東アジア産エラブウミヘビ亜科とウミヘビ亜科を対象とした集団遺伝学的解析と分類に関する研究

Doctoral Thesis of Philosophy

Population genetics and taxonomic study of laticaudine and hydrophiine sea snakes in the islands of East Asia

September 2013

by

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A dissertation submitted to the Graduate School of
Engineering and Science, University of the Ryukyus,
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Marine and Environmental Sciences
Graduate School of Engineering and Science
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Abstract

This study is divided into two main parts, concerning two different groups of marine snakes, laticaudine sea kraits and hydrophiine sea snakes. First, sequences of mitochondrial cytochrome *b* (mtDNA cyt *b*) gene were examined to infer the population genetic structures among two species of amphibious sea kraits, *Laticauda laticaudata* (n=136) and *L. semifasciata* (n=177), in the Ryukyu-Taiwan region. The results of the molecular analyses revealed a total of 4 and 16 haplotypes for *L. laticaudata* and *L. semifasciata*, respectively. The pairwise F_{ST} values revealed distinctive genetic differentiations between subregions, suggesting that deep waters serve as obstacles for dispersal and gene flow in both species. In *L. laticaudata*, the results of the analysis of molecular variance (AMOVA) further revealed genetic differences even between the islands within the same subregion, suggesting its limited dispersal ability across the sea.

In Chapter 2, sequences of mtDNA cyt *b* gene obtained from few *Laticauda* specimens collected from the main islands of Japan were examined to infer their origins. Molecular analyses revealed that the origins of the Mie specimen was the Central Ryukyus while the Oita specimen was most likely from the Southern Ryukyus or further south. The results confirmed the presence of occasional accidental drifters to the main islands of Japan by the Kuroshio Current. This finding may contradict the restricted gene flows among the Ryukyu subregions, which is demonstrated in Chapter 1. Probably, the putative drifters are unable to exit the Kuroshio at will. As such, the Kuroshio poses as threats, even for a vagile marine species without planktonic larval stages, such as the sea kraits, as the current may carry them out of their distributional range and suitable habitats.

In Chapter 3, genetic assessments were conducted to resolve taxonomic confusions and verify the taxonomic status of 2 species of hydrophiine sea snakes, *Hydrophis melanocephalus* and *H. cyanocinctus*, in the Ryukyu Archipelago. Sequences of mtDNA cyt *b* gene were obtained from *Hydrophis* spp. specimens from the Central and Southern Ryukyus, and Thailand (n=37), and they were compared with published sequences of congeneric specimens from Southeast Asia and Australia. Results of the phylogenetic analyses suggested that two separate taxonomic entities exist in the Ryukyu Archipelago. However, the Ryukyu *Hydrophis* spp. samples exhibited a wide range of morphological

variations and we were unable to distinguish the two entities based on any of the examined morphological characteristics.

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General Introduction

Situated in the northwestern Pacific, the islands of East Asia are comprised of continental islands of varying sizes, the most prominent is the Ryukyu Archipelago, which consists of approximately 140 subtropical islands extending from Kyushu, Japan, to Taiwan. The current land configurations are the results of series of complex geohistorical events, involving more than one period of land-bridge connections with adjacent landmasses. These events are believed to play important roles in the range expansion and geographic isolation of terrestrial organisms, which are reflected in the current geographic faunal patterns of the region (Ota, 1998). In addition, the region's subtropical climate is governed in part by the Kuroshio Current. The Kuroshio transports warm water northward from the Philippines, along the eastern coast of Taiwan, past the Ryukyu Archipelago, to the southeastern coast of mainland Japan. It plays an important role in the dispersal of marine organisms (e.g., Mukai et al., 2009; Yasuda et al., 2009). The combination of the unique geohistorical, geographical, and oceanographical features are regarded as important factors in enhancing and maintaining the biodiversity within the region. Consequently, the region has long served as an interesting backdrop for numerous extensive research pertaining to the biodiversity and biogeography of both terrestrial (e.g. Ota, 1998, 2000 for amphibians and reptiles) and marine organisms (e.g. Benzie, 1998; Bohonak, 1999; Bernardi, 2000; Kyle and Boulding, 2000; Kojima et al., 2006). Nonetheless, with the exception of sea turtles (Hatase et al., 2002; Roberts et al., 2004; Bowen et al., 2005), notably few studies have been conducted on marine reptiles, such as sea snakes.

