri Lanka and Myanmar); 2) a PT cluster (Phuket, Thailand) 3) a SE Asian cluster (East Java, West Java, Sulawesi and Vietnam) and 4) 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.

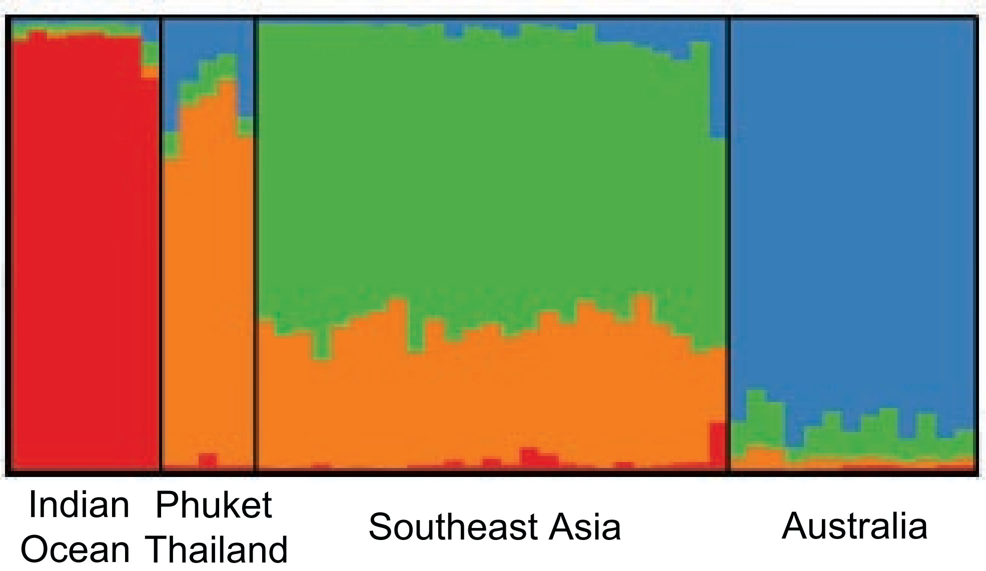

Fig. 4 Bayesian population assignment test of 51 *Hydrophis curtus* individuals based on 10 microsatellite loci. The four clusters that partition the data are displayed with different colours. Each vertical line represents one individual and its assignment likelihood (Y-axis from 0 to 1.0) into the four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

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 19.6% 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 (Rogers & Harpending 1992; Slatkin & Hudson 1991). 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).

Table 3. Historical demographic analyses of geographically delimited mitochondrial clades.

Test	Clade	
	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 - 3.359	-4.953 - 4.548
Tajima’s D (all)	**-1.599**	**-2.231**
Tajima’s D (all) 95% CI	-1.599 - 2.045	-2.414 - 1.791
Tajima’s D (synonymous)	**-**	**-2.126**
Tajima’s D (nonsynomous)	-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 significantly (*P*<0.05) values

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) only for the SE Asian clade. It was not significant (*P*>0.05) for both clades for the nonsynonymous 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 value 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.

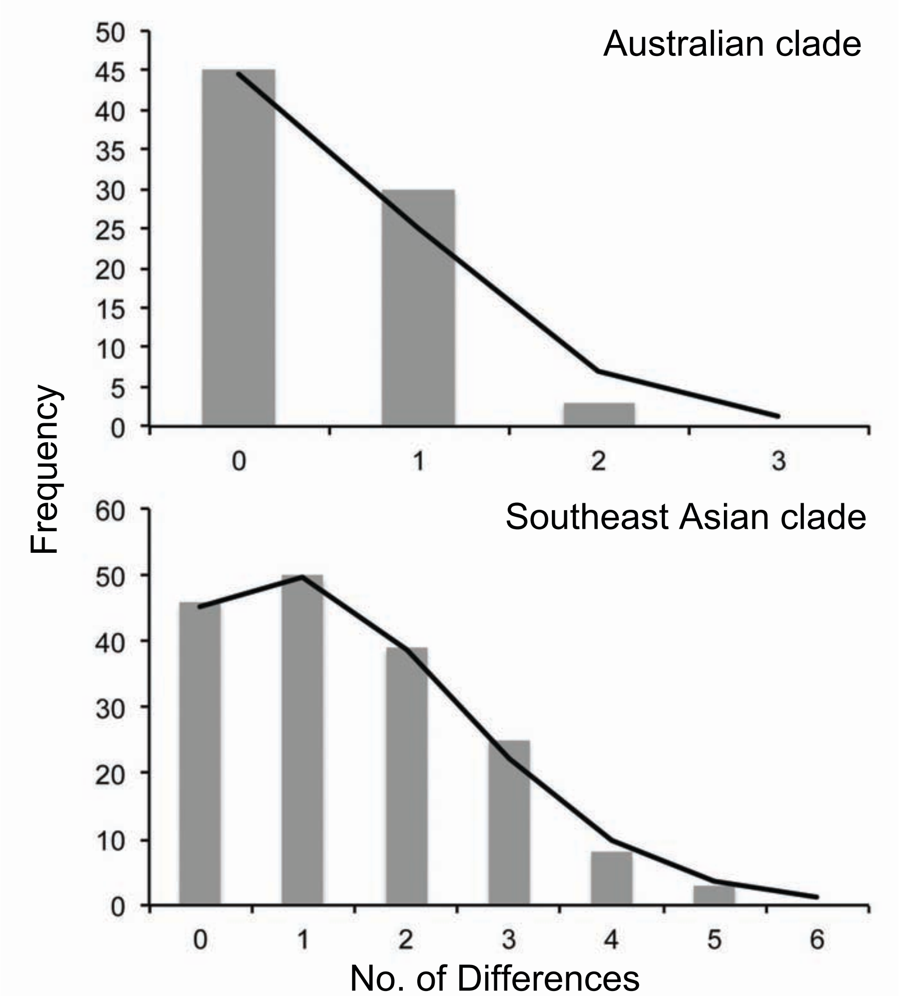

Fig. 5 Mismatch distributions for the *Cytb* gene in each mitochondrial clade. The grey bars depict the observed pairwise distributions, and black lines show the distribution simulated under a model of sudden/recent population expansion.

Isolation by Distance (IBD)

Spearman's rank correlation tests revealed a significant positive correlation between the genetic and geographical distances for both *Cytb* (ρ=0.534, *P*=0.003) and the microsatellite markers (ρ=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 (ρ=0.818, *P*=0.006) but not for *Cytb* (ρ=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, Southeast 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 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: 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 (Lambeck *et al.* 2002; Voris 2000). Hence it is possible that the Sunda shelf/Indo-pacific 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 (Gaither *et al.* 2011; Hobbs *et al.* 2009; Marie *et al.* 2007). 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 northwestern 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 neighboring WP populations.

Phylogeographic structure within the West Pacific

Mitochondrial (Fig. 2) and microsatellite data (Fig. 3) revealed further population substructure within the WP, however microsatellite data indicated more frequent gene flow between SE Asian and PT populations compared to the Australian population. This is consistent with 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 ~3km deep, 80 km wide and carries the Indonesian throughflow current (Fig. 1). The great expanse of deep sea (>200m) 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) (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 (Gaither *et al.* 2011; Leray *et al.* 2010) and invertebrates (Crandall *et al.* 2008a; Crandall *et al.* 2008b) 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 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.* 2008; Lukoschek *et al.* 2007). 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 10000 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 recognised 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 and 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 IWP. 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 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 IWP.

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Supporting Information

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

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Field number	Museum voucher number	Sampling Locality	*Cytochrome b*	*ND4* and *tRNA*	*16S rRNA*	*G1888*	*G1894*
-	NTM R36639	South Groote, NT, Australia	TBA	TBA	TBA	TBA	TBA
-	NTM R36641	South Groote, NT, Australia	TBA	TBA	TBA	TBA	TBA
-	NTM R36644	South Groote, NT, Australia	TBA	TBA	TBA	TBA	TBA
-	NTM R36645	South Groote, NT, Australia	TBA	TBA	TBA	TBA	TBA
-	NTM R36648	South Groote, NT, Australia	TBA	TBA	TBA	TBA	TBA
-	ABTC55605	South Groote, NT, Australia	EU547085	EU547036	EU547183	TBA	TBA
MW04486	BGF	Weipa, Qld, Australia	KC014452	KC014525	TBA	TBA	TBA
MW04489	BGF	Weipa, Qld, Australia	KC014453	KC014527	TBA	TBA	TBA
MW04497	BGF	Weipa, Qld, Australia	TBA	TBA	TBA	TBA	TBA
MW04673	BGF	Weipa, Qld, Australia	TBA	TBA	TBA	KC014140	KC014205
MW04674	BGF	Weipa, Qld, Australia	TBA	TBA	TBA	TBA	TBA
MW04675	BGF	Weipa, Qld, Australia	TBA	TBA	TBA	TBA	TBA
MW04687	BGF	Weipa, Qld, Australia	TBA	TBA	TBA	TBA	TBA
-	FMNH249634	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA
-	FMNH249635	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA
-	FMNH249637	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA
-	FMNH249640	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA
-	FMNH249650	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA
-	CAS204975	Ayeyarwady Divison, Myanmar	TBA	TBA	TBA	TBA	TBA
KLS0015	2013.11.01.NH	Pulmodai, Sri Lanka	TBA	TBA	TBA	TBA	TBA
KLS0045	2013.12.01.NH	Pulmodai, Sri Lanka	TBA	TBA	TBA	TBA	TBA
KLS0047	2013.13.01.NH	Kirinda, Sri Lanka	TBA	TBA	TBA	TBA	TBA
KLS0048	2013.14.01.NH	Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA
KLS0049	2013.15.01.NH	Pulmodai, Sri Lanka	TBA	TBA	TBA	TBA	TBA
KLS0108	2013.16.01.NH	Trincomalee, Sri Lanka	TBA	TBA	TBA	TBA	TBA
KLS0119	2013.17.01.NH	Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA

India	-	Kerala, India	TBA	TBA	TBA	TBA	TBA
GS138		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MT223		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MT160		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MT175		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MT181		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MT182		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MT183		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MT199		Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA
MW04730	MZB 4195	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	JQ217172 JQ217173	JQ217197 JQ217198
MW04626		Pelebuhanratu, West Java, Indonesia		KC014526	KC014374	KC014139	
MW04627		Pelebuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MW04628		Pelebuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MW04629		Pelebuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MW04630		Pelebuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA
-	MZB Ophi 3888	Pelebuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MT225	MZB 4158	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MT226		Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MT227		Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MT228	MZB 4161	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	KC014137	KC014204
MT229		Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MT230		Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MT231	MZB 4164	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	KC014138	TBA
MT232		Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA
MT234		Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA
	ZRC CDM1678	Vietnam	TBA	TBA	TBA	KC014136	TBA

Abbreviations
ABTC: Australian Biological Tissue collection, South Australian Museum, Adelaide, Australia
BGF: Bryan G. Fry private collection, Venom Evolution laboratory, University of Queensland, Brisbane, Australia
CAS: California Academy of Sciences, San Francisco, USA
FMNH: Field Museum of Chicago, Chicago, USA
NH: Zoology Department, National Museum of Sri Lanka, Colombo, Sri Lanka
NTM: Northern Territory Museum, Darwin, Australia
MZB: Museum of Zoology, Bogor, Indonesia
ZRC: Zoological reference collection, Raffles Museum, Singapore
TBA: To Be Accessioned

CHAPTER 5: Colonisation and species diversification across the Indo-West Pacific by a rapid marine snake radiation (Elapidae: Hydrophiinae)

STATEMENT OF AUTHORSHIP

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Author Contributions

Kanishka D. B. Ukuwela

Collected specimens, co-developed the research concept, generated and analysed data and co-authored the manuscript.

I hereby certify that the statement of contribution is accurate.

Signature………………………………….. Date……………………………………...

Michael S. Y. Lee

Co-developed the research concept, assisted with analysing data and contributed to writing the manuscript.

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Anslem de Silva

Collected specimens and contributed to writing the manuscript.

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Signature………………………………….. Date…14th November, 2013…………….…

Mumpuni

Collected specimens and contributed to writing the manuscript.

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Bryan G. Fry

Collected specimens and contributed to writing the manuscript.

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Kate L. Sanders

Co-developed the research concept, collected specimens, assisted with generating data and contributed to writing the manuscript.

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Colonisation and species diversification across the Indo-West Pacific by a rapid marine snake radiation (Elapidae: Hydrophiinae)

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Abstract

There are several competing hypotheses to explain the high species richness of the Indo-Australian Archipelago (IAA) marine biodiversity hotspot, centered within Southeast Asia. A novel perspective on this problem is provided by the viviparous sea snakes, a group with high species richness in the IAA that is highly distinct from other taxa previously studied, both phylogenetically (Reptilia, Amniota) and biologically (e.g. viviparity and direct development). Phylogenetic analyses and biogeographic reconstructions indicate that viviparous sea snakes underwent rapid speciation after colonizing SE Asia during the last 3 million years. Most of SE Asian diversity is the result of *in-situ* speciation, supporting the "centre of origin" model for biodiversity hotspots. There is also speciation at the periphery, or outside of, SE Asia: however, contrary to predictions of the "accumulation" and "overlap" models, these new outlying taxa do not preferentially disperse back into SE Asia. Instead, lineages are equally likely to disperse either into or away from SE Asia. Thus, in sea snakes, high biodiversity in SE Asia (and thus the IAA) is mostly explained by *in-situ* speciation rather than accumulation or overlap.

Keywords: biodiversity hotspot, biogeography, evolutionary radiation, Indo-Australian Archipelago, Pleistocene, sea level changes

Introduction

The Indo-Australian Archipelago (IAA), situated between the Indian and Pacific Oceans (Figure S1), supports an exceptionally rich concentration of marine biodiversity [1], with more fish and coral species reported than for any other region [2, 3]. A pattern of declining diversity with latitudinal and longitudinal distance from the central IAA in many taxa [4, 5] suggests that a common process explains this biodiversity hotspot. Theories proposed to explain the exceptional IAA marine diversity typically view the region as either: (1) a centre of origin/speciation, where new species form rapidly and are subsequently exported to peripheral areas [6]; (2) a centre of accumulation of diversity, with speciation in isolated locations at the periphery of the IAA and subsequent dispersal of new taxa into the region [7]; or alternatively (3) a region of overlap for marine biodiversity that originated in the Pacific and Indian Oceans, i.e. outside the IAA [8].

A plethora of studies on different taxa from the region have provided support for the ‘centre of origin’ [4, 5, 9], ‘centre of accumulation’[10-12] and ‘region of overlap’[13] models. Taken together, these studies suggest that all three processes could contribute towards higher IAA marine biodiversity in different taxa [14-16] and have led to a ‘biodiversity feedback model’ under which the IAA and other tropical marine biodiversity hotspots act as centres of speciation, accumulation and/or overlap [17].

Distinguishing alternative diversification scenarios for the origins of IAA marine biodiversity, and determining their relative importance, requires study groups that contain large numbers of species, span the Indo-Pacific, and can be reasonably well sampled for phylogenetic analysis. The viviparous sea snakes (Elapidae: Hydrophiinae) offer high species diversity, with 62 species that share a terrestrial Australian ancestor only ~10.6-6.5 million years ago (mya) [18-20]. They occupy shallow-marine habitats throughout the tropical and subtropical Indian and Pacific Oceans, but like many other marine groups in the Indo-Pacific, reach peak diversity in the IAA hotspot [21]. Moreover, at least 75% of sea snake species are part of a single, explosively speciating ‘core *Hydrophis* clade’, which is dated at less than 3 million years old [22, 23] and is widespread throughout the IAA.