Sea snakes are marine reptiles belonging to the family Elapidae and subfamilies Laticaudinae and Hydrophiinae, which have secondarily and independently evolved a marine lifestyle from different terrestrial ancestors (Heatwole, 1999). The most prominent evidence of the convergent adaptation to the marine environment is the possession of flattened, paddle-like tail. Sea snakes are widely distributed in the tropics and subtropics; from east Africa, along the coastal waters of the Persian Gulf, eastward into the tropical waters of Asia, and Australia, the islands of the southwestern Pacific, Japan and northwestern China. The highest diversity is observed in the waters of northern Australia, Indonesia, and Malaysia (Heatwole, 1999). In Japan, five species of hydrophiines (*Emydocephalus ijimae*, *Hydrophis ornatus*, *H. melanocephalus*, *H. cyanocinctus*, and *Pelamis platurus*) and three species of laticaudines

(*Laticauda laticaudata, L. semifasciata*, and *L. colubrina*) have been reported, mostly from the Ryukyu Archipelago (Toriba, 1994). In Taiwan, 10 species of hydrophiines (*E. ijimae*, *H. melanocephalus*, *H. cyanocinctus*, *H. ornatus*, *P. platurus*, *Acalyptophis peronii*, *Astortia stokesii*, *Kerilia jerdonii*, *Lapemis curtus*, and *Praescutata viperina*) and three species of laticaudines (*L. laticaudata*, *L. semifasciata*, and *L. colubrina*) have been reported (Xiang and Li, 2009).

In the Ryukyu-Taiwan region, two species of laticaudine sea kraits, *L. laticaudata* and *L. semifasciata*, are sympatrically distributed throughout the entire range of the region (Ota, 2008). Despite the fact that they are morphologically adapted to the marine environment and aquatic locomotion, members of the subfamily Laticaudinae (sea kraits) are amphibious. They retained several primitive character states, such as oviparity, enlarged ventral scales, absence of nostril flaps, and anterolateral placement of the nostrils, suggesting that they are less well adapted to the marine environment and are more terrestrial compared to the true sea snakes of the subfamily Hydrophiinae (Slowinski, 1989). Furthermore, previous studies have demonstrated that they exhibit a behavioral trait known as philopatry or site fidelity (e.g., Shetty and Shine, 2002; Brischoux et al., 2009). Their strong terrestrial affinity raises questions regarding the extent of their dispersal ability and their population genetic structures within the region.

Among the hydrophiines, two out of the five species, namely *H. melanocephalus* and *H. cyanocinctus*, are still taxonomically confused. In the recent past, there have been several reports of both fatal and nonfatal sea snake bites in the Ryukyus. Most of the reported cases involved either *H. melanocephalus* (Asanuma et al., 1998) or *H. cyanocinctus* (Higa et al., 1990). Due to their medical importance, it is crucial that the issues regarding the taxonomic status and the distinguishing morphological characteristics for each species be resolved.

In recent years, the applications of molecular techniques have been commonly used as the tools for assessing biogeographical patterns as well as solving various taxonomical issues. In this study, a molecular technique, specifically mitochondrial DNA sequencing, was utilized to assess the population genetic structures of two species of laticaudine sea kraits, *L. laticaudata and L. semifasciata*, and the taxonomic status of two species of hydrophiine sea snakes, *H. melanocephalus* and *H. cyanocinctus*, in the Ryukyu-Taiwan region.

Chapter I

Geographic genetic structure in two laticaudine sea kraits, *Laticauda laticaudata* and *Laticauda semifasciata* (Serpentes: Elapidae), in the Ryukyu-Taiwan region as inferred from the mitochondrial cytochrome *b* sequences

1.1. Introduction

Dispersal is a fundamental process in the evolutionary dynamics of organismal populations. It generally results in gene flow, which alters the effects of localized selection and genetic drifts, and ultimately determines the genetic structure of populations (Ross, 2003). The majority of the marine taxa, including both benthic and pelagic organisms, have been considered to be passively transported during the larval stage, by which the ranges of their dispersal are grossly determined (Levin, 2006; Bradbury et al., 2008). Indeed, numerous authors had previously clarified the relationships between dispersal distances in larvae and certain biological traits in various marine organisms (e.g. Benzie, 1998; Bohonak, 1999; Bernadi, 2000; Kyle and Boulding, 2000; Kojima et al., 2006; Imron et al., 2007). In contrast to marine invertebrates and fishes, extant marine tetrapods that had secondarily entered the marine environment, such as whales, penguins, and sea snakes, lack pelagic larval stages in their life cycles. Instead, they may rely on regular or accidental migrations by juveniles or adults for dispersals. In highly mobile, social organisms, such as marine mammals, young individuals are accompanied by adults (Valsecchi et al., 2002) and their genetic clues to population traits are relatively well studied. Although there are intraspecific variations to some extent, marine mammals usually display complex, fine-scale population structure, most probably governed by social and behavioral factors (Hoelzel, 1998; Natoli et al., 2004). In marine reptiles, however, migrations by juveniles, which may either be passive or active, are not well understood, with the exceptions of loggerhead (Hatase et al., 2002; Bowen et al., 2005) and green turtles (Roberts et al., 2004). Particularly, the distances to which they disperse from their natal sites and the extent of gene flows among populations remain unclear.

Sea kraits of the genus *Laticauda* (Elapidae: Laticaudinae) secondarily entered marine environments from terrestrial elapids independently from the other group of marine snakes including true sea snakes of the elapid subfamily Hydrophiinae (Heatwole, 1999). Both sea kraits and true sea snakes exhibit convergent adaptations for aquatic locomotion as characterized by the use of compressed, paddle-like tail. However, only sea kraits are amphibious. Even though only female sea kraits are obligated to come on land to lay eggs, both sexes frequently come on land to rest, digest meals, as well as to mate (Heatwole, 1999). In fact, *Laticauda* retains several primitive character states, such as oviparity, enlarged

ventral scales, absence of nostril flaps, and anterolateral placement of the nostrils, suggesting that the members of this particular genus are less adapted to the marine environment and are more terrestrial compared to true sea snakes (Slowinski, 1989). Such strong terrestrial affinity of sea kraits raises questions regarding the degree of their dispersal ability and the distance to which they can disperse across bodies of waters.

Regarding the dispersal aspects of the sea kraits, several recent authors have studied a few laticaudine species. For instance, Shetty and Shine (2002) demonstrated the homing ability and philopatry of the adult *Laticauda colubrina*: individuals of this sea krait consistently stayed in the vicinity of their home island, and when displaced, they returned home rapidly. The authors tentatively attributed such site fidelity in this sea krait to intrinsic behavioral mechanisms rather than to any obstacle to dispersal (Shetty and Shine, 2002). Another study on the fine scale site fidelity in two other congeneric species, *L. saintgironsi* and *L. laticaudata*, also revealed their philopatry, but with differential intensities: *L. laticaudata* exhibited narrower site fidelity compared to *L. saintgironsi* (Brischoux et al., 2009). In these and other studies, dispersal in sea kraits seems to be somewhat limited. However, since these authors adopted direct approaches, with the exception of Lane and Shine (2011a), actual dispersal abilities of the sea kraits may be underestimated.

Although dispersal estimates obtained from direct approaches, such as mark-recapture and translocation methods used in the aforementioned studies, are useful in assessing the current pattern of dispersal, they are often challenging, involving certain limitations and difficulties in obtaining data in large quantity. Since population genetic structure reflects dispersal ability to some extent, indirect approaches, such as those using genetic markers and genetic differentiation estimates, are advantageous in inferring dispersal by a relatively small number of individuals over periods of thousands of generations (Koenig et al., 1996). Genetic inferences are also effective in detecting less frequent, long-distance dispersal. Hitherto, however, there have been only a few studies using indirect approaches to infer dispersal in sea snakes, two of which were studies of the phylogeography and population genetic structure of the viviparous hydrophiine sea snake, *Aipysurus laevis* (Lukoschek et al., 2007, 2008). In addition, Lane and Shine (2011b) elucidated intraspecific variations in the direction and degree of sex-specific dispersal in two laticaudine sea kraits, namely *L. laticaudata* and *L. saintgironsi*, on the basis of genetic data. Nonetheless, gene flow between any given pairs

of localities is often complex and not easy to evaluate, especially when there are many islands (i.e. possible nesting sites) around the localities of interest.

The Ryukyu-Taiwan region is located in the northwestern Pacific and is composed of nearly 200 subtropical, continental islands, which are arranged in an arch extending from northeast to southwest, between mainland Japan (Kyushu) and Taiwan (Fig. 1.1). It is characterized by island connections of varying distances, ranging from less than a kilometer to more than 150 km apart. These islands are classified into four subregions, Northern Ryukyus, Central Ryukyus, Southern Ryukyus, and Taiwan, on the basis of characteristics in terrestrial fauna and inferred geohistorical events. The Northern and Central Ryukyus are separated by the Tokara Tectonic Strait, which is approximately 900 m deep and 160 km wide. Southwardly, the Kerama Strait, approximately 1,000 m deep and 193 km wide, lies between the Central and Southern Ryukyus. Lastly, the Southern Ryukyus and Taiwan are separated by the prominent Yonaguni (or East Taiwan) Strait, which is approximately 1,900 m deep at its greatest depth and 160 km wide (Moroz and Bogdanov, 2007). The separating bodies of waters between islands within a single subregion are shallower than those between subregions, except for the sea between Lyudao and Lanyu, the two islands of the Taiwan subregion (>2,000 m). Such geographic features of the Ryukyu-Taiwan region serve as an ideal backdrop to examine the dispersal ability of sea kraits, *L. laticaudata* and *L. semifasciata*, both of which are sympatrically distributed throughout the region (Ota 2008). In this study, we examined sequence variations of mitochondrial cytochrome *b* gene in *L. laticaudata* and *L. semifasciata* in the Ryukyu-Taiwan region to assess the extent of gene flows between localities. We also discuss the historical implications of the current population structures of the two laticaudine sea kraits in this region.

1.2. Materials and Methods

1.2.1. Sampling sites

Specimens of *Laticauda laticaudata* were collected from one island in the Northern Ryukyus, five islands in the Central Ryukyus, five islands in the Southern Ryukyus, and two islands in Taiwan. Specimens of *L. semifasciata* were collected from one island in the

Northern Ryukyus, five islands in the Central Ryukyus, five islands in the Southern Ryukyus, and one island in Taiwan (Fig. 1.1). Sample sizes from each island are given in Table 1.1.