The majority of sea snake diversification, including the rapid core *Hydrophis* radiation, coincided with major climatic and geological events [24, 25] that drove vicariant population and species divergence in many of the region’s marine groups (reviewed in [26]).Viviparous sea snakes might be particularly susceptible to ‘soft’ biogeographic barriers (such as incomplete and thus permeable land bridges) because they undergo direct development (i.e. give birth to live young) and thus lack the dispersing planktonic larval stage that is expected to promote population connectivity in most other marine groups (e.g. many fish and invertebrates) [27]. Population genetic analyses have indeed shown strong intraspecific genetic structure in several species that corresponds to deep-water and historical land barriers [28-30]. However, broad scale biogeographic patterns and the role of geo-climatic events in sea snake diversification have not previously been investigated in a phylogenetic context.

In this study we aimed to resolve the biogeographic history of viviparous sea snakes using a multi-locus time-calibrated phylogeny for ~70% of described species, many sampled from multiple localities. We then compared rates and temporal concordance of inferred vicariance and dispersal events between marine basins in Australasia, Southeast Asia and the Indian Ocean. Specifically, our objective was to test whether viviparous sea snake diversity in the IAA is best explained by *in-situ* speciation, accumulation, peripheral speciation and accumulation, or external speciation and subsequent overlap. We use several approaches including new Bayesian analyses that allow for clade-specific and event-specific dispersal rates.

Although numerous studies have investigated the biogeography of Indo-Pacific marine taxa, most of these have involved a single [31, 32] or few species [33-35], and many have been restricted to sub-regions/single marine basins [28, 36, 37]. The few broad scale biogeographic studies of species-rich, widely distributed groups have focused primarily on reef fish [13, 15]. Our study of sea snakes thus represents a novel contribution towards understanding the biogeographic processes that have shaped this important marine region.

Materials and Methods

Sampling, DNA amplification and sequencing

The study sampled a total of 320 individuals from 42 species of viviparous sea snakes from Australia, Indonesia, Myanmar, Malaysia, Vietnam, Thailand, Bangladesh, Sri Lanka, India and Iran (see Figure S1). Liver/muscle tissue samples preserved in 90% Ethanol/Iso-propanol were obtained from specimens collected primarily as fisheries by-catch (233 individuals, 36 species) and from specimens accessioned in museums (57 individuals, 22 species). Additional mitochondrial and nuclear sequences were also obtained from Genbank (30 individuals, 16 species). Specimen collection localities and museum voucher numbers are provided in the supplementary Appendix S2.

We amplified and sequenced a total of 5792 base pairs (bp) from three mitochondrial markers (*Cyt-b*, *ND4* and adjacent *tRNA* region, *16SrRNA*), two nuclear coding genes (*c-mos, RAG-1*) and three nuclear anonymous markers (*G1888*, *G1894*, *G1914*) to reconstruct sea snake phylogeny. Details of DNA extraction, PCR amplification and sequencing are available in the supplementary methods section (see Appendix S1). The sequences generated in this study are deposited in the Genbank (see Appendix S2).

Phylogenetic analyses and divergence dating

Time-calibrated sea snake phylogenies were inferred using Bayesian and Maximum likelihood (ML) analyses of the concatenated mitochondrial and nuclear alignment (See Appendix S1 for details). The Australasian terrestrial elapid *Hemiaspis damielli* was used as an outgroup because there is strong molecular and morphological evidence that *Hemiaspis* is a close relative of the viviparous sea snakes [18, 38, 39]. Bayesian analyses with estimation of the divergence times were done in MrBayes 3.2 [40]. Since there are no known Hydrophiini fossils that could be used to calibrate the tree, uniform secondary calibrations of 6.5 my to 10.6 my and 4.5 my to 7.9 my were applied to the root divergence and the *Aipysurus-Hydrophis* lineage split, respectively. These dates correspond to the 95% posterior distributions estimated for the two divergences using long nuclear sequences and several reliable squamate fossil calibrations [18, 20, 41]; while these dates are reasonable, the biogeographic reconstructions employed here require only relative rather than absolute dates (e.g. root age could have been set to 1). Bayes Factors were used to choose the optimal clock model. Convergence of the independent runs in topology assessed by examining similar clade (split) frequencies across runs (standard deviation <0.05); convergence in numerical parameters was assessed though essentially identical distributions with high effective sample sizes (>100) as shown by Tracer v1.5 [42]. Maximum Likelihood analyses (undated, no clock) were implemented in RAxML v7.2.8 [43].

In addition to the dated analyses, we estimated the amount of genetic divergence between sister lineages in different Ocean basins: corrected (HKY) pairwise sequence divergence was calculated for the mitochondrial *Cyt-b* gene using Geneious Pro 5.4 software [44].

Dispersal Dynamics and Ancestral Area Reconstruction (AAR)

Ancestral areas were reconstructed to examine the biogeographic history of sea snakes. Three oceanic regions/ancestral areas were delimited on the basis of endemism, species distribution maps, distribution of reciprocally monophyletic geographic lineages across separate taxa, and known dispersal barriers (e.g. deep-sea trenches). The three regions (Figure 1 inset map) are the (1) Indian Ocean, (2) Southeast Asia (comprising ~70% of the IAA) and (3) Australasia (which includes the eastern end of the IAA). Ancestral area reconstructions were done using the dated consensus tree (from the MrBayes analysis) using Bayesian inference in BEAST 1.8 [45], parsimony as implemented in Mesquite ver.2.75 [46], and maximum-Likelihood as implemented in Lagrange [47]. For all analyses each sample (tip) was assigned to one of the three Oceanic regions based on the collection locality (See Appendix S1 for details of all analyses).

The BEAST analyses implemented novel methods to test whether rates of dispersal varied across lineages (clades) and/or events: the most appropriate model, selected using Bayes Factors, was adopted for Ancestral Area Reconstruction (see above). To test the importance of lineage-specific dispersal rates, we tested a model where different lineages (clades) were permitted different rates (using a "random local clock") and one, which assumed a uniform dispersal rate across all lineages (a "strict clock"). To test whether the different dispersal events occurred at different rates, we tested 4 different dispersal models of differing complexity: (1) a "time-irreversible" model which assumed that 6 dispersal events occurred at different rates (Australia→SE Asia; Indian Ocean→SE Asia; Australia→Indian Ocean and the reverse), (2) a "time-reversible" model which assumed three such rates (Australia↔SE Asia; Indian Ocean ↔ SE Asia; Australia↔ Indian Ocean), and (3) a single rate (unordered) model which assumed a single common rate for all 6 events. We further evaluated (4) a single-rate "ordered" model, which permitted only dispersals between adjacent regions (Australia↔SE Asia; Indian Ocean↔SE Asia). There is no direct continental shelf connection between Australasia and the Northern/Western Indian Ocean, hence the "ordered" model evaluates the hypothesis that sea snakes (with the possible exception of the pelagic, planktonic *H. (Pelamis) platurus*) moving between these regions must generally pass through SE Asia. A posterior probability of >0.7 for a region for a node was considered as strong support. These analyses also directly recorded the exact number of each of the 6 dispersal events occurring in each sampled tree (inferring events by examining the reconstructed states at nodes will underestimate events if there are multiple events along single branches). We also tested the fit of these 4 event-specific dispersal rates in Bayestraits [48], under assuming a uniform dispersal rate across lineages (Bayestraits does not implement lineage-specific dispersal rates).

The parsimony analyses in Mesquite 2.75 [46], optimised regions and dispersals on the tree using an ordered model, which was the best-supported model identified in model testing (see above).

Maximum-Likelihood was implemented in the Dispersal-Extinction-Cladogenesis (DEC) model in Lagrange [47] with unordered and an ordered dispersal models. Likelihood ratio tests did not strongly favour one model over the other, but provided generally similar results for both models. Thus we provide only the results of the most biologically plausible ordered model (see above). Range inheritances scenarios >2 log-likelihood units from all other possible scenarios were considered as strong support for reconstructions at each node.

Even though the *Hydrophis* lineage is the most diverse marine tetrapod clade, this diversity was insufficient to permit statistical tests of relationship between geographic areas and speciation rate, with robust results requiring at least "roughly one or two hundred tip species" [49].

Results and Discussion

Phylogenetic relationships and divergence times

Bayesian (dated) and ML (undated) analyses of the concatenated alignment recovered similar topologies, branch lengths and levels of support (Figure S2), and were consistent with a previous study that used concatenated mitochondrial, concatenated nuclear and multi-locus species tree analyses [22]. Both of our analyses recovered every sampled species as monophyletic with strong support (posterior probabilities (PP) >0.9 and bootstrap values (BS) >70%) (Figure S2). Phylogenetic analyses recovered strongly supported (PP >0.9 and BS >70%) geographically distinct reciprocally monophyletic clades within species that correspond to the Indian Ocean and SE Asia/West Pacific for *Microcephalophis (Hydrophis) gracilis*, *Hydrophis caerulescens, H. (Lapemis) curtus*, *H. (Enhydrina) schistosus* and *H. (Thalassophina) viperinus* (Figure S2). *Hydrophis curtus* further showed population divergence within the West Pacific with distinct clades in SE Asia and Australasia. The analysis also recovered distantly related cryptic lineages of *H. cyanocinctus* and *H. ornatus* with allopatric distributions in the Indian Ocean or West Pacific/SE Asia (Figure S2). However, widely distributed species *H. (Astrotia) stokesii*, *H. (Acalyptophis) peronii* and *H. platurus* did not display clear geographic genetic structure.

Divergence time estimates indicate that the speciation of the *Aipysurus* (clade containing the species of the genera *Aiypusurus* and *Emydocephalus)* and the core *Hydrophis* (clade containing the species of the genus *Hydrophis sensu* Sanders et al. [22]) lineages commenced about 3.5 mya (Figure S2, Figure 1). However, the majority of the divergence time estimates between sister species and sister lineages ranged from 2.34 to 0.53 mya (2.878-0.343 95% HPD) indicating a rapid late Pliocene or Pleistocene diversification (Table 1). The high speciation rates in the Pleistocene, particularly within core *Hydrophis*, are contemporaneous with, and likely linked to, the sea level changes during the last 2.5 million years [24, 50] that created and removed barriers forming isolated marine basins (e.g. [51, 52]).

Corrected pairwise genetic distances between sister lineages in the Indian Ocean and SE Asia ranged between 9.96-2.36%, and between 0.72-0.78% for sister lineages in Australasia and SE Asia (Table 1); this was again consistent with speciation during the late Pliocene-Pleistocene.

Table 1. Percentage pairwise corrected genetic divergences and mean divergence times (millions of years) between sister species/lineages in different Ocean basins.

Species/Lineage	Genetic divergence	Mean divergence time	95% HPD
A. eydouxii-A. mosaicus	7.10-7.39	2.297	2.878-1.679
H. atriceps-H. fasciatus	2.02-2.92	1.027	1.366-0.654
H. caerulescens (IO-SEA)	2.36-2.91	0.965	1.292-0.651
H. curtus (IO-WP)	8.64-9.96	2.337	2.895-1.698
H. curtus (SEA-AUS)	0.72-0.78	0.289	0.411-0.174
H. cyanocinctus (IO-WP)*	4.01-4.96	-	-
H. ornatus (IO-SEA)*	3.33-4.12	-	-
H. schistosus (IO-SEA)	4.05-4.96	0.716	0.967-0.490
H. lamberti-H. ornatus (IO)	1.04-1.30	0.526	0.718-0.343
H. viperina (IO-SEA)	4.05-4.85	0.708	0.977-0.457
M. gracilis (IO-SEA)	4.53-5.44	1.270	1.756-0.841

Abbreviations: IO- Indian Ocean, SEA- SE Asia, AUS-Australasia, WP-West Pacific

*These species are considered to be single species. However, molecular analyses indicate that they are two cryptic and closely related groups that do not show a sister species/lineage relationship.

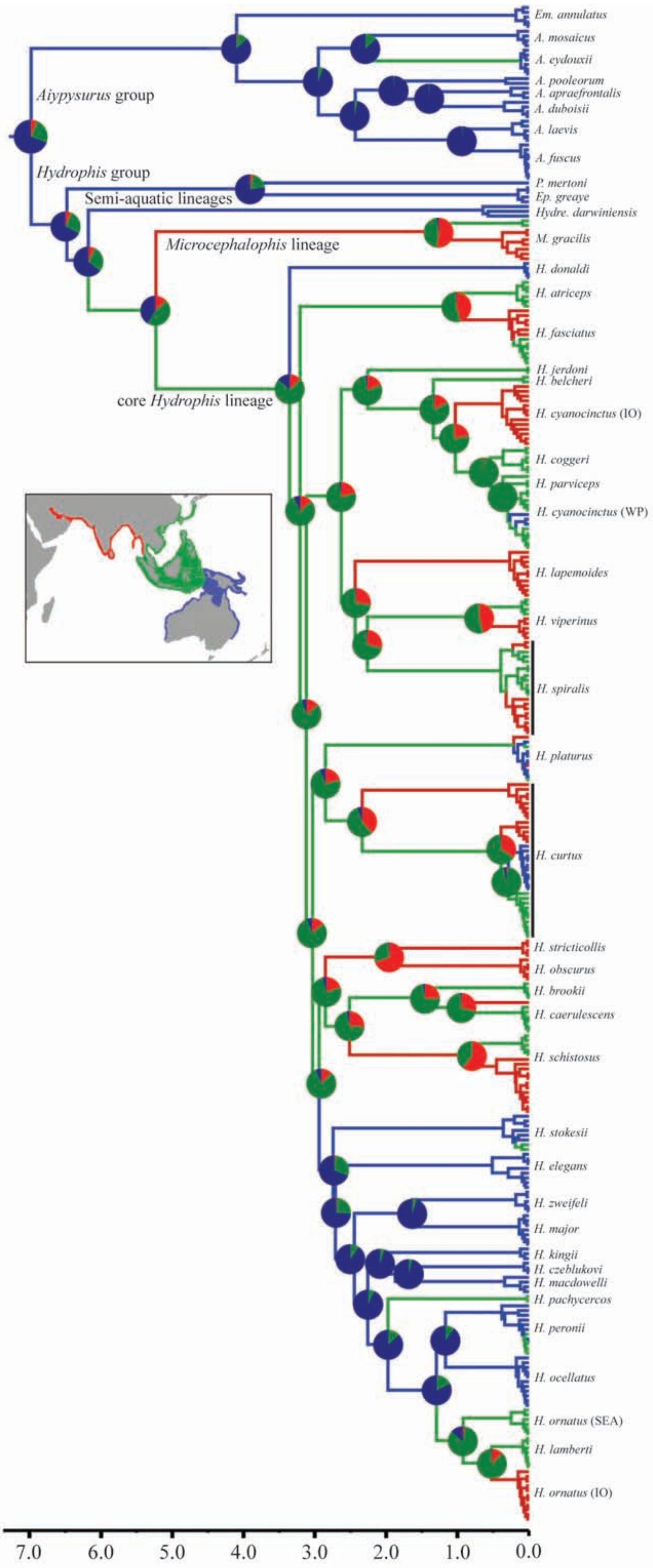
Em. annulatus
A. mosaicus
A. eydouxii
A. pooleorum
A. apraefrontalis
A. duboisii
A. laevis
A. fuscus
P. mertoni
Ep. greaye
Hydre. darwiniensis
M. gracilis
H. donaldi
H. atriceps
H. fasciatus
H. jerdoni
H. belcheri
H. cyanocinctus (IO)
H. coggeri
H. parviceps
H. cyanocinctus (WP)
H. lapemoides
H. viperinus
H. spiralis
H. platurus
H. curtus
H. stricticollis
H. obscurus
H. brookii
H. caerulescens
H. schistosus
H. stokesii
H. elegans
H. zweifeli
H. major
H. kingii
H. czeblukovi
H. macdowelli
H. pachycercos
H. peronii
H. ocellatus
H. ornatus (SEA)
H. lamberti
H. ornatus (IO)
Aiypysurus group
Hydrophis group
Semi-aquatic lineages
Microcephalophis lineage
core Hydrophis lineage
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Figure 1. Bayesian time calibrated ultra-metric tree with Bayesian (BEAST) ancestral area reconstructions of the Indo-Pacific viviparous sea snakes. Time scale is in millions of years before present. Colours of the branches indicate the ancestral area reconstructions and correspond to the biogeographic/ancestral regions (Red: Indian Ocean, Green: Southeast Asia, Blue: Australasia) shown in the map. The pie charts depict the Bayesian estimations of the relative probability of having the ancestral area at each node and colours correspond to the regions shown in the map.