1.2.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted using Qiagen's DNeasy Blood and Tissue Kit, following instructions from the manufacturer. Target fragment (mtDNA cytochrome *b*) was amplified in polymerase chain reactions (PCR) (see Table 1.2 for primer details). Amplification reactions were performed using a cycling profile consisting of an initial denaturation step at 94°C for 90 s followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 45 s, and final extension at 72°C for 5 min. PCR products were verified using 1% agarose gel electrophoresis and purified with either PEG Precipitation methods or ExoSAP-IT (USB). Purified PCR products were cycle sequenced with BIGDYE v 3.1(Applied Biosystems) using a cycling profile of initial denaturation at 96°C for 60 s followed by 35 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. Direct sequencing was performed by a commercial firm (Invitrogen) using automated capillary sequencers: ABI 3130x1 Genetic Analyzer and ABI 3730x1 DNA Analyzer (Applied Biosystems). The obtained sequences were deposited in the DNA Data Bank of Japan.

1.2.3. Data analyses

Prior to analyses, sequenced fragments were manually screened and edited using 4Peaks software (Griekspoor and Groothuis, 2005). Edited sequences were assembled and aligned using Geneious v5.5 (Drummond et al., 2010). Thereafter, population statistical analyses, i.e. genetic diversity, nucleotide diversity, F_{ST} values for genetic differentiation, were conducted using Arlequin 3.5 software (Excoffier and Lischer, 2010). In the analyses, a series of specimens collected from the same island was treated as a sample. Haplotype networks (unrooted) were drawn from Arlequin's tree file data (NEXUS) using TreeView software (Page, 1996). All specimens were included in the haplotype networks. However, samples with single specimens were excluded from the population statistical analyses. In order to evaluate the sources of genetic variations, the analysis of molecular variance (AMOVA) was conducted (Excoffier et al., 1992), where three hierarchical categories were

established as among subregions, among samples (i.e. islands) within subregion, and among individuals. In addition, the analyses of isolation by distance were performed for each species using IBD software (Isolation by Distance, Web Service) (Jensen et al., 2005), where the correlations between genetic distances (F_{ST}) and geographic distances (shortest distances between islands) were assessed using the Mantel tests for matrix correlation with 1,000 randomizations.

1.3. Results

Cytochrome *b* sequences obtained from 313 *Laticauda* specimens revealed four haplotypes, Lati-1 to Lati-4 (DDBJ/EMBL/GenBank accession numbers AB701325-328), for *L. laticaudata* (n = 136; 962 bp) and 16 haplotypes, Semi-1 to Semi-16 (DDBJ/EMBL/GenBank accession numbers AB701329-344), for *L. semifasciata* (n = 177; 1,029 bp) (Tables 1.3 and 1.4). In *L. laticaudata*, the highest number of haplotypes detected from a single locality was two (Kuchinoerabu, n = 16; Iriomote, n = 39; Lanyu, n = 25) (Table 1.3). In addition, the highest gene diversity ($h \pm SE = 0.5128 \pm 0.0188$) and nucleotide diversity ($\pi \pm SE = 0.0011 \pm 0.0008$) were observed in the Iriomote sample (Table 1.5). In *L. semifasciata*, the highest number of haplotypes detected from a single locality was six (Kodakara, n = 45), followed by five haplotypes from Amamioshima (n = 30) and Miyako (n = 33) (Table 1.4). The highest gene diversity (0.6000 ± 0.1305) and nucleotide diversity (0.0034 ± 0.0021) were observed in the Ishigaki sample (Table 1.5).

In *L. laticaudata*, there were two major haplotypes, Lati-1 and Lati-2, which differed from each other by two substitutions. They were linked through a hypothetical haplotype, which exhibited a single substitution from those two haplotypes. Haplotype Lati-1 was shared among the Northern and Southern Ryukyu, as well as Taiwan samples, but was not detected from any of the Central Ryukyu samples. There were two additional minor hyplotypes, Lati-3 and Lati-4, each of which was supposedly derived from haplotype Lati-1 by single substitution. Of these, haplotype Lati-3 was detected in all four specimens from Kodakara, but was not detected from any other islands. Haplotype Lati-4 was detected in two of the 16 Kuchinoerabu specimens. All samples from the Central Ryukyus, exclusive of Kodakara, invariably had haplotype Lati-2 (16/16). This particular haplotype was also detected at relatively high frequencies in the Iriomote (19/39) and Lanyu samples (22/25), but was

completely absent from the remaining Southern Ryukyu (0/25) and Lyudao (0/11) samples (Fig. 1.2, Table 1.3).

In *L. semifasciata*, the total number of haplotypes (16) was much larger and the structure of the haplotype network was more complicated compared to *L. laticaudata* (Fig. 1.2 and 1.3). The 16 haplotypes (Semi-1 to 16) detected from *L. semifasciata* samples were classified into two distinct groups, of which, the smaller group (Group 1) contained a major, central haplotype (Semi-3) and two minor, derived haplotypes (Semi-4 and 14). Of these, haplotype Semi-3 was predominant in the Taiwan's Lanyu sample (27/28) and was also detected in some Southern Ryukyu samples, but at relatively low frequencies (Iriomote: 1/9; Ishigaki: 3/10; Miyako: 1/33). Haplotypes Semi-1 and Semi-14 were detected in single specimens from the Miyako and Lanyu samples, respectively (Fig. 1.3, Table 1.4). The larger haplotype group (Group 2) consisted of three major haplotypes, namely haplotypes Semi-1, Semi-5, and Semi-2, which were linked by single substitutions. Of these, haplotype Semi-5 was detected in samples from all three subregions of the Ryukyus at substantial frequencies, but absent from Taiwan's Lanyu sample (0/28). Haplotype Semi-5 was linked with haplotype Semi-3 of Group 1 by no less than six substitutions and through five hypothetical haplotypes. Five minor, derived haplotypes independently diverged from haplotype Semi-5 by single substitutions. These were haplotypes Semi-8 from Kodakara (3/45) of the Central Ryukyus, Semi-9 from Kodakara (1/45) and Yoron (1/2) of the Central Ryukyus, Semi-16 from Kuchinoerabu (3/11) of the Northern Ryukyus, and Semi-11 (1/30) and Semi-12 (1/30) from Amamioshima of the Central Ryukyus. Another minor haplotype, Semi-15 from Kuchinoerabu, diverged from Semi-16 by a single substitution. Haplotype Semi-1 was most common in, and confined to, the Southern Ryukyus (37/59), with an exception of one individual from Ikei of the Central Ryukyus. Two minor haplotypes, Semi-6 and Semi-7, both diverged from haplotype Semi-1 by single independent substitutions, were detected in relatively low frequencies in the Miyako (1/33) and Ishigaki (1/10) samples, respectively. Haplotype Semi-2 was unique to the Central Ryukyus (53/79). From this major haplotype, two minor haplotypes, Semi-10 (1/10 from Amamioshima and 2/45 from Kodakara) and Semi-13 (1/45 from Kodakara), both from the Central Ryukyus, diverged by single substitutions.

In both *L. laticaudata* and *L. semifasciata*, F_{ST} analyses revealed statistically significant genetic differentiation (Bonferroni-corrected $P < 0.001$ and $P < 0.002$ for *L. laticaudata* and *L. semifasciata*, respectively) in the majority of the pairwise comparisons of samples from different subregions (Tables 1.6 and 1.7). Additionally, *L. laticaudata* also exhibited significant, relatively large genetic differentiation among samples within each subregion, where more than one sample, consisting of more than two specimens, was available (i.e., with the exception of the Northern Ryukyus). For instance, significant F_{ST} values were observed between *L. laticaudata* samples from Kodakara and Amamioshima of the Central Ryukyus ($F_{ST} = 1.000$), between those from Ishigaki and Iriomote of the Southern Ryukyus ($F_{ST} = 0.370$), and between those from Lyudao and Lanyu of Taiwan ($F_{ST} = 0.831$) (Table 1.6). On the contrary, in *L. semifasciata*, within-subregion genetic differentiation was not observed (Table 1.7). The analyses of molecular variance (AMOVA) revealed that genetic variations in *L. laticaudata* were predominated by variations among islands of the same subregions (76.29%), while the majority of genetic variations in *L. semifasciata* were derived from those between subregions (75.84%) (Table 1.8). In the analyses of isolation by distance, significant correlation between genetic and geographic distances was detected in *L. semifasciata* ($P < 0.001$), but not in *L. laticaudata* ($P = 0.162$).

1.4. Discussion

1.4.1. Dispersal abilities of *Laticauda laticaudata* and *L. semifasciata*

The molecular analyses revealed that, in the Ryukyu-Taiwan region, populations of both *Laticauda laticaudata* and *L. semifasciata* are genetically structured to some extent, as was evident in the F_{ST} values in nearly all pairwise comparisons of samples from the four subregions, i.e. Northern, Central, and Southern Ryukyus, as well as Taiwan (Tables 1.6 and 1.7). These results indicate that gene flow between subregions is restricted in both species. As previously mentioned, the straits separating neighboring subregions of the Northern, Central, and Southern Ryukyus, and Taiwan are approximately 900 m deep and at least 160 km wide (Moroz and Bogdanov, 2007) (Fig. 1.1). The above results suggest that moderately deep and wide straits can serve as obstacles against dispersals for both *L. laticaudata* and *L. semifasciata* despite their morphological and physiological adaptations to the marine environment, such as flattened, paddle-like tail and relatively large lungs (Dunson, 1975).

Lukoschek et al. (2007) argued for the scarcity of gene flow among populations of the hydrophiine sea snake *Aipysurus laevis*, which were separated by deep waters in the Timor Sea and the Great Barrier Reef of Australia. With the exception of the pelagic *Pelamis platurus*, all elapid sea snakes, including laticaudines, are associated with benthic habitats and must surface to breathe in intervals (Dunson, 1975). Thus, deep expanses of waters are expected to act as effective anti-dispersal barriers for both sea kraits and true sea snakes. Our results further support this idea.