Dispersal, Speciation and the Drivers of Indo-Pacific Biodiversity

The best-fitting model, as evaluated in BEAST, allowed lineage-specific dispersal rates, and permitted dispersal only between adjacent regions ("ordered" model), with a single common rate for all 4 possible dispersal events (Australasia↔SE Asia; Indian Ocean↔SE Asia) (Table 2). Dispersal rates are relatively similar across most lineages, but as expected, the pelagic, planktonic *H. platurus* exhibits great (~eightfold) increase in dispersal rate (Figure 1, and 2. Figure S3; see below). The preferred model with single common rate for all 4 possible dispersal events suggests there is no significant bias in direction of dispersal: thus, contrary to predictions of the overlap or accumulation models, taxa are not more likely to disperse into, rather than out of, the IAA. Bayestraits, which tested the 4 event-specific dispersal models but had to assume a common dispersal rate across lineages, could not distinguish between the ordered, 3-rate and 6-rate models (BF <5 compared to best model) but rejected the unordered model (BF=14.1).

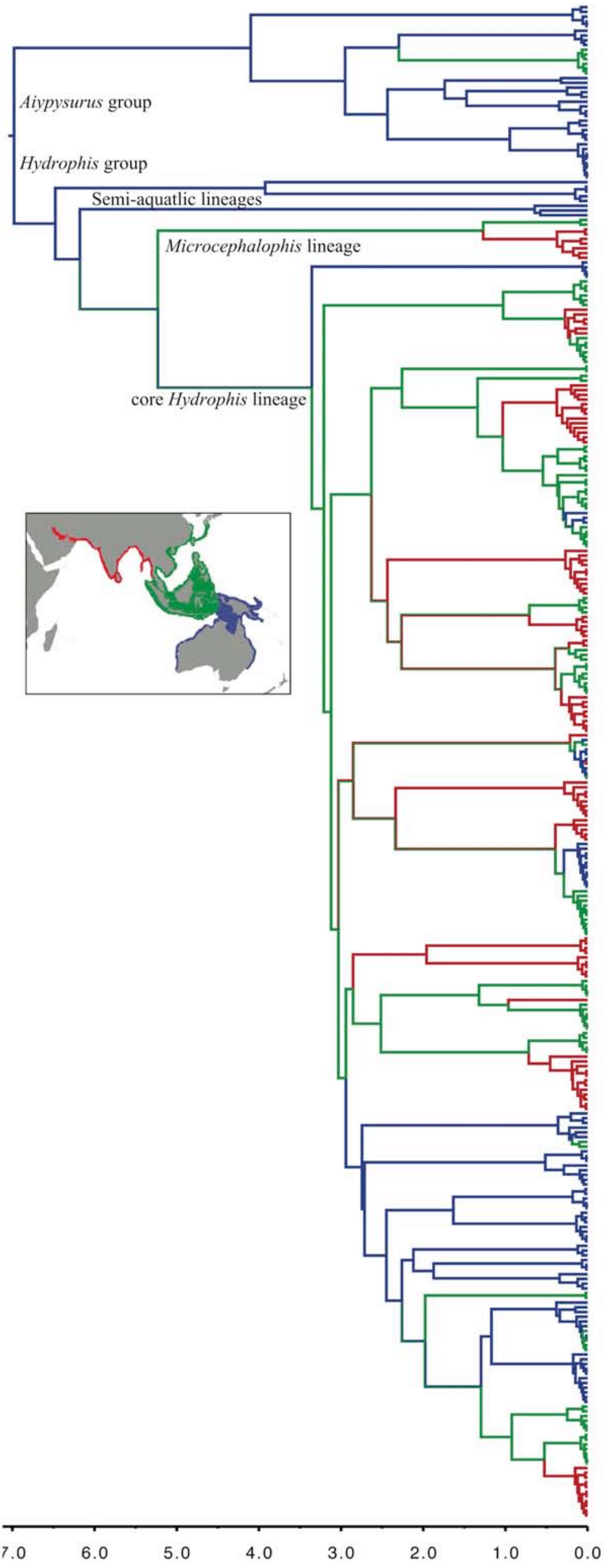
Aiypysurus group
Hydrophis group
Semi-aquatlic lineages
Microcephalophis lineage
core *Hydrophis* lineage
7.0
6.0
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Figure 2. Bayesian time calibrated ultra-metric tree with ordered Parsimony ancestral area reconstructions of the Indo-Pacific viviparous sea snakes. Time scale is in millions of years before present. Colours of the branches indicate the ancestral area reconstructions and correspond to the biogeographic/ancestral regions (Red: Indian Ocean, Green: Southeast Asia, Blue: Australasia) shown in the map.

All three AAR methods (Bayesian, parsimony, DEC) recover an Australasian origin for viviparous sea snakes, approximately 6.9 million years ago (Figures 1, 2 and Figure S3). Similarly, all three analyses indicate that the *Aipysurus* group also originated in Australasia, and subsequently diverged mostly within this region. Of the *Aipysurus* group species, only the specialist fish egg-eaters *Emydocephalus ijimae and E. szczerbaki* (not sampled in the present study) and *A. eydouxii* have colonized SE Asia and none have reached the Indian Ocean beyond the coast of Western Australia. Parsimony and DEC analyses support an Australasian origin for the two semi-aquatic lineages and an early transition (~5 mya) to SE Asian habitats for the *Microcephalophis* lineage. However, BEAST analysis did not strongly recover the origins of the two semi-aquatic and *Microcephalophis* lineages. BEAST analyses strongly indicated (probability=0.73) a SE Asian origin for the MRCA of the core *Hydrophis* group, which accounts for ~75% of extant species richness. Parsimony and DEC analyses are consistent with either an Australasian or SE Asian origin for this group.

All three AAR methods indicated that the core *Hydrophis* group initially diversified primarily in SE Asia, with subsequent dispersals into the Indian Ocean and re-colonisation of Australasia. The core *Hydrophis* radiation has been shown to have an accelerated rate of speciation, with other viviparous sea snakes and their terrestrial sister groups having a slower background rate [23]. All the basal speciation events, and the majority of subsequent speciation events, are reconstructed as occurring in SE Asia. In the BEAST AAR (Figure 1), for instance, there are 34 divergences between lineages >0.5 my old (candidate speciation events); 22 of these occur in SE Asia, 10 in Australasia, and 2 in the Indian Ocean (Figure 3). This suggests that most of the sea snake diversity in the SE Asia is derived from a period of rapid *in-situ* diversification. Thus, although viviparous sea snakes originated in Australasia, SE Asia (which comprises most of the IAA) appears to be their primary ‘centre of speciation’.

Table 2. Inferred dispersal events from the three ancestral area reconstruction methods (A-C) and the fit of alternative dispersal models (D), which assume uniform or variable dispersal rates across lineages (clades) and events.

A: BEAST (variable rates across lineages, ordered)

From\To	Australasia	SE Asia	Indian Ocean
Australasia	-	17.1 (8)	* (1^{A})
SE Asia	18.2 (5)	-	17.7 (3)
Indian Ocean	*	11.6 (13)	-

B: Parsimony (ordered)

From\To	Australasia	SE Asia	Indian Ocean
Australasia	-	5	*
SE Asia	4	-	7
Indian Ocean	*	1	-

C: Lagrange (ordered, interspecific events only)

From\To	Australasia	SE Asia	Indian Ocean
Australasia	-	4	*
SE Asia	3	-	2
Indian Ocean	*	0	-

D: Fit of alternative dispersal models in BEAST

Dispersal models	-LognL	BayesFactor
Variable rates across lineages, 1 event rate (ordered)	113.506	0 (best)
Variable rates across lineages, 1 event rate (unordered)	121.073	-15.134
Variable rates across lineages, 3 event rates (reversible)	117.398	-7.784
Variable rates across lineages, 6 event rates (irreversible)	118.378	-9.744
Uniform rates across lineages, 1 event rate (ordered)	122.758	-18.504

* = set to zero (see model testing in Appendix S1). In the BEAST table, the actual events inferred in each MCMC sample are listed first; the events "inferred" by only examining nodal reconstructions in the Bayesian consensus tree are shown in parentheses (^{A}one of these inferred events is a "forbidden" direct Australasia-to-Indian Ocean dispersal, which would

have been represented in the actual MCMC reconstruction through two successive dispersal events along one branch: Australasia-to-SEA and SEA-to-Indian Ocean).

In ‘D’ the preferred model assumes variable rates across lineages, and a common rate for all dispersals, but only dispersals between adjacent regions (ordered).

Viviparous sea snakes provide little support for the ‘region of accumulation hypothesis': there are few instances of peripheral speciation *followed by subsequent recolonisation*. Peripheral speciation is here identified as cladogenesis where one of the two resultant lineages is inferred to have (primitively) a SE Asian distribution, and the other, an external (Australasian or Indian Ocean) distribution. BEAST, Parsimony, and DEC analyses indicated two such speciation events between Australasia and SE Asia (*A. mosaicus*-*A. eydouxii* and within *H. curtus*) and six such events between the Indian Ocean and SE Asia (*H. ornatus*-*H. lamberti* and within *M. gracilis*, *H. caerulescens, H. curtus, H. schistosus,* and *H. viperinus*) (Figures 1, 2, 3 and S3). These findings support a role of geographic/historical isolation at the periphery of the IAA in generating overall species/genetic diversity [7]. However, these events do not increase diversity in the SE Asia (i.e. the IAA): the ancestral lineage in each species pair is inferred to be from SE Asia, the speciation event thus adds a new species to the diversity in the adjacent area (Australasia or Indian Ocean), but there is no evidence of secondary range expansion of these species back into the SE Asia.

A smaller proportion of SE Asia/IAA sea snake diversity appears to be consistent with the "overlap" model: speciation outside of SE Asia and subsequent recolonisation. In all three AARs, the only major external contribution appears to be from the *H. ornatus* clade (sensu Sanders *et al*., 2013); a few lineages from this predominantly Australasian *H. ornatus* clade have secondarily extended their ranges back into SE Asia (*H. stokesii*, *H. peroni,* and the *H. ornatus*-*H. lamberti* clade). The Indian Ocean fauna has made little or no contribution to the SE Asian sea snake diversity (the only possible recolonisations involve *H. fasciatus* and *H. spiralis*). The majority of sampled Indian Ocean species and lineages have a SE Asian origin and the regional sea snake fauna seems to be mainly derived from direct dispersal from SE Asia, with few dispersals in the other direction. Altogether, these findings indicate that considerable speciation does occur outside of the IAA; however, subsequent inward dispersal into the IAA is not a major driver of species richness there.

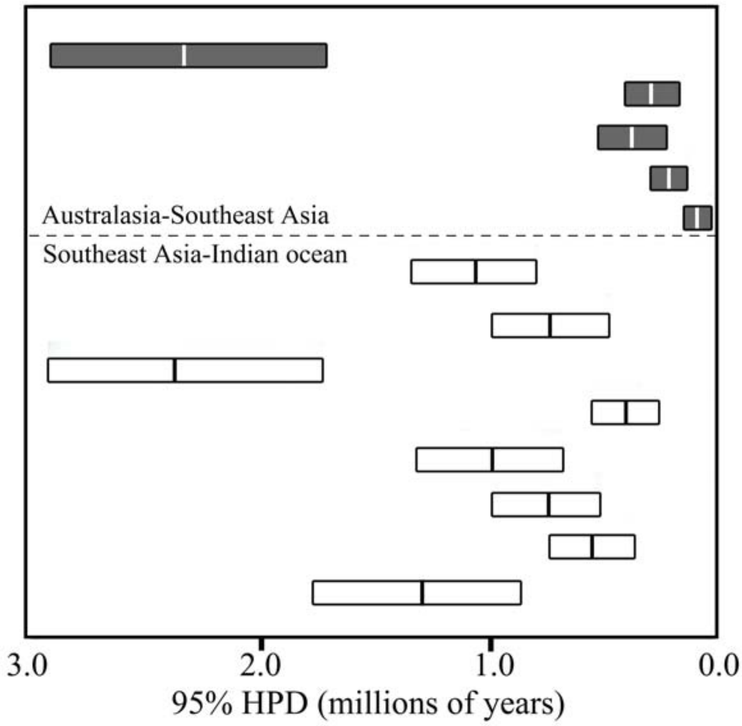

Figure 3. Divergence times and 95% highest posterior distributions of species/population divergences and dispersal events. The black and white vertical bars depict the species/population divergence time and horizontal bars indicate the 95% highest posterior densities of the divergence time estimates.

DEC analysis estimated an overall dispersal rate of 0.156 events per lineage per my (and an extinction probability of 0.016 per my) whereas BEAST analyses suggest dispersal rates ranging from 0.31-0.34 per lineage per my in most lineages, up to 2.38 in *H. platurus*. The slower rate in the DEC analysis might be due to the fact that it only evaluates rates in interspecific branches (intraspecific dispersals, which are often recent, are not considered). Alternatively, the loose prior in the BEAST analysis might have allowed fast rates (see

Appendix S1). Consistent with the inferences from node reconstructions discussed above, dispersals into SE Asia were not more frequent (and often less frequent) than outward dispersals; thus, there is no evidence that high species diversity in SE Asia is derived from preferential inward dispersal of peripheral or external clades. All analyses suggested dispersals between SE Asia and Australasia occurred approximately as frequently in both directions (Table 2). The BEAST analyses suggested that dispersals between SE Asia and the Indian Ocean also occurred approximately the same frequency in both directions; however, parsimony and DEC indicated that dispersals from SE Asia to the Indian Ocean were more frequent than the reverse. However, the DEC analysis reconstructed very few events in total, by only considering events between rather than within species.

Caveats and Conclusions

Incomplete taxon sampling can affect biogeographic reconstructions and inferred dispersal patterns [53]. In this study ~70% of known species of viviparous sea snakes were sampled: sampling was more complete for Australasian and Indian Ocean taxa (both >75%), but less complete for SE Asia (<60%). This would tend to bias results against reconstructing SE Asia for ancestral nodes. Despite this potential bias, our AARs nevertheless recovered a SE Asian distribution for all basal, and most subsequent, speciation events in the core *Hydrophis* group. Hence, the importance of SE Asia as a centre of speciation for viviparous sea snakes is likely to remain and perhaps be amplified with additional species sampling.

The drivers of this elevated speciation rate in the core *Hydrophis* group still need to be identified. They could involve extrinsic (geographical) factors, such as the formation of transient barriers in the Plio-Pleistocene [54], or intense competition [17, 55], or divergent selection in a highly heterogeneous and biodiverse environment [56]. Alternatively, they could be intrinsic: recent studies have suggested that plasticity of head size evolution contributed to rapid speciation in this group [29]. Evaluation of whether the core *Hydrophis* group exhibits different diversification rates in different regions would answer this question, but robust inferences would require far more species than exist: at least 100-200 [49] or >300 [57]. However, pooling phylogenies of sea snakes and other vertebrate groups (fish) spanning this region might provide sufficient sample size [49].

Distinguishing alternative diversification scenarios for the origins and maintenance of extraordinary marine biodiversity in the IAA remains a central goal in marine biogeography. Analyses of viviparous sea snakes suggest that SE Asia, which includes most of the IAA, has functioned mainly as a ‘centre’ or a ‘cradle’ of speciation for viviparous sea snakes: the core *Hydrophis* group underwent rapid and largely *in-situ* diversification during the last 3 my in SE Asia. Speciation either at the periphery (or outside) of SE Asia, followed by biased inwards range expansion, does not appear to be an important contributor of marine snake biodiversity of the IAA.