Within a single subregion, statistically significant F_{ST} values were observed between several combinations of samples in *L. laticaudata*, but not in *L. semifasciata* (Table 1.6 and 1.7). This difference was also evident in the results of AMOVA, where genetic variations between islands within a subregion were predominant in *L. laticaudata* whereas in *L. semifasciata*, the greatest source of genetic variations was the differences between subregions (Table 1.8). Indeed, actual gene flow in *L. laticaudata* was limited even between neighboring islands of the same subregion, such as Kodakara and Amamioshima of the Central Ryukyus (maximum depth and width of the separating strait were 800 m and 84 km, respectively) (Marsset et al., 1987), Ishigaki and Iriomote of the Southern Ryukyus (20 m and 17 km), and Lyudao and Lanyu of Taiwan (>2,000 m and 61 km) (Liang et al., 2003). Among neighboring islands, Ishigaki and Iriomote deserve particular attention because both islands are located in the same lagoon system with smaller islets (e.g. Taketomi and Kuroshima; Fig. 1.1), where *L. laticaudata* occurs, between them (Map No. NG-51-12-18, Geographical Survey Institute of Japan). Persistence of genetic heterogeneity in assemblages of *L. laticaudata* from Ishigaki and Iriomote, regardless of geographic and topographic relationships, implies that this species of sea krait does not disperse between closely located islands within the same lagoon, even with the presence of intervening islets, which seemingly allow dispersal in a stepping-stone manner. This result may reflect stronger site fidelity or higher degree of philopatry in *L. laticaudata* compared to *L. semifasciata*.

Lillywhite et al. (2008) revealed that three species of East Asian sea kraits, *L. laticaudata*, *L. semifasciata*, and *L. colubrina*, differed in the degrees of terrestrial dependency and tolerance. These authors, as well as some others (e.g. Shine and Shetty, 2001; Bonnet et al., 2005), revealed that *L. laticaudata* exhibited a relatively high degree of adaptation to the terrestrial environment and is actually dependent on terrestrial resources,

such as freshwater and resting sites. In contrast, *L. semifasciata* is considered to be most adapted to the marine environment (Lillywhite et al., 2008). It is probable that, among laticaudines, the extent of terrestrial adaptation and dependency is more or less correlated with the degree of site fidelity, which consequently leads to interspecific differences in the extent of gene flows within sympatric area.

Our discussion developed here is still premature because it is based only on the results of the indirect approach using a mtDNA marker. Although the extent of female-mediated gene flow can be inferred from the results of this study, we should not ignore male-mediated gene flow or occasional long-distance migrations without reproduction in females. Lane and Shine (2011b) suggested the presence of sexually differential patterns of dispersal in two species of sea kraits, *L. laticaudata* and *L. saintgironsi*. Moreover, in green turtles, ocean basin-specific mtDNA patterns are known to be maintained even though females occasionally migrate to distant places from their natal beaches (Roberts et al., 2004). Further studies using nuclear gene markers and more sophisticated comparisons of genetic compositions of different age groups (cohort samplings) are essential in order to verify our hypothesis.

1.4.2. Overall genetic structures of *L. laticaudata* and *L. semifasciata* and their historical implications

Differences in the geographic genetic patterns in the Ryukyu-Taiwan region between *L. laticaudata* and *L. semifasciata* may also be caused partially by differences in their population histories. In the Ryukyu-Taiwan region, many phylogeographical and faunistic studies have been conducted for various terrestrial ectotherm tetrapods (Ota, 1998, 2000; Toda et al., 1999; Matsui et al., 2005a, b), each of which showed striking faunistic and genetic differences among the subregions. For example, Matsui et al. (2005b) detected 8.4% nucleotide divergence in the mitochondrial 12S and 16S rRNA genes between the ranid frogs, *Odorrana supranarina* and the *O. amamiensis-narina* clade, which supposedly reflects the divergence between the Southern and Central Ryukyus. They estimated the isolation period between the two subregions as 2.3-5.0 million years before present (MyBP) accordingly. In addition, 3.0% nucleotide divergence was detected between *O. swinhoana* from Taiwan and *O. utsunomiyaorum* from the Southern Ryukyus, which suggests the isolation period of the two subregions to be 1.9-4.1 MyBP. Compared to these results, genetic

differentiations among the subregional samples of laticaudine sea kraits revealed in this study appear subtle: in both *L. laticaudata* and *L. semifasciata*, overall geographic genetic divergences are much smaller (nucleotide diversity of 0-0.3%, Table 1.5). It is, therefore, probable that the putative long isolation among the landmasses has not much affected divergences and present-day population structures in each *Laticauda* species, whose divergence, if any, obviously reflects more recent isolations.

In *L. laticaudata*, most of the island assemblages were fixed to a single haplotype, suggesting that their effective population sizes have been very small. Molecular analyses revealed a total of only four haplotypes and the haplotype network displayed a close relationship among them. Although the haplotype compositions of the Central and Southern Ryukyu populations were different, the two predominant haplotypes (Lati-1 and 2) were shared even by most distant island samples across the Ryukyu-Taiwan region (e.g., by the Kuchinoerabu and Lanyu samples, Fig. 1.2). Judging from these results, *L. laticaudata* assemblages in the entire region probably shared the same gene pool until recently. As demonstrated above, however, gene flows are currently much restricted, even between nearby islands. This suggests an occurrence of very recent fragmentation of a region-wide reproductive unit into many localized populations, possibly as the results of severe population declines accompanied by bottleneck events.

Laticauda semifasciata populations in the Ryukyu-Taiwan region maintain a much higher haplotype diversity, compared to *L. laticaudata*, and most of the island samples were genetically polymorphic. Moreover, island samples in each subregion shared more than one haplotype at similar frequencies. These lines of evidence suggest that, in *L. semifasciata*, each subregional assemblage maintains genetic unity through relatively frequent gene flow. On the other hand, the haplotype network suggests that genetic diversification has progressed among the three Ryukyu subregions to some extent. Samples of the Central and Southern Ryukyus exhibited major haplotypes unique to, and common within, each subregion (i.e., Semi-2 for the Central Ryukyus and Semi-1 for the Southern Ryukyus), and both of these haplotypes were located at positions as independently deriving from the common haplotype, Semi-5 (Fig. 1.3). There were a few haplotypes further derived from Semi-2 or Semi-1, which were also unique to each subregion. The Kuchinoerabu sample, the sole representative of the Northern Ryukyu assemblage of *L. semifasciata*, also exhibited a similar pattern by

possessing two unique, derived haplotypes, Semi-16 and Semi-15. This phylogeographical haplotype pattern implies that, in the Ryukyus, genetic divergence was initiated among the three subregions. Superficially, this may be in contradiction with the result of the Mantel test, which supported the isolation-by-distance pattern for the entire region. However, when samples from three distinct, genetically diverged entities, geographically arranged in a line, are examined together, it is possible that a significant correlation between genetic and geographic distances among samples can be detected. In fact, neighboring pairs of samples, each of which belongs to different subregions, displayed relatively high F_{ST} values (0.515 for Kuchinoerabu and Kodakara [approx. 157 km apart], and 0.478 for Kuchinoerabu and Amamioshima [approx. 216 km apart]). These support the idea of the predominating between-subregion divergences, which is evident in the AMOVA results discussed above. Although we could not specify any historical events that seemingly triggered this subregional divergence, environmental fluctuations or changes in land configurations, or both, during the recent glacial-inter glacial periods may have been the factors.

Interestingly, a small proportion of the Southern Ryukyu samples (6 specimens) had haplotypes that were much diverged from the remaining Ryukyu haplotypes and identical with the major Taiwan (Lanyu) haplotype Semi-3 (5/6), or close to it but with a single substitution (haplotypes Semi-4, possessed by a Miyako specimen; Fig. 1.3). One possible interpretation is that genetic divergence has occurred between Taiwan (Lanyu) and the Ryukyus, followed by gene influx from the former to the Southern Ryukyus (i.e., geographically closest subregion of the Ryukyus to Taiwan), but not in the other direction. Nonetheless, we cannot exclude another scenario that the second group of haplotypes was derived from areas further south, i.e., the Philippines, where *L. semifasciata* also occurs (Bacolod, 1983). Further studies using samples from areas further south of the Ryukyu-Taiwan region are needed to address this particular issue.

From the results of this study, it is worth mentioning that populations of both *L. laticaudata* and *L. semifasciata* in the Ryukyu-Taiwan region are structured to some extent, especially in *L. laticaudata*, where the population size is small and fragmented on an island-based scale. Since both species are locally exploited and utilized as traditional food materials and are listed as 'nearly threatened' species in the Red Data Book of Okinawa (Masunaga,

2005), the above findings should be taken into consideration for future establishment and implementation of additional conservation measures.

Table 1.1. Localities and sample sizes (n) of *L. laticaudata* and *L. semifasciata* used in the present study.

Locality	Sample Size (n)	
	L. laticaudata	***L. semifasciata***
Northern Ryukyus		
1. Kuchinoerabu Is	16	11
Central Ryukyus		
2. Kodakara Is	4	45
3. Amamioshima Is	7	30
4. Yoron Is	-	2
5. Okinawajima Is	6	1
6. Ikei Is	-	1
7. Tsuken Is	2	-
8. Zamami Is	1	-
Southern Ryukyus		
9. Ikema Is	-	5
10. Miyako Is	1	33
11. Tarama Is	-	2
12. Ishigaki Is	15	10
13. Taketomi Is	4	-
14. Iriomote Is	39	9
15. Kuroshima Is	5	-
Taiwan		
16. Lyudao (Green) Is	11	-
17. Lanyu (Orchid) Is	25	28
Total	136	177

Table 1.2. Primers used to generate PCR products and DNA sequences.