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SUPPLIMENTARY FIGURES

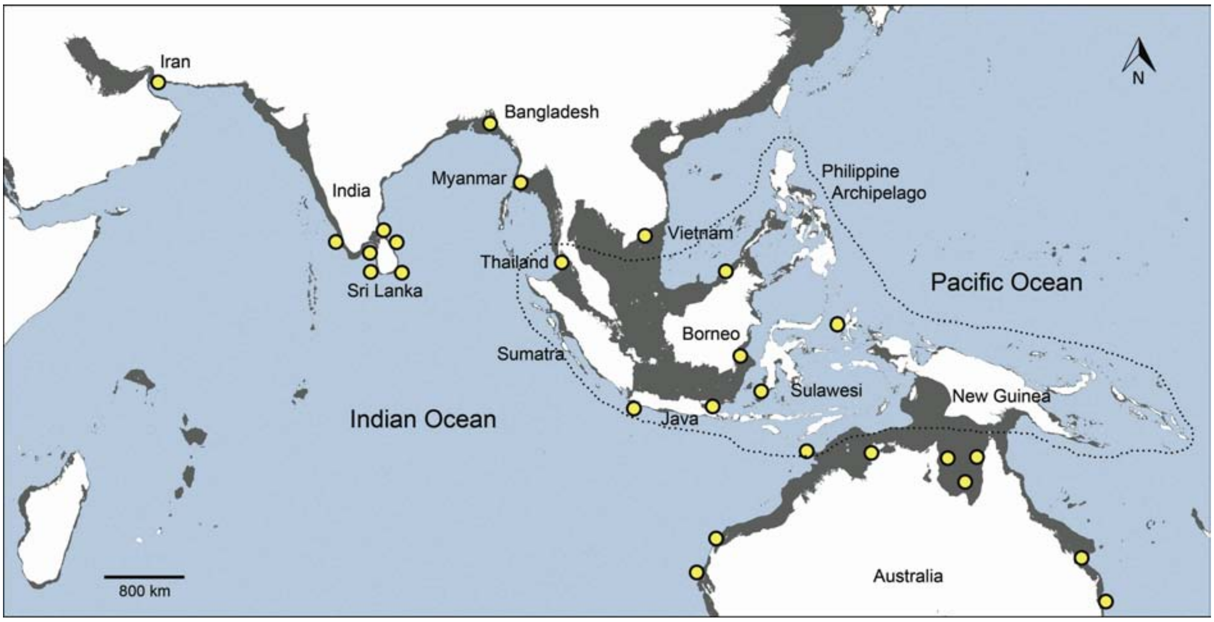
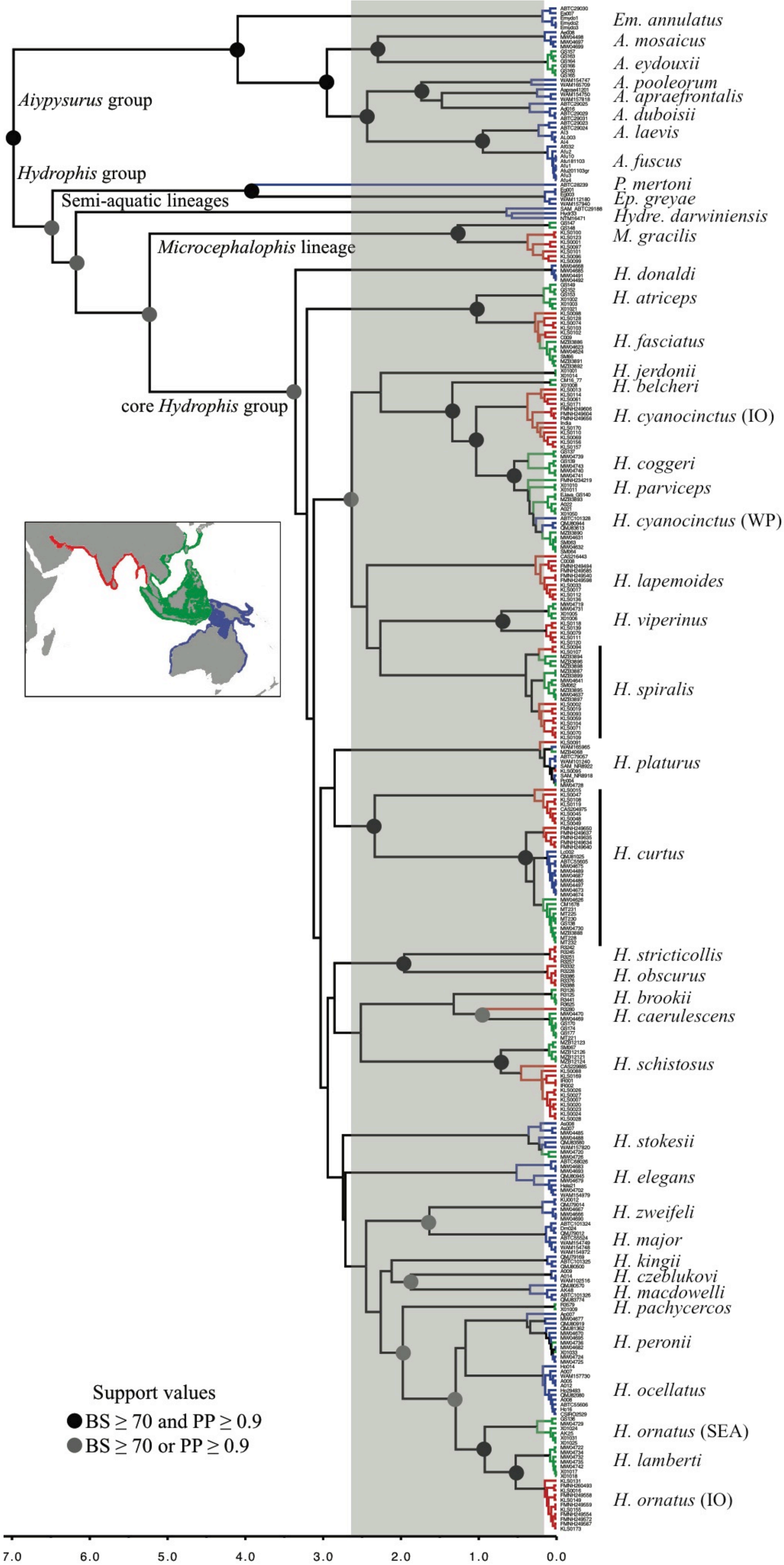


Figure S1. The sampling locations (yellow circles) in the Indo-West Pacific marine biogeographic region with the Indo-Australian Archipelago demarcated by the thin dotted line. The grey areas denote the 120m isobath which indicates the extent of land that formed the Sunda shelf/Indo-Pacific barrier when sea levels were ~120 m below present levels during Pleistocene glacial maxima. Bathymetric data are from GEBCO (http://www.gebco.net/).


Em. annulatus
A. mosaicus
A. eydouxii
A. pooleorum
A. apraefrontalis
A. duboisii
A. laevis
A. fuscus
P. mertoni
Ep. greyae
Hydre. darwiniensis
M. gracilis
H. donaldi
H. atriceps
H. fasciatus
H. jerdonii
H. belcheri
H. cyanocinctus (IO)
H. coggeri
H. parviceps
H. cyanocinctus (WP)
H. lapemoides
H. viperinus
H. spiralis
H. platurus
H. curtus
H. stricticollis
H. obscurus
H. brookii
H. caerulescens
H. schistosus
H. stokesii
H. elegans
H. zweifeli
H. major
H. kingii
H. czeblukovi
H. macdowelli
H. pachycercos
H. peronii
H. ocellatus
H. ornatus (SEA)
H. lamberti
H. ornatus (IO)
Aipysurus group
Hydrophis group
Semi-aquatic lineages
Microcephalophis lineage
core Hydrophis group
Support values
● BS ≥ 70 and PP ≥ 0.9
● BS ≥ 70 or PP ≥ 0.9
7.0
6.0
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1.0
0.0

Figure S2. Bayesian time calibrated ultra-metric tree showing the phylogenetic relationships and divergence times of Indo-Pacific viviparous sea snakes. Node support is indicated with black and grey circles. Time scale is in millions of years before present. Grey vertical bar depicts the Pleistocene era (~2.588-0.011 mya). Colours (Red: Indian Ocean, Green: Southeast Asia, Blue: Australasia) of tips indicate the sampling locations and correspond to the regions shown in the map. (SEA-Southeast Asia, WP- West Pacific, IO-Indian Ocean)

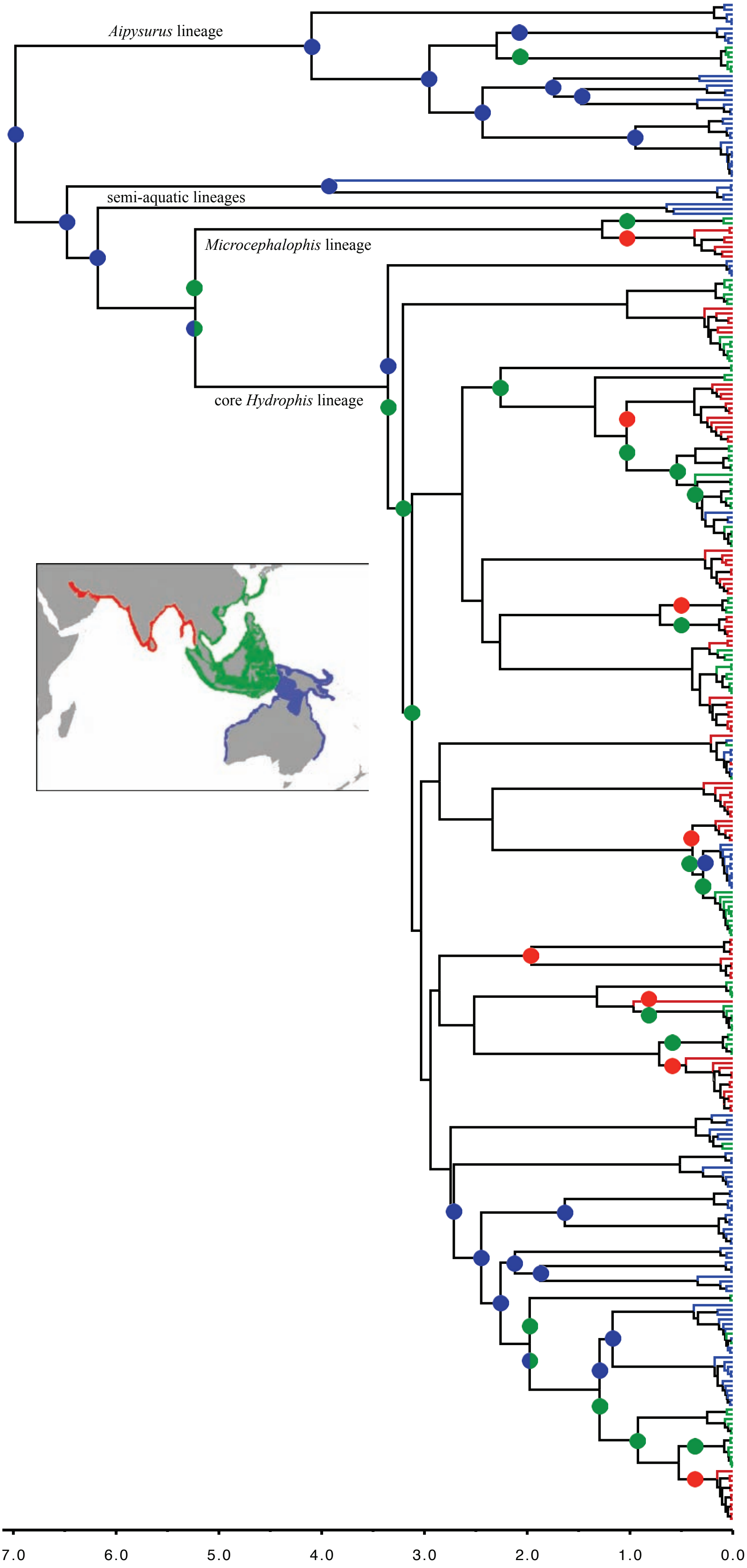
Aipysurus lineage
semi-aquatic lineages
Microcephalophis lineage
core *Hydrophis* lineage
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Figure S3. DEC ancestral area reconstruction of viviparous sea snakes. Most probable range inheritance scenarios are shown at each node. The tip labels are the same as for Figure 1. The colours correspond to ancestral areas shown in the inset map (Red: Indian Ocean, Green: SE Asia, Blue: Australasia). Nodes having more than one probable range inheritance scenarios are not shown. The circles above and below each node represent the range inherited by each lineage/species and circles on the nodes indicate that both branches/lineages/species inherited the same ancestral areas. Bicolored circles represent broad ranges containing two geographic states. Time scale is in millions of years before present.

Appendix S1: SUPPLIMENTARY MATERIALS AND METHODS

Specimen and Taxon Sampling

The study sampled a total of 320 individuals from 42 species of viviparous sea snakes. A total of 233 specimens from 36 species of viviparous sea snakes were obtained from fisheries by-catch during field trips to Australia, Indonesia, Vietnam, Thailand, Bangladesh, Sri Lanka, India and Iran (see Figure S1 for map). The specimens were (provisionally) identified following descriptions and diagnoses of Smith [1] and Rasmussen [2]. Liver/muscle tissue samples preserved in 90% Ethanol/Iso-propanol were collected from the specimens for DNA analysis. 52 additional tissue samples from 22 species from Australia, Myanmar, Malaysia, Thailand and Vietnam were obtained from specimens accessioned in museums and research institutions. Additional mitochondrial and nuclear sequences that were generated in previous studies were also obtained from the Genbank (www.ncbi.nlm.nih.gov/genbank/). Specimen collection localities and museum voucher numbers are provided in the Appendix S2.

DNA extraction, amplification and sequencing

Whole genomic DNA was extracted from liver/muscle tissues using standard Proteinase K protocols (Puregene™ DNA Isolation Tissue Kit, Gentra Systems). We amplified and sequenced a total of 5792 base pairs (bp) from three mitochondrial, two nuclear coding and three nuclear anonymous markers to reconstruct viviparous sea snake phylogeny. Mitochondrial markers were a 1095bp fragment of *Cytochrome b* (*Cyt-b*) gene [3], 838bp of *NADH dehydrogenase subunit 4* (*ND4*) and the adjacent *tRNA* region [4], and 531bp of *16S small subunit of ribosomal RNA* (*16SrRNA*) [5] gene. The two nuclear protein coding genes were a 918bp fragment of *Oocyte maturation factor* (*c-mos)* [6] and a 1066bp fragment of *Recombination activation gene* (*RAG-1*) [7]; and the three anonymous nuclear markers were *G1888* (428bp), *G1894* (422bp) and *G1914* (494 bp) isolated from a 454 shotgun sequencing run [8, 9]. All markers were amplified using standard PCR protocols with HotMaster^{TM}

Taq reagents (Applied Biosystems, Foster city, CA, USA) and 34 cycles; annealing temperatures were 52ºC for mitochondrial markers, 57ºC for *c-mos*, 56ºC for *RAG-1* and 59ºC for the three anonymous nuclear markers. Sequencing of the cleaned PCR products was outsourced to the Australian Genome Research facility (AGRF) in Adelaide. Visualisation of chromatograms from forward and reverse sequences and the generation of the consensus sequences were done in Geneious Pro 5.4 software [10]. Consensus sequences were aligned using Geneious Pro 5.4 [10] and then manually edited and refined by eye. Aligned sequences of the protein coding genes were translated into amino acid sequences to determine the correct reading frame and check for premature stop codons that might indicate amplification of pseudogenes. The sequences generated in this study are deposited in the Genbank (see Appendix S2).

Phylogenetic analyses and divergence dating

Time-calibrated viviparous sea snake phylogenies were inferred using Bayesian and Maximum likelihood (ML) analyses of the concatenated mitochondrial and nuclear alignment. *Hemiaspis damielli* was used as an outgroup in the analyses because there is strong molecular and morphological evidence that *Hemiaspis* is a close terrestrial relative to the viviparous sea snakes [11-13]. Partitioning schemes and best-fit substitution models for the concatenated dataset was assessed using the Bayesian information criterion (BIC) implemented in PartitionFinder ver. 1.0.1 [14]. BIC selected the following five-partition scheme and substitution models for the concatenated alignment: **partition 1** - *Cyt b* codon position 1, *ND4* codon position 1, *tRNA* region and *16SrRNA* (GTR+I+G); **partition 2** - *Cyt b* codon position 2, *ND4* codon position 2 (HKY+I+G); **partition 3** -*Cyt b* codon position 3, *ND4* codon position 3 (GTR+I+G); **partition 4** - *RAG-1* codon positions 1 and 2, *c-mos* codon position 1 (HKY+G); **partition 5** - *c-mos* codon positions 2 and 3, *RAG-1* codon position 3, *G1888, G1894, G1914* (HKY+I+G).

Multilocus coalescent (MC) methods did not converge in either MrBayes or BEAST despite extensive attempts, probably because of the large number of terminals (>300), and the less precise implementation of these methods in other packages appears inferior to concatenated approaches, at least with simulated data [15].

Bayesian analyses of the concatenated data, with estimation of the divergence times. were done in MrBayes 3.2, which implements heating and multiple chains to improve convergence [16]; analyses with BEAST did not converge adequately. Since there are no known Hydrophiini fossils that could be used to calibrate the tree, uniform secondary calibrations of 6.5 my to 10.6 my and 4.5 my to 7.9 my were applied to the root divergence and the *Aipysurus-Hydrophis* lineage split, respectively. These dates correspond to the 95% posterior distributions estimated for the two divergences using long nuclear sequences and several reliable squamate fossil calibrations [13, 17, 18]. We tested a strict versus relaxed (independent gamma rates) clock model of branch rate variation for the partitioned, concatenated dataset. The clock models used the default parameters in MrBayes, except that the prior on overall rate was set to encompass a broad range, with a lower bound of zero and an upper 95% bound of 17.4% per lineage per million years, ~10X the "typical" rate of mtDNA (normal distribution with mean 0.01 substitutions per million years and a standard deviation of 0.1, truncated at 0). Bayes factors were used to test support for the two clock models with $\log_{10}$ Bayes factor >3 considered as strong evidence for one model over another [19].

The analyses were conducted using the preferred (strict) clock model employing 7 million generations with two independent runs, four chains and a temperature of 0.1 with sampling every 5000 generations. Convergence of the independent runs was assessed by examining split frequencies of clades across runs and effective sample sizes (ESS values) of the likelihood plots through time in Tracer v1.5 [20], and the first 25% of sampled trees were discarded as burn-in.

Maximum Likelihood analyses were implemented in RAxML v7.2.8, which only implements the GTR substitution model (Stamatakis, 2006). The GTR+G substitution model was thus applied to the same partitions as for the Bayesian analyses, and performing 200 independent ML searches from random starting trees. Branch support in ML was estimated using 1000 bootstrap pseudoreplicates.

Genetic divergence

To estimate the amount of genetic divergence between sister lineages in different Ocean basins, corrected (HKY) pairwise sequence divergence was calculated for the mitochondrial *Cytochrome b* gene using Geneious Pro 5.4 software [10]. We used the highly variable mitochondrial gene *Cytochrome b* to estimate genetic divergence since it is the longest and the most complete mtDNA marker of all three used in this study. Furthermore the gene is probably the one most extensively used for phylogeographic divergence studies, so the values obtained here can be assessed in the context of many other taxa.

Ancestral Area Reconstruction (AAR): Additional Discussion of Bayesian and Likelihood analyses.

Three oceanic regions were delimited on the basis of endemism, species distribution maps, distribution of reciprocally monophyletic lineages across independent taxa and dispersal barriers such as deep-sea trenches (e.g. Timor trench). The three regions (Figure 1 inset map) are the Indian Ocean, SE Asia and Australasia (also see [21]). The Indian Ocean region in this study includes the seas of the Arabian Gulf through the coasts of Iran to India, and from Sri Lanka to Phuket-Thailand. The SE Asian region comprises majority (~70%) of the IAA and includes the coasts of peninsular Malaysia from south of Phuket-Thailand, seas surrounding the Greater Sunda Islands, Philippines, southern China, and up to Timor and the Moluccas. The Australasian region includes the seas east of the Moluccas with New Guinea, northern

Australia, Solomon islands, Vanuatu and New Caledonia broadly encompassing the eastern end of the IAA. Ancestral area reconstructions were done using Bayesian methods in BEAST [22], parsimony as implemented in Mesquite ver. 2.75 [23], and maximum-Likelihood as implemented in the Dispersal-Extinction-Cladogenesis (DEC) model in Lagrange [24]. The consensus tree from the MrBayes analysis was used for the ancestral area reconstructions. The first two methods assume fixed regions, whereas the third method accommodates potential fission and fusion of biogeographic regions through time. For all analyses, each sample was assigned to one of the three Oceanic regions based on the sampling locality (see colour-coding of tips in Figure S2).

The BEAST analyses implemented novel methods to test whether rates of dispersal varied across lineages (clades) and/or events. To test the importance of lineage-specific dispersal rates, we tested a model where different lineages (clades) were permitted different rates (using a random local clock: [25]) and one which assumed a uniform dispersal rate across all lineages (a "strict clock"). The random local clock estimates both the number, and phylogenetic position, of rate shifts on a phylogenetic tree (or pool of trees): this model returned a median of 3 shifts (i. e. 4 different clade-specific dispersal rates across the tree), and was preferred over a model assuming a common dispersal rate across all taxa (Table 2(D)).

Event-specific dispersal rates were also tested: i.e. whether particular classes dispersal events occurred at a faster rate than others. We tested 4 different dispersal models of differing complexity (see main text), by implemeting the relevant stepmatrix in BEAST (i.e. fixing certain classes of changes to be equal, or to be zero). The preferred model (Table 2) allowed dispersal only between adjacent regions, with all 4 permissible events happening at the same rate α:

	Australia	SE Asia	Indian Ocean
Australia	-	α	0
SE Asia	α	-	α
Indian Ocean	0	α	-

We focus discussion on the results of this "ordered" model, and also use this same model in the parsimony and likelihood analyses. BEAST analyses used the dated tree from the MrBayes analysis, and the standard Lewis [26] model for k-state discrete data, with the "ordered" stepmatrix above enforced using RateMatrix. The actual numbers of dispersals for each class of events (4 possible classes in the above matrix) were recorded for every sampled tree, using markovJumpsTreeLikelihood. There was a deliberately wide exponential prior (mean=0.1) for overall dispersal rate (i.e. averaged across clades and event classes). To ensure convergence, 4 separate BEAST runs were performed. Each used 120 million steps, sampling every 10000 steps, with a burnin of 20 million. The burnin fraction and convergence of the parameters were confirmed using Tracer v1.5 [20]. The post-burnin samples of four runs were combined in LogCombiner v1.62 [27] and summary statistics visualised in Tracer [20]. Bayes factors were calculated as twice the difference in harmonic mean $\log_{10}$-likelihood [19].

As in BEAST analysis we tested 2 different dispersal models in DEC analysis: (1) an unordered model that allowed dispersal between any region in equal rates despite the adjacency and (2) an ordered model that allowed dispersal only between adjacent areas in equal rates (i.e. fixing Australia↔Indian Ocean to be zero). The allowed maximum possible range size for a certain species was three, which included all three areas (only *H. platurus* is distributed in all three regions). However, we did not include the combination of Australasia-Indian Ocean as these areas were not adjacent to each other (allowed ranges were: Indian Ocean, SE Asia, Australasia, Indian Ocean-SE Asia-Australasia, Indian Ocean-SE Asia and SE Asia-Australasia). Each model was run for three times to test the consistency of the outcomes. All three runs in each model provided identical results and likelihood scores. Likelihood ratio tests did not strongly favour the unordered or the ordered models, but provided generally similar results for both models. Thus we provide only the results of the most biologically plausible ordered model (see above). Range inheritances scenarios >2 log-likelihood units from all other possible scenarios were considered as strong support for reconstructions at each node.

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Appendix S2. Details of the Specimens, voucher numbers and the respective Genbank accession numbers used in the molecular phylogenetic analysis

Species	Voucher number	Locality	Genbank Acession Number							
			Cytb	*ND4*	*16srRNA*	*G1888*	*G1894*	*G1914*	*Rag1*	*c-mos*
Aipysurus apraefrontalis	WAM157818	Exmouth, WA, Australia	JX002974	JX002981	JX002987	JX423466	JX423439	KC014212	KC014257	KC014288
Aipysurus apraefrontalis	WAM154750	Bundegi Reef, WA, Australia		JX423410	JX423421	JX423464	JX423438			
Aipysurus apraefrontalis	Aaprae41201	WA, Australia	KC014380		JX423420	KC014071				
Aipysurus duboisii	ABTC29025	Hibernia Reef, WA, Australia	JX423392	JX423411	JX423422	JX423468	JX423440	KC014213		
Aipysurus duboisii	ABTC29031	Hibernia Reef, WA, Australia	JX423393		JX423423	JX423469	JX423442			
Aipysurus duboisii	ABTC29029	Hibernia Reef, WA, Australia	KC014381	JX423412		KC014072	KC014149			
*Aipysurus duboisii**	Ad016	Scott reef, WA, Australia	DQ233907		DQ233983				FJ587086	FJ587164
Aipysurus eydouxii	GS160	Pasuruan, East Java, Indonesia	JX423395		JX423424	JX423475	JX423448			
Aipysurus eydouxii	GS165	Pasuruan, East Java, Indonesia	JX423398			JX423478	JX423452			
Aipysurus eydouxii	GS166	Pasuruan, East Java, Indonesia		JX423415	JX423425	JX423479	JX423453 / JX423454			
Aipysurus eydouxii	GS164	Pasuruan, East Java, Indonesia	JX423397	JX423414		JX423477	JX423450			KC014287
Aipysurus eydouxii	GS163	Pasuruan, East Java, Indonesia	JX423396	JX423413		JX423475	JX423448			
Aipysurus eydouxii	GS157	Pasuruan, East Java, Indonesia	JX002975	JX002982	JX002988	JX423474	JX423447	KC014214	KC014258	
Aipysurus fuscus	Afu181103	WA, Australia	KC014382		KC014309					
Aipysurus fuscus	Afu10	WA, Australia	JX423401		JX423429	JX423482				
Aipysurus fuscus	Afu1	WA, Australia	JX423402		JX423430	JX423480		KC014215		
Aipysurus fuscus	Afu3	WA, Australia	JX423404		JX423432	JX423483				
Aipysurus fuscus	Afu4	WA, Australia								
Aipysurus fuscus	Afu2	WA, Australia	JX423403		JX423431	JX423481				
Aipysurus fuscus	Afu201103gr	WA, Australia	KC014383		KC014310					
*Aipysurus fuscus**	Af032	Scott reef, WA, Australia	DQ233912	EF506635	DQ233988				FJ587090	FJ587168
Aipysurus laevis	ABTC29023	Cartier Islet, WA, Australia	EU547083	EF506673	EU547181			KC014216	EU546906	EU546945
Aipysurus laevis	ABTC29024	Cartier Islet, WA, Australia	KC014384	JX423419		JX423485	JX423456			
Aipysurus laevis	Al4	Australia	JX423406		JX423434	JX423487	JX423459			
Aipysurus laevis	Al3	Australia	JX423405		JX423433	JX423486	JX423458			
*Aipysurus laevis**	AL003	Ashmore reef, WA, Australia	DQ233913		DQ233989				FJ587087	FJ587169
Aipysurus mosaicus	MW04498	Weipa, QLD, Australia	JX423399	JX423417	JX423426	JX423471	JX423444			
Aipysurus mosaicus	MW04697	Weipa, QLD, Australia		JX423415	JX423425	JX423479	JX423453 / JX423454			
Aipysurus mosaicus	MW04699	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
*Aipysurus mosaicus**	Ae008	Groote Eylandt, NT, Australia	DQ233910	EF506637	DQ233986				FJ587092	FJ587166
Aipysurus pooleorum	WAM165709	Shark Bay, WA, Australia	KC014389	KC014461	KC014313	KC014080	KC014155			
Aipysurus pooleorum	WAM154747	Shark Bay, WA, Australia	KC014388	KC014460	KC014312	KC014079	KC014154	KC014219	KC014260	KC014289

*Emydocephalus annulatus**	Ea007	Cartier Islet, WA, Australia	DQ233940	FJ593196	DQ234001				FJ587094	FJ587172
Emydocephalus annulatus	Emydo1	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Emydocephalus annulatus	Emydo2	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Emydocephalus annulatus	Emydo3	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Emydocephalus annulatus	ABTC29030	Hibernia Reef, WA, Australia	EU547087	EU547038	EU547185	JX423488	JX423460	KC014222	EU546908	EU546947
Ephalophis greyae	WAM157940	Yanrey, WA, Australia	JX002976	JX002983	JX002989	KC014085				
Ephalophis greyae	WAM112180	Onslow, WA, Australia	KC014392	KC014466	KC014317	JX987153	JX987154	KC014223		
*Ephalophis greyae**	Eg001	Australia		FJ593197	FJ587208				FJ587095	FJ587173
*Ephalophis greyae**	Eg003	Australia		FJ593198	FJ587209				FJ587096	FJ587174
Hydrophis atriceps	X01002		KC014395		FJ587209					
Hydrelaps darwiniensis	ABTC29188	Dinah Beach, NT, Australia								FJ587176
Hydrelaps darwiniensis	NTM16471	Bing bong station, NT, Australia	EU547182	EU547035	EU547084				EU546907	EU546946
Hydrelaps darwiniensis	Hydr33	Australia	KC014413		KC014333					
Hydrophis atriceps	X01021	Vietnam	KC014396							
Hydrophis atriceps	X01003	Phan Tiet, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis atriceps	GS149	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis atriceps	GS152	Pasuruan, East Java, Indonesia		KC014470	KC014320	JQ217162	JQ217182	JQ217183		
Hydrophis atriceps	GS153	Pasuruan, East Java, Indonesia	JQ27206	JQ272152	JQ27216	KC014087			KC014262	KC014291
Hydrophis belcheri	CM1677		KC014397	KC014472	KC014321			KC014227		
Hydrophis belcheri	X01008	Phat Thien, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis brooki	R3126		TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis brooki	R3441		TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis brooki	R3625	Thale Luang, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis brooki	R3125		TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis caerulescens	R3280	Chittatgong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis caerulescens	MW04470	Anganna, E. Kalimantan, Indonesia	KC014405	KC014478	KC014327	KC014095				
Hydrophis caerulescens	MW04469	Anganna, E. Kalimantan, Indonesia	KC014404	KC014477	KC014326		KC014164			
Hydrophis caerulescens	GS170	Pasuruan, East Java, Indonesia	KC014403	KC014476	KC014325	JQ217164	JQ217186	KC014231		
Hydrophis caerulescens	MT221	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis caerulescens	GS177	Pasuruan, East Java, Indonesia				KC014094	KC014163			
Hydrophis caerulescens	GS174	Pasuruan, East Java, Indonesia	JG217208	JQ217154	JG217218	KC014093	KC014162		KC014266	KC014294
Hydrophis coggeri	MW04739	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis coggeri	GS137	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis coggeri	GS139	Makassar, Sulawesi, Indonesia	KC014406	KC014479		KC014096	KC014165		KC014267	
Hydrophis coggeri	MW04743	Makassar, Sulawesi, Indonesia	KC014408	KC014481	KC014330	KC014099	KC014167			
Hydrophis coggeri	MW04741	Makassar, Sulawesi, Indonesia	KC014407	KC014480	KC014329	KC014098	KC014166			KC014295

Hydrophis coggeri	MW04740	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	KLS0048	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	KLS0049	Pulmoddai, Trincomallee, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	CAS204975	Mwe Hauk, Ayeyarwady, Myanmar	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	KLS0015	Pulmoddai, Trincomallee, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	KLS0045	Pulmoddai, Trincomallee, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	KLS0047	Kirinda, Hambanthota, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	KLS0108	Uppaveli, Trincomalee, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	KLS0119	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MW04626	Pelabuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MT225	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	FMNH249637	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	FMNH249634	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	FMNH249640	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	FMNH249635	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	FMNH249650	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	GS138	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MW04687	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MW04673	Weipa, QLD, Australia				KC014140	KC014205			
Hydrophis curtus	MW04497	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MW04486	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MW04674	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	ABTC55605	Gulf of Carpentaria, Australia	EU547085	EU547036	EU547183				EU366437	EU366453
*Hydrophis curtus**	QMJ81025	Groote Eylandt, NT, Australia		FJ593231					FJ587123	FJ587200
*Hydrophis curtus**	Lc002	Yongala, QLD, Australia	DQ233968	FJ593227	DQ234040				FJ587122	FJ587199
Hydrophis curtus	MW04675	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MW04489	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	CM1678	Vietnam				KC014136				
Hydrophis curtus	MW04730	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MT232	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MZB(Ophi)3888	West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis curtus	MT228	Pasuruan, East Java, Indonesia				KC014137	KC014204			
Hydrophis curtus	MT231	Pasuruan, East Java, Indonesia				KC014138				
Hydrophis curtus	MT230	Pasuruan, East Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0110	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0069	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Hydrophis cyanocinctus	KLS0156	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0157	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0170	Uppaveli, Trincomalee, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	FMNH249604	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	FMNH249606	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	FMNH249656	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0061	Kirinda, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0171	Kumana, Ampara, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0013	Kirinda, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	KLS0114	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	India	Thiruvanathapuram, Kerala, India	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	FMNH234219	Sabah, Malaysia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	A021	Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	A022	Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	MZB3893	Pelebuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	GS140	Pasuruan, East Java, Indonesia	KC014409	KC014482	KC014331	KC014100	KC014169	KC014232	KC014268	KC014296
Hydrophis cyanocinctus	X01050	Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	MW04632	Pelabuhanratu, West Java, Indonesia	KC014411	KC014484						
Hydrophis cyanocinctus	MW04631	Pelabuhanratu, West Java, Indonesia	KC014410	KC014483						
Hydrophis cyanocinctus	SM064	Pelabuhanratu, West Java, Indonesia				KC014101				
Hydrophis cyanocinctus	SM063	Pelabuhanratu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis cyanocinctus	MZB3890	Pelabuhanratu, West Java, Indonesia	KC014412	KC014485	KC014332	JQ217166	JQ217189			
Hydrophis cyanocinctus	ABTC101328	Mornington Island, QLD, Australia								
*Hydrophis cyanocinctus**	QMJ83613	Gulf of Carpentaria, QLD, Australia		FJ593226					FJ587121	FJ587198
*Hydrophis cyanocinctus**	QMJ80944	Gulf of Carpentaria, QLD, Australia		FJ593224					FJ587119	FJ587196
Hydrophis czeblukovi	A014	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis czeblukovi	WAM102516	Eighty Mile Beach, WA, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis czeblukovi	A009	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis donaldi	MW04491	Weipa, QLD, Australia	JQ217202	JQ217147	JQ217211	JQ217159	JQ217180	KC014256	KC014285	KC014308
Hydrophis donaldi	MW04685	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis donaldi	MW04492	Weipa, QLD, Australia	JQ217203	JQ217148	JQ217212	JQ217160	JQ217181			
Hydrophis donaldi	MW04668	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis elegans	ABTC68026	Gulf of Carpentaria, Australia	KC014414	KC014486	KC014334		KC014170			
Hydrophis elegans	MW04693	Weipa, QLD, Australia								
Hydrophis elegans	MW04683	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
*Hydrophis elegans**	QMJ80945	QLD, Australia		FJ593217					FJ587114	FJ587191

Hydrophis elegans	Hele21	Australia	TBA	TBA	TBA	TBA			KC014269	
Hydrophis elegans	MW04702	Weipa, QLD, Australia		KC014487	KC014335		KC014171	KC014233		KC014297
Hydrophis elegans	MW04679	Weipa, QLD, Australia	KC014416	KC014489	KC014337					
Hydrophis elegans	WAM154979	Shark Bay, WA, Australia	KC014415	KC014488	KC014336	KC014103				
Hydrophis fasciatus	MZB3886	Pelebuharatu, West Java, Indonesia		KC014491	KC014338	KC014104	KC014174			
Hydrophis fasciatus	MW04623	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis fasciatus	SM66	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis fasciatus	MZB3892	Pelebuharatu, West Java, Indonesia	KC014418	KC014493	KC014340					
Hydrophis fasciatus	MW04624	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis fasciatus	MZB3891	Pelebuharatu, West Java, Indonesia	KC014417	KC014492	KC014339	KC014105	KC014175	KC014234	KC014270	KC014298
Hydrophis fasciatus	C009	Phuket, Thailand					KC014173			
Hydrophis fasciatus	KLS0074	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis fasciatus	KLS0098	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis fasciatus	KLS0102	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis fasciatus	KLS0103	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis fasciatus	KLS0128	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis jerdonii	X01014	Phan Tiet, Vietnam	KC014450	KC014524	KC014373					
Hydrophis jerdonii	X01001	Phan Tiet, Vietnam	KC014449	KC014523	KC014372	KC014135	KC014203	KC014249	KC014283	
*Hydrophis kingii**	QMJ79169	Gulf of Carpentaria, QLD, Australia		FJ593207					FJ587105	FJ587183
*Hydrophis kingii**	QMJ80500	Gulf of Carpentaria, QLD, Australia		FJ593208					FJ587106	FJ587184
Hydrophis kingii	ABTC101325	Cape Hillsborough, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lamberti	MW04734	Makassar, Sulawesi, Indonesia				KC014108	KC014179			
Hydrophis lamberti	MW04722	Makassar, Sulawesi, Indonesia	KC014421	KC014496	KC014343	KC014107	KC014178	KC014236	KC014272	KC014300
Hydrophis lamberti	MW04732	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lamberti	MW04735	Makassar, Sulawesi, Indonesia	KC014422	KC014497	KC014344	KC014109	KC014180			
Hydrophis lamberti	X01017	Ham Tan, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lamberti	X01018	Ham Tan, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lamberti	MW04742	Makassar, Sulawesi, Indonesia				KC014110	KC014181			
Hydrophis lapemoides	FMNH249540	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lapemoides	FMNH249598	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lapemoides	FMNH249585	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lapemoides	FMNH249494	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lapemoides	C0008	Phuket, Thailand	KC014425	KC014500	KC014347		JQ217191	KC014237		
Hydrophis lapemoides	KLS0017	Shanthipuram, Mannar, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lapemoides	KLS0033	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lapemoides	KLS0112	Inbaratti, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Hydrophis lapemoides	KLS0136	Polikandi, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis lapemoides	CAS216443	Kanythaya, Rahkhine st., Myanmar	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis major	WAM154972	Monkey mia, WA, Australia	KC014428	KC014503	KC014350					
Hydrophis major	ABTC55524	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis major	WAM154749	Exmouth Gulf, WA, Australia	KC014427	KC014502	KC014349					
Hydrophis major	WAM154748	Shark Bay, WA, Australia	KC014426	KC014501	KC014348	JQ217169	JQ217193	KC014238		
Hydrophis major	ABTC101327	Gulf of Carpentaria, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
*Hydrophis major**	Dm024	Mornington island, NT, Australia	DQ233937	FJ593210	DQ234018				FJ587108	FJ587186
*Hydrophis major**	QMJ79012	QLD, Australia		FJ593209					FJ587107	FJ587185
Hydrophis mcdowelli	AK48	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
*Hydrophis mcdowelli**	QMJ80570	Port Headland, WA, Australia		FJ593220					FJ587115	FJ587192
*Hydrophis mcdowelli**	QMJ83774	Gulf of Carpentaria, QLD, Australia		FJ593221					FJ587116	FJ587193
Hydrophis mcdowelli	ABTC101326	Moondalbee Island, QLD, Australia	JX002978	JX002985	JX002991	JQ217171	JQ217194			
Hydrophis obscurus	R3228	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis obscurus	R3386	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis obscurus	R3388	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis obscurus	R3376	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis obscurus	R3332	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ocellatus	A012	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ocellatus	A005	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ocellatus	WAM157730	Cottesloe, WA, Australia	KC014435	KC014508	KC014357	KC014122	KC014189	KC014242	KC014277	
Hydrophis ocellatus	Ho29493	Australia	KC014433		KC014355					
Hydrophis ocellatus	ABTC55606	Gulf of Carpentaria, Australia	KC014434	KC014507	KC014356	KC014121				KC014302
Hydrophis ocellatus	Ho16	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ocellatus	A008	Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ocellatus	A007	Australia	KC014432							
*Hydrophis ocellatus**	QMJ82080	Gulf of Carpentaria, NT, Australia		FJ593222					FJ587118	FJ587195
*Hydrophis ocellatus**	Ho014	Mornington Island, NT, Australia	DQ233962	FJ593223	DQ234027				FJ587117	FJ587194
*Hydrophis ocellatus**	CSIRO2529	Mornington Island, NT, Australia	DQ233962	FJ593223	DQ234027				FJ587117	FJ587194
Hydrophis ornatus	FMNH249567	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	FMNH249572	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	FMNH249558	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	FMNH260493	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	FMNH249554	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	FMNH249559	Phuket, Thailand	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	KLS0016	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Hydrophis ornatus	KLS0131	Inparasiddy, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	KLS0149	Inbaratti, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	KLS0155	Inparasiddy, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	KLS0173	Kirinda, Hambanthota, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	AK25	Vietnam?	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	X01024	Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	X01031	Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	X01025	Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis ornatus	MW04729	Makassar, Sulawesi, Indonesia					KC014191			
Hydrophis ornatus	GS136	Makassar, Sulawesi, Indonesia	KC014436	KC014509	KC014358	KC014123	JQ217195	KC014243	KC014278	
Hydrophis pachycercos	R3579	Phat Thien, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis pachycercos	X01009	Phat Thien, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis parviceps	X01011	Ham Tan, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis parviceps	X01010	Ham Tan, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
*Hydrophis peronii**	Ap007	Ashmore reef, WA, Australia	DQ233925	FJ593204	DQ234006				FJ587102	FJ587180
*Hydrophis peronii**	QMJ80919	Gulf of Carpentaria, QLD, Australia		FJ593202					FJ587100	FJ587178
Hydrophis peronii	MW04695	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis peronii	MW04682	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis peronii	MW04725	Makassar, Sulawesi, Indonesia	JQ217200	JQ217209	JQ217145	JQ217156				
Hydrophis peronii	MW04736	Makassar, Sulawesi, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis peronii	MW04724	Makassar, Sulawesi, Indonesia				KC014077	KC014152			
Hydrophis peronii	X01033	Ham Tan, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis peronii	MW04670	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
*Hydrophis peronii**	QMJ81362	Gulf of Carpentaria, NT, Australia		FJ593203					FJ587101	FJ587179
Hydrophis peronii	MW04677	Weipa, QLD, Australia					KC014151			
Hydrophis platura	MZB4068	Ternate, Indonesia	KC014454	KC014530						
Hydrophis platura	WAM165965	Cottesloe, WA, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis platura	WAM101240	Floreat beach, WA, Australia	KC014455	KC014531	KC014377	JQ217174	JQ217199			
Hydrophis platura	ABTC79057	Broken Bay, NSW, Australia		KC014528	KC014375	KC014142				
Hydrophis platura	KLS0091	Hikkaduwa, Galle, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis platura	KLS0095	Thali-Mannar, Mannar, Sri Lanka	TBA	TBA	TBA	TBA	TBA	KC014253		
Hydrophis platura	MW04728	Makassar, Sulawesi, Indonesia		KC014529	KC014376	KC014143				
*Hydrophis platura**	Pp004	South Ballina, NSW, Australia	DQ233978		DQ234052					
*Hydrophis platura**	NR8918	Richmond river, NSW, Australia		FJ593233					FJ587124	FJ587201
*Hydrophis platura**	NR8922	South Ballina, NSW, Australia		FJ593234					FJ587125	FJ587202
Hydrophis schistosa	CAS229885	Ka Net Thiri, Tanintharyi, Myanmar	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Hydrophis schistosa	IR001	Strait of Hormuz, Iran	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	IR002	Strait of Hormuz, Iran	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	KLS0007	Pubudugama, Puttlam, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	KLS0020	Kudiramale, Puttlam, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	KLS0023	Kudiramale, Puttlam, Sri Lanka		JX987170	JX987140		JX987157		JX987181	
Hydrophis schistosa	KLS0024	Kudiramale, Puttlam, Sri Lanka	JX987163	JX987171	JX987141	JX987148	JX987158		JX987182	KC014290
Hydrophis schistosa	KLS0026	Kudiramale, Puttlam, Sri Lanka	JX987164	JX987172	JX987142	JX987149				JX987186
Hydrophis schistosa	KLS0027	Pubudugama, Puttlam, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	KLS0028	Pubudugama, Puttlam, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	KLS0088	Puttlam laggon, Puttlam, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	KLS0169	Negambo, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	MZB12121	Anganna, E. Kalimantna, Indonesia	JX987161	JX987168	JX987138				JX987180	
Hydrophis schistosa	MZB12123	Anganna, E. Kalimantna, Indonesia	JX987162	JX987169	JX987139	JX987146	JX987160			
Hydrophis schistosa	SM067	Pelebuhanratu, West Java, Indonesia				JX987147	JX987159			
Hydrophis schistosa	MZB12126	Anganna, E. Kalimantna, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis schistosa	MZB12124	Anganna, E. Kalimantna, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0002	Kirinda, Hambanthota, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0019	Pulmodai, Trincomallee, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0059	Kirinda, Hambanthota, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0070	Kirinda, Hambanthota, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0071	Pulmodai, Trincomallee, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0093	Negambo, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0094	Nadukuda, Mannar, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0104	Negambo, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0107	Vankalei, Mannar, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	KLS0109	Vankalei, Mannar, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	MZB3898	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	MZB3896	Pelebuharatu, West Java, Indonesia	KC014443	KC014515	KC014364					
Hydrophis spiralis	MZB3894	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	MZB3899	Pelebuharatu, West Java, Indonesia	KC014445	KC014517	KC014366	KC014130	KC014198	KC014247	KC014281	KC014305
Hydrophis spiralis	MZB3887	Pelebuharatu, West Java, Indonesia	KC014444	KC014516	KC014365	KC014129	KC014197			
Hydrophis spiralis	SM062	Pelebuharatu, West Java, Indonesia	KC014446	KC014518	KC014367	KC014131	KC014199			
Hydrophis spiralis	MW04641	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	MZB3897	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	MZB3895	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis spiralis	MW04637	Pelebuharatu, West Java, Indonesia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Hydrophis stokesii	MW04720	Makassar, Sulawesi, Indonesia	KC014391	KC014463	KC014315	KC014082	JQ217178	KC014220		
Hydrophis stokesii	MW04726	Makassar, Sulawesi, Indonesia		KC014464	KC014316	KC014084	KC014157			
Hydrophis stokesii	WAM157820	Exmouth Gulf, WA, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis stokesii	MW04488	Weipa, QLD, Australia	KC014390	KC014462	KC014314		KC014156			
*Hydrophis stokesii**	QMJ83580	Gulf of Carpentaria, NT, Australia		FJ593205					FJ587103	FJ587181
*Hydrophis stokesii**	As008	Ashmore reef, WA, Australia	DQ233929	FJ593206	DQ234010				FJ587104	FJ587182
*Hydrophis stokesii**	As007	Vanderlin island, NT, Australia	DQ233928		DQ234009					
Hydrophis stokesii	MW04485	Weipa, QLD, Australia	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis stricticollis	R3242	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis stricticollis	R3245	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis stricticollis	R3251	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis stricticollis	R3257	Chittagong, Bangladesh	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis viperina	KLS0079	Valvettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis viperina	KLS0111	Inbaratti, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis viperina	KLS0118	Inbaratti, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis viperina	KLS0120	Thikam, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis viperina	KLS0139	Polikandi, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis viperina	MW04719	Makassar, Sulawesi, Indonesia	JX002980	JX002986	JX002993	KC014144	KC014209	KC014254	KC014284	KC014307
Hydrophis viperina	MW04731	Makassar, Sulawesi, Indonesia		KC014532	KC014378	KC014145	KC014211			
Hydrophis viperina	X01005	Phan Tiet, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis viperina	X01006	Phan Tiet, Vietnam	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hydrophis zweifeli	KU0012	Weipa, QLD, Australia		JX987176	JX987145				JX987179	JX987184
Hydrophis zweifeli	QMJ79014	Repulse Bay, QLD, Australia	FJ587155	FJ593211	FJ587210	JX987150	JX987155		FJ587109	FJ587187
Hydrophis zweifeli	MW04667	Weipa, QLD, Australia	JX987166	JX987174					JX987177	JX987185
Hydrophis zweifeli	MW04666	Weipa, QLD, Australia	JX987165	JX987173	JX987143	JX987151				JX987183
Hydrophis zweifeli	MW04690	Weipa, QLD, Australia	JX987167	JX987175	JX987144	JX987152			JX987178	
Microcephalophis gracilis	GS147	Pasuruan, East Java, Indonesia	KC014419	KC014494	KC014341	KC014106	KC014176	KC014235	KC014271	KC014299
Microcephalophis gracilis	GS148	Pasuruan, East Java, Indonesia	KC014420	KC014495	KC014342		KC014177			
Microcephalophis gracilis	KLS0001	Kirinda, Hambanthota, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microcephalophis gracilis	KLS0096	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microcephalophis gracilis	KLS0097	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microcephalophis gracilis	KLS0099	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microcephalophis gracilis	KLS0100	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microcephalophis gracilis	KLS0101	Velettithurai, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microcephalophis gracilis	KLS0123	Thikam, Jaffna, Sri Lanka	TBA	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Parahydrophis mertoni	ABTC28239	Palmerston boatramp, NT, Australia	JX002979	FJ593201	JX002992	JQ217155	JQ217175	KC014251 /	FJ587099	FJ587177

*Hemiaspis damieli**	Genbank	Australia	EU547073	EU547025	EU547171		EU546896	EU546935

Abbreviations

ABTC- Australian Biological Tissue collection, South Australian Museum, Adelaide, Australia; **WAM**- Western Australian Museum, Perth, Australia; **CAS**- California Academy of Science, San Fracisco, USA; **FMNH**-Field Museum of Chicago, Chicago, USA; **QM**- Queensland Museum, Brisbane, Australia; **MZB**- Museum of Zoology, Borgor, Indonesia

CSIRO- National Wildlife Collection, Canberra, Australia; **CM**- Raffles Museum of Biodiversity research,National University of Singapore, Singapore

Specimens bearing **KLS** numbers will be deposited in the National Museum of Sri Lanka, Colombo, Sri Lanka

TBA- To Be Accessioned

*Sequences downloaded from the Genbank

CHAPTER 6: Concluding Discussion and Future Directions

This thesis consists of four research chapters that have been published as research papers, accepted for publication, or ready to be submitted for publication. Each of these chapters have their own detailed discussion on the research outcomes. However, in this chapter I summarise the overall findings of this study, discuss their broader implications and highlight future directions.

6.1. Summary of aims of thesis

In this this thesis I examined the systematics, evolution and biogeography of the viviparous sea snakes. Specifically, I described a previously undiscovered species of viviparous sea snake from northern Australia, examined species limits, phylogenetic relationships and morphological convergence in the medically important beaked sea snakes of the genus *Enhydrina*, assessed how past and present barriers to gene flow in the Indo-Pacific marine biogeographic region have influenced population structure and speciation in the viviparous sea snake *Hydrophis curtus*, and investigated broad-scale biogeographic and temporal patterns of diversification in viviparous sea snakes in the Indo-Pacific. The following discussion will summarise the major findings, and discuss broader implications and recommendations for future research.

6.2. Systematics of the Indo-Pacific viviparous sea snakes

6.2.1. Viviparous sea snake phylogeny and generic boundaries

Systematics of the viviparous sea snakes has always been challenging due to a lack of a robust phylogenetic hypothesis and hence many species have been variously placed in numerous monotypic genera (Smith, 1926; McDowell, 1972; Voris, 1977; Rasmussen, 1997;

Kharin, 2004). The 8-gene sea snake timetree reconstructed in chapter five represents the most taxonomically complete phylogeny for the group to date. The evolutionary relationships and divergence times estimated in this study are consistent with the recently published findings of Sanders *et al.* (2013a), which are based on much of the same molecular data and calibrations but with far sparser intra-specific sampling: the current study strongly supports monophyletic *Aipysurus* and *Hydrophis* groups sensu Smith (1926), plus four major lineages within the *Hydrophis* group: *Ephalophis-Parahydrophis, Hydrelaps*, *Microcephalophis* and the core *Hydrophis* radiation. In addition, the present phylogeny recovered the paraphyly of the genus *Hydrophis,* with the short and weakly resolved backbone of the core *Hydrophis* group consistent with its recent rapid radiation (Lukoschek and Keogh, 2006; Sanders *et al*., 2013a). Although this study and Sanders *et al.* (2013a) were able to include ~70% of all known viviparous sea snakes, the relationships of 14 species of viviparous sea snakes are yet to be investigated using molecular data. Future analyses including these missing taxa are needed to more fully understand the systematics and the evolutionary history of this group of snakes.

The five nuclear sequence markers used in this study provided few variable sites even at the inter-specific level and yielded highly discordant gene trees when they were analysed separately (see chapter 3 and Sanders *et al.,* 2013a). The discordant gene trees retrieved are probably due to low signal (few variable sites) in the nuclear markers, as well as slow rates of lineage sorting and the rapid divergences and short speciation intervals found in sea snakes. Consequently low statistical support for these nodes (even for fast-evolving mitochondrial markers) mean that the evolutionary relationships of the core *Hydrophis* group remain poorly resolved (this study and Sanders *et al.,* 2013a). Therefore, identifying many additional polymorphic nuclear loci developed using next generation sequencing methods, or testing of the utility of other available nuclear loci (eg: (Portik *et al.*, 2012), should be a priority to further resolve the relationships of the core *Hydrophis* sea snakes.

6.2.2. Species diversity, cryptic species, population genetic structure and regional endemism

As a part of the systematics portion of this thesis I described the morphologically highly distinct viviparous sea snake species, *Hydrophis donaldi*, from northern Australia (Chapter 2). Phylogenetic analysis of mitochondrial and nuclear sequences revealed this new species to be a deeply divergent lineage within the *Hydrophis* group. Recently another previously overlooked species of viviparous sea snake was described from northern Australian waters (Sanders *et al.*, 2012). This species (*Aipysurus mosaicus*) showed a long history of isolation and clear morphological differentiation from allopatric populations in Southeast Asia (*A. eydouxii*). Both new species were collected from the Gulf of Carpentaria (GoC), the most intensively surveyed region for sea snakes in Australia due to their high levels of mortality as bycatch in prawn trawls (Redfield *et al.*, 1978; Wassenberg *et al.*, 1994). This highlights how little is known about the diversity of viviparous sea snakes even in a region that has been relatively well explored and emphasizes the importance of continued taxonomic studies in these regions, let alone less well surveyed regions.

In the third chapter of this thesis I showed that the ‘beaked’ sea snakes that were termed *Enhydrina schistosa* (*Hydrophis schistosus*) and thought to be distributed from the Arabian Gulf to Australia in fact consist of two species that are not even closest relatives. Due to morphological similarity, Australian population was tentatively assigned the to *Enhydrina zweifeli* (*Hydrophis zweifeli*), a species described from a single specimen from northern New Guinea but without reference to other *Enhydrina* specimens from the Australo-Papuan region (Kharin, 1985). Based on molecular evidence, I postulated that the external morphological similarity of these two unrelated species is due to ecological convergence as a result of dietary specialization. However, closer examination of the morphological characters in these two species revealed notable differences in scalation and skull morphology between the Australasian and Asian species. This result is especially significant because beaked sea snakes are responsible for the majority of sea snake bites worldwide, especially to fishermen handling nets, and the only sea snake anti-venom is raised against the Asian population of this species. If it were not for the extremely streamlined nature of sea snake venoms (Fry *et al.*, 2003), it is likely that conflating these ‘beaked’ forms as a single species would have had serious medical consequences. The striking phenotypic convergence of these species also highlights the importance of molecular methods in systematics as decisions based solely on morphology can yield inaccurate relationships where unrelated species have developed similar adaptations to similar environments or ecological selection pressures (Futuyama, 1998).

In the fourth chapter of this thesis I showed that the widely distributed spine-bellied sea snake, *Hydrophis curtus* (formally *Lapemis curtus*) consists of two long-isolated lineages consistent with cryptic species in the Indian Ocean and West Pacific. Historically *H. curtus* was considered to comprise two distinct species in the Indian Ocean (*Lapemis curtus*) and the West Pacific (*Lapemis hardwickii*) (Smith, 1926). Due to the lack of morphological variation between these two species, they were synonymized to a single species (Gritis & Voris, 1990). Since our genetic analyses indicate the presence of at least two species with limited recent gene flow, a re-evaluation of morphological traits that may separate these lineages is needed. Furthermore, intensive sampling is necessary especially around Myanmar and the Andaman coast of Thailand in order to locate the possible contact zone for individuals from the Indian Ocean and West Pacific lineages. Quantifying and characterizing hybridization dynamics in this region would potentially shed light on the mechanisms (biological or environmental) that maintain species boundaries and facilitate speciation in sea snakes.

The population level findings presented in chapter 4 are highly consistent with the broad scale phylogenetic analyses done in chapter 5: Here I analysed samples from widespread species spanning different Ocean basins (Indian Ocean, Southeast Asia, Australasia) to reveal the presence of geographically divergent cryptic lineages in many species. Five species showed strongly supported and reciprocally monophyletic lineages that correspond to the Indian Ocean and Southeast Asia/West Pacific, while some species showed further cryptic divergence within the West Pacific between lineages in Southeast Asia and

Australasia. This suggests that the actual diversity and regional endemism of viviparous sea snakes is higher than currently known. However, broader sampling across each species' range and morphological studies are needed to delimit geographic ranges and cryptic species boundaries. Although intraspecific corrected pairwise genetic divergences in the *Cytochrome b* gene ranged between 2.5% and 9.5%, many species show little or no corresponding morphological divergence and as a result it is presently difficult to diagnose new species. In particular, our analyses recovered distantly related but cryptic lineages within both *Hydrophis cyanocinctus* and *H. ornatus* in the Indian Ocean and West Pacific. *Hydrophis cyanocinctus* sampled from the Indian Ocean formed the sister lineage to a clade that contained three highly morphologically divergent species, including *H. cyanocinctus* from West Pacific (Southeast Asia and Australasia), *H. parviceps* and *H. coggeri* which was also consistent with a previous study (Sanders *et al.*, 2013b). Similarly, *H. ornatus* in Southeast Asia was sister to a clade that contained Southeast Asian species *H. lamberti* and *H. ornatus* from the Indian Ocean. Whether the paraphyly of *H. ornatus* reflects phenotypic convergence or is due to incomplete lineage sorting of molecular markers needs to be tested using additional independent nuclear markers. Moreover, in addition to identifying morphological characters that may separate these cryptic lineages, it would be worthwhile to test for the presence of gene flow between them, which would provide further evidence that could be used in species delimitation.

Although the cryptic lineages discovered in this study are not yet confirmed as valid species, they at least qualify as 'Evolutionary significant units' (Ryder, 1986; Moritz, 1994). Comparative phylogeographic data from many species of viviparous sea snakes suggest the presence of at least two ESU's that correspond to the Indian Ocean and Southeast Asia/West Pacific. The purpose of defining ESU's are to ensure that the genetic diversity and evolutionary heritage of a species is recognized and protected (Moritz, 1994). Divergence dating indicates that the viviparous sea snakes spanning the Indian Ocean and West Pacific have long (between 2.5-0.5 million years) and complex evolutionary histories in the region with a unique conservation value. Marine protected area networks currently ignore sea snakes due to a lack of data for these potentially vulnerable species. Further, there are currently no conservation policies that target sea snakes in terms of fishing practices, trade restrictions or protected areas (Livingstone, 2009). On a broader scale, results from this study indicate significant limits to genetic connectivity among sea snake populations (eg: *H. curtus*) in the Indo-West Pacific (IWP) as well as within the West Pacific. Identification of these distinct phylogeographic regions through comparative phylogeographic approaches is critical for regional scale marine conservation planning in the IWP (Sala *et al.*, 2002; Reaka & Lombardi, 2011). Thus, findings on the systematics and population genetic structure of viviparous sea snakes in this thesis can be used not only to inform future sea snake targeted conservation but may also be used to inform marine reserve planning in the IWP as a whole.

6.2.3. Species concepts for viviparous sea snakes

The findings on viviparous sea snake systematics highlighted in this thesis and the recent discovery of *Hydrophis donaldi* (Ukuwela *et al.*, 2012) and *Aipysurus mosaicus* (Sanders *et al.*, 2012) demonstrate the efficacy of molecular methods in identifying and delineating morphologically cryptic species and distinct evolutionary lineages. More and more systematists are using molecular methods in conjunction with morphology in species delimitation because of their ability to recover species that are morphologically indistinct (Oliver *et al.*, 2009; Vieites *et al.*, 2009; Strutzenberger *et al.*, 2011). Molecular methods have revolutionized biodiversity research and have increased the speed of the taxonomic process. As a result the number of species delimited with the aid of molecular methods have rapidly increased during the last decade (Bickford *et al.*, 2007). The current best practice of species delimitation is based on the reciprocal monophyly of subgroups in gene trees from multiple independent loci within a putative species (Avise, 1994; Sites & Marshall, 2004). Currently, DNA based taxonomy mainly has, at the minimum, an important role in validating hypotheses for alpha taxonomy based on morphology. Due to its rising popularity in recent years, a few authors have proposed a more primary or even a fundamental role in taxonomy (Hebert *et al.*, 2003; Tautz *et al.*, 2003). However, many have argued that rather than replacing the conventional method of morphological taxonomy by molecular taxonomy, both should be used in conjunction and mutually supplement each other (Lee, 2004). Taking this in to consideration, I have refrained from delimiting viviparous sea snake species based solely from reciprocally monophyletic molecular clades that correspond to different Ocean basins. However, this information can provide a useful framework for investigating other data sources eg: morphology, ecology, behavior, population genetics, distribution patterns, which could be used together with molecules to delimit species in this taxonomically challenging group of marine reptiles.

6.3. The evolutionary history of viviparous sea snakes in the Indo-Pacific

As mentioned at the beginning of this thesis viviparous sea snakes represent a rapidly diverged adaptive radiation. However, the underlying drivers of such rapid speciation are not well understood. Palaeoclimatic and past geological events have been implicated in accelerating speciation in many Indo-Australian marine groups (Hewitt, 2003; Janis, 2003), but their influence in sea snake speciation has rarely been investigated (Lukoschek *et al.*, 2008). For this reason, I examined the role of palaeoclimatic and past geological events in the speciation of viviparous sea snakes both at the population level and the broad-scale. First, I evaluated how past and present barriers to dispersal caused by historical geoclimatic events in the Indo-Pacific marine biogeographic region have influenced fine-scale population genetic structure and speciation in a fully marine snake using the widely distributed spine-bellied sea snake *Hydrophis curtus* as a representative example. Then, I used a phylogenetic and a comparative phylogeographic approach to examine geographical and temporal patterns of diversification in viviparous sea snakes across the Indo-Pacific. The following section summarises the overall findings of these studies and discusses their broader implications.

6.3.1. Speciation at the population level

In the fourth chapter of the thesis I showed how past and present barriers to gene flow caused by the contemporary deep-water Timor Trench and historical geoclimatic events (specifically sea level fluctuations) in the IWP have contributed to the genetic and species diversity in the widely distributed spine-bellied sea snake, *Hydrophis curtus*. Data on mitochondrial and nuclear sequences and microsatellite variation of this species sampled from India to Australia revealed strong and concordant geographic structure with a prominent genetic break between populations broadly distributed in the Indian Ocean and the West Pacific. Mitochondrial DNA lineages and microsatellite population genetic assignment tests indicated the presence of four distinct populations of *H. curtus* in the IWP. Divergence dating suggested that the two main populations in the Indian Ocean and West Pacific diverged in the late Pliocene or early Pleistocene (~2.8 mya). Interestingly, microsatellite admixture analyses indicated very limited recent gene flow between these oceanic regions despite the current lack of barriers to dispersal. The three West Pacific populations (Phuket-Thailand, Southeast Asia and Australia) indicated a mid-late Pleistocene divergence and also showed molecular evidence of recent range expansions. Due to the absence of a strong pattern of isolation by distance within the West Pacific, I hypothesized that the historical vicariance caused by the Pleistocene Sunda shelf and Timor Trench biogeographic barriers (Pillans *et al.*, 1998; Voris, 2000) have caused the genetic divergence seen in mitochondrial and nuclear DNA. Previous studies have also demonstrated the effect of historical isolation caused by the Sunda shelf/Indo-Pacific biogeographic barrier on other marine taxa (reviewed in (Carpenter *et al.*, 2011; Lohman *et al.*, 2011). Signals of recent demographic or range expansion observed in *H. curtus* populations in Southeast Asia and Australia are also consistent with other marine taxa that show range expansions onto continental shelves as sea levels rose at the end of the Pleistocene (Lukoschek *et al.*, 2007; Crandall *et al.*, 2008; Gaither *et al.*, 2010).

Although the Sunda shelf posed a historical or a ‘soft’ biogeographic barrier for dispersal and gene flow in the past for *H. curtus* and other marine taxa in the IWP, it disappeared when sea levels reached current levels ~10000 years ago. However, the presence of four distinct populations with limited among-region gene flow indicates the presence of cryptic barriers for contemporary gene flow in the IWP or other isolating mechanisms. Most striking is the deep genetic divergence in *H. curtus* populations in the Indian Ocean and Phuket-Thailand. This deep genetic divergence may suggest the presence of a cryptic barrier somewhere between Myanmar and Phuket-Thailand or an unknown mechanism that limits gene flow between these two populations. Interestingly, admixture analysis indicated asymmetrical gene flow from Phuket-Thailand to populations in Southeast Asia. This suggests that there is asymmetrical dispersal from Phuket-Thailand to Southeast Asia, probably due to an oceanic surface current that may carry individuals from Phuket-Thailand to Southeast Asia (Lumpkin, 2013). Phylogeographic breaks in the West Pacific suggest that the Timor trench and the Halmahera eddy may represent possible dispersal barriers for *H. curtus* populations. Previous studies also report this geographic feature (Ovenden *et al.*, 2009; Sanders *et al.*, 2012) and the Halmahera eddy (DeBoer *et al.*, 2008) as possible barriers for dispersal and gene flow between populations in Australasia and Southeast Asia. However, more extensive sampling is required to determine the exact boundaries and contact zones of each lineage/population in order to better characterise contemporary barriers and other isolating mechanisms that prevent present day dispersal and gene flow. The findings of this study indicate an important role for Plio-Pleistocene vicariance in generating population genetic and species diversity in *H. curtus*. Further, the results imply a significant role for the contemporary but subtle barriers that separate and limit the gene flow between the populations that diverged in the Plio-Pleistocene.

6.3.2. Speciation and Pleistocene sea level changes

The time-calibrated phylogenies presented in chapter five reveal a rapid upturn of viviparous sea snake lineage accumulation in the last 2.5-0.3 my. However, this rapid speciation characterizes just the highly diverse core *Hydrophis* group; in contrast the less diverse *Aipysurus* group shows only a moderate rate of Pliocene-Pleistocene speciation within Australasia. These findings are also consistent with the previous study of Sanders *et al.* (2013a). Thus the results indicated that in both the *Aipysurus* and core *Hydrophis* groups the majority of lineages have diversified over the last 2.5 my and consequently most sister-species are less than 2.4 my old. Cyclic sea level changes in the Pleistocene during the last 2.5 million years in the Indo-Australian Archipelago (IAA) (Voris, 2000; Lambeck *et al.*, 2002) are believed to have facilitated speciation of marine fauna via vicariance in isolated marine basins (e.g. (De Bruyn & Mather, 2007; Crandall *et al.*, 2008). The upturn of diversification rates in the last 2.5 my, presence of sister-species less than 2.4 my old and the divergence of distinct geographic lineages between the Indian Ocean, Southeast Asian and Australasian marine biogeographic regions during the last 2.34-0.53 my supports the Pleistocene vicariance hypothesis. The phylogeographic and temporal patterns uncovered for Indo-Pacific sea snakes are also consistent with other species of marine invertebrates (Lavery *et al.*, 1996; Williams & Benzie, 1998; Benzie, 1999; Duda & Palumbi, 1999) and fish (Drew & Barber, 2009; Leray *et al.*, 2010; Gaither *et al.*, 2011) studied in this region. However, studies on other marine taxa from this region indicate that many taxa currently recognized at the species level and above pre-date the Pleistocene (Barber & Bellwood, 2005; Renema *et al.*, 2008; Williams & Duda Jr, 2008; Cowman & Bellwood, 2013). Thus, while Pleistocene vicariance appears to have played an important role in generating cryptic species and genetic diversity in Indo-Pacific viviparous sea snakes and other marine taxa, its contribution to the alpha diversity of viviparous sea snakes remains unknown. This is largely because these morphologically indistinct cryptic species/lineages are not currently considered as species and also due to the lack of information on the effect of this phenomenon on deeper internal nodes of the viviparous sea snake phylogeny.

6.3.3. Non-geographic speciation drivers

As mentioned in the above section, allopatric or geographic speciation does not entirely explain the rapid lineage accumulation and alpha diversity in viviparous sea snakes though it coincides with the Pleistocene transgressions. Many non-geographic factors have been proposed to describe elevated speciation rates in marine fish and corals in the IAA region. The Sunda and Sahul shelves which underlies the IAA are the largest tropical continental shelf area on earth (Hanebuth *et al.*, 2000). Thus the availability of a highly heterogeneous environment means that divergent selection pressures (Rocha & Bowen, 2008) and intense competition (Briggs, 2005; Bowen *et al.*, 2013) might be responsible for accelerating speciation in the region. Complex oceanic currents (Barber *et al.*, 2006), geological complexity (Hall, 1998) and eustatic sea-level changes (Emery & Niino, 1963) in the region are also thought to reduce gene flow and isolate populations. These hypotheses may also explain the high speciation rates in viviparous sea snakes of the core *Hydrophis* group in the IAA. The presence of highly divergent ecomorphs with specialized dietary adaptations in the core *Hydrophis* group (Voris & Voris, 1983) may indicate that divergent selection pressures coupled with intense competition have accelerated speciation. The low species and ecomorph diversity and reduced speciation rates in the mainly Australasian *Aipysurus* group is consistent with this hypothesis. A recent study indicated that rapid evolution of head and fore-body size variation in viviparous sea snakes of the core *Hydrophis* group is a likely contributor to the explosive speciation (Sanders *et al.*, 2013b). The findings of the present study only provide a timeframe for speciation and a possible link between the Pleistocene sea level changes and speciation of viviparous sea snakes. However, it is important to note that the rapid radiation of core *Hydrophis* sea snakes considerably post dates the initial transition to Southeast Asian habitats (in *Microcephalophis*, ~2 my earlier); also *Hydrophis* species subsequently re-colonised Australasia to generate seven endemic species there. Further studies exploring the ecological, genetic and physiological basis of speciation may provide more insights to the rapid speciation of viviparous sea snakes in the core *Hydrophis* group.

6.4. Biogeography and colonisation history of viviparous sea snakes across the Indo-Pacific

In the fifth chapter of this thesis I examined the biogeographic origins and spatio-temporal patterns of broad-scale diversification in viviparous sea snakes. Since the diversity of the viviparous sea snakes also peaks at the IAA marine biodiversity hotspot, the findings on viviparous sea snakes may also provide important insights to the evolutionary origins and biogeographic history of this region. Explaining the origins and maintenance of high marine biodiversity in the IAA remains a central goal in marine biogeography and evolutionary biology (Barber, 2009). Most of the studies that have investigated the biogeography of Indo-Pacific marine taxa involved a single (Williams & Benzie, 1998; Gaither *et al.*, 2011) or few species (Williams, 2007; Gaither *et al.*, 2010) or focused primarily on fish (Barber & Bellwood, 2005; Gaither & Rocha, 2013). Thus the study of viviparous sea snakes presented in this thesis provides a novel perspective and represents a major contribution towards understanding the evolutionary and biogeographic history of this important marine region.

Results from this study indicated an Australasian origin for extant viviparous sea snakes. Further, the findings show that the *Aipysurus* group and semi-aquatic lineages also originated and subsequently diverged in Australasia during the Plio-Pleistocene with only *Emydocephalus* and *A. eydouxii* having colonised Southeast Asia. Most significantly the study indicates a transition to Southeast Asian habitats for the *Microcephalophis* lineage and the core *Hydrophis* group with the initial rapid diversification in the latter occurring in Southeast Asia approximately 2.5 mya.

The finding that the majority of the viviparous sea snake diversity in Southeast Asia is predominantly derived from an *in-situ* radiation that diversified from a single colonisation from Australasia strongly support the idea that the IAA is a ‘centre of origin/speciation’ for viviparous sea snakes. Most importantly this rapid radiation gave rise to the most ecomorphologically diverse group that comprise more than 60% of global viviparous sea snake diversity within a short time frame in Southeast Asia. Elevated speciation rates are consistent with the 'centre of speciation' hypothesis, however as discussed in section 6.3.2., results from this study do not reveal a specific mechanism or reasons for rapid speciation. Analyses show that Southeast Asia exported diversity to Indian Ocean and Australasia several times, but also received sea snake diversity from adjacent regions (mainly Australasia). Thus the centre of origin scenario does not entirely explain the high diversity of *Hydrophis* in the IAA. Collectively, these findings indicate that IAA has acted both as a sink and a source for viviparous sea snakes, a finding that is consistent with the 'biodiversity feedback' model under which hotspots export diversity to adjacent regions and also accumulate species with origins in peripheral areas (Bowen *et al.*, 2013).

The 'centre of overlap' hypothesis suggests that the isolation of conspecific populations across the Indo-Pacific by the shallow Sunda and Sahul shelves of Indonesia, Malaysia and Northern Australia (Sunda shelf or Indo-Pacific biogoegraphic barrier) in the Pleistocene led to allopatric speciation contributing to distinct Pacific and Indian Ocean faunas (Woodland, 1983). The relaxation of the this biogeographic barrier following sea-level rise resulted in the dispersal of sister taxa that were formally separated between the Pacific and Indian Oceans leading to the overlap of geographic ranges at the IAA (Woodland, 1983). Although it was believed that sea level fluctuations in the Pleistocene were responsible in isolating the populations in the Indian and Pacific Oceans leading to allopatric speciation, subsequent phylogenetic studies indicated much older divergence times for most allopatric sister species (Barber & Bellwood, 2005; Renema *et al.*, 2008; Cowman & Bellwood, 2013). The findings in the chapters four and five in this thesis show Plio-Pleistocene divergence across the Indian and Pacific Oceans in five species of sea snakes but without any strong indication of post divergence colonisation of the IAA by Indian Ocean populations. However, many species of marine invertebrates (Crandall *et al.*, 2008; Kochzius *et al.*, 2009) and fish (Craig *et al.*, 2007; Gaither *et al.*, 2011) are known to show post-divergence colonisation of the IAA or vice versa and similarly many sister species of fish overlap in their ranges at the IAA (reviewed in (Gaither & Rocha, 2013) supporting the ‘centre of overlap’ hypothesis (Woodland, 1983). The lack of genetic signals of post divergence colonisation of the IAA could be due to the low dispersal ability of the viviparous sea snakes (Heatwole, 1999; Lukoschek *et al.*, 2007; Lukoschek *et al.*, 2008; Lukoschek & Shine, 2012) compared to marine fish and invertebrates that have a highly dispersal larval stage. The young age of sea snakes (Sanders *et al.*, 2008; Sanders *et al.*, 2013a) compared to other groups in the region (Renema *et al.*, 2008) can also affect post divergence colonisation since there is less time for range changes following divergence. However, to further investigate the contribution of the ‘centre of overlap’ hypothesis on the origins of IAA marine biodiversity, more viviparous sea snake sampling is needed from the IAA to accurately delimit species ranges, and examine patterns of secondary colonisation, gene flow and introgression.

Compelling evidence for alternative hypotheses in different marine groups in the IAA has led to the conclusion that more than a single process is involved in the origins and maintenance of biodiversity in this region (Bowen *et al.*, 2013). Although the findings in this thesis similarly show complex geographic and temporal patterns of diversification, biogeographic reconstructions strongly indicate that Southeast Asia represents a centre or a ‘cradle’ of speciation for the viviparous sea snakes of the rapidly speciating *Hydrophis* group. Specifically, the contribution towards the accumulation of species that originated at the periphery is of minor importance compared to the rapid increase of species that resulted from the in-situ radiation in Southeast Asia. It has been proposed that the centres of origin have three developmental stages: initial accumulation through migration from other regions, *in-situ* diversification and dispersal to adjacent areas (Mironov, 2006). However, in viviparous sea snakes, *in-situ* diversification and dispersal has preceded the initial accumulation stage and accumulation has only occurred at a much later stage during their diversification.

6. 5. Concluding remarks

Results presented in this thesis have made a significant contribution towards the understanding of systematics, diversity, speciation and biogeographic history of viviparous sea snakes. The detection of cryptic lineages and the new species described here suggest a need for more basic systematic studies on viviparous sea snakes. The discovery of remarkable morphological convergence among medically important Asian and Australian beaked sea snakes further highlights the significance of molecular methods in species delimitation and phylogeny reconstruction. Temporal and phylogeographic diversification patterns suggest that viviparous sea snakes are a promising group to further study the relationship between Pleistocene sea level changes and speciation. Although historical geographic barriers explain cryptic species and genetic diversity in some species, it does not account for the rapid speciation observed in this young radiation. Therefore, other avenues of research (e.g. adaptation genomics) should be explored to study and explain the high species diversity in this rapidly diverged adaptive radiation.

CHAPTER 7: References for Chapters 1 & 6

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