Region	Name	Sequence (5' – 3')
Cytochrome *b*	Glu-5'eeg (Suzuki and Hikida, 2010)	TGATATGAAAAACCACCGTTG
	H16064 (Burbrink et al., 2000)	CTTTGGTTTACAAGAACAATGCTTTA

Table 1.3. Haplotype frequency for *Laticauda laticaudata* populations.

	North	**Central**					**South**				**Taiwan**	
Haplotype	Kuchinoerabu (n=16)	Kodakara (n=4)	Amamioshima (n=7)	Zamami (n=1)	Okinawajima (n=6)	Tsuken (n=2)	Miyako (n=1)	Ishigaki (n=15)	Taketomi (n=4)	Iriomote (n=39)	Kuroshima (n=5)	Lyudao (n=11)	Lanyu (n=25)
Lati-1	14						1	15	4	20	5	11	3
Lati-2			7	1	6	2				19			22
Lati-3		4											
Lati-4	2												

Table 1.4. Haplotype frequency for *Laticauda semifasciata* populations.

	North	Central					South				Taiwan
Haplotype	Kuchinoerabu (n=11)	Kodakara (n=45)	Amamioshima (n=30)	Yoron (n=2)	Okinawajima (n=1)	Ikei (n=1)	Tarama (n=2)	Miyako (n=33)	Ikema (n=5)	Ishigaki (n=10)	Iriomote (n=9)	Lanyu (n=28)
Semi-1						1	1	19	5	6	6	
Semi-2		32	20	1								
Semi-3								1		3	1	27
Semi-4								1				
Semi-5	7	6	7		1		1	11			2	
Semi-6								1				
Semi-7										1		
Semi-8		3										
Semi-9		1		1								
Semi-10		2	1									
Semi-11			1									
Semi-12			1									
Semi-13		1										
Semi-14												1
Semi-15	1											
Semi-16	3											

Table 1.5. Population summary statistics for *Laticauda laticaudata* and *L. semifasciata*. Note that localities with less than 3 specimens were excluded from the calculations of gene diversity and nucleotide diversity.

	L. laticaudata				***L. semifasciata***			
Locality	Sample Size (n)	No. of Haplotypes (N)	Gene Diversity ($h \pm$ SE)	Nucleotide Diversity ($\pi \pm$ SE)	Sample Size (n)	No. of Haplotypes (N)	Gene Diversity ($h \pm$ SE)	Nucleotide Diversity ($\pi \pm$ SE)
Northern Ryukyus								
Kuchinoerabu Is	16	2	0.2333 ± 0.1256	0.0002 ± 0.0003	11	3	0.5636 ± 0.1340	0.0007 ± 0.0006
Central Ryukyus								
Kodakara Is	4	1	0	0	45	6	0.4798 ± 0.0851	0.0006 ± 0.0006
Amamioshima Is	7	1	0	0	30	5	0.5149 ± 0.0889	0.0006 ± 0.0006
Yoron Is	-	-	-	-	2	2	-	-
Zamami Is	1	1	-	-	-	-	-	-
Okinawajima Is	6	1	0	0	1	1	-	-
Tsuken Is	2	1	-	-	-	-	-	-
Ikei Is	-	-	-	-	1	1	-	-
Southern Ryukyus								
Tarama Is	-	-	-	-	2	2	-	-
Miyako Is	1	1	-	-	33	5	0.5720 ± 0.0628	0.0013 ± 0.0009
Ikema Is	-	-	-	-	5	1	0	0
Ishigaki Is	15	1	0	0	10	3	0.6000 ± 0.1305	0.0034 ± 0.0021
Taketomi Is	4	1	0	0	-	-	-	-
Iriomote Is	39	2	0.5128 ± 0.0188	0.0011 ± 0.0008	9	3	0.5556 ± 0.1653	0.0018 ± 0.0013
Kuroshima Is	5	1	0	0	-	-	-	-
Taiwan								
Lyudao (Green) Is	11	1	0	0	-	-	-	-
Lanyu (Orchid) Is	25	2	0.2200 ± 0.0995	0.0005 ± 0.0005	28	2	0.0714 ± 0.0652	0.0001 ± 0.0002

Table 1.6. Population pairwise F_{ST} (lower diagonal) and P (upper diagonal) values for *Laticauda laticaudata*. Bold values represent significant F_{ST} values (Bonferroni-corrected $P < 0.001$). Note that localities with less than 3 specimens were excluded from the population pairwise F_{ST} analyses.

	Kuchinoerabu	Kodakara	Amamioshima	Okinawajima	Ishigaki	Iriomote	Kuroshima	Taketomi	Lyudao	Lanyu
Kuchinoerabu	-	0	0	0	0.496	0	0.991	0.991	0.477	0
Kodakara	**0.835**	-	0	0.009	0	0	0	0.027	0	0
Amamioshima	**0.923**	**1.000**	-	0.991	0	0.018	0	0.009	0	0.604
Okinawajima	**0.919**	1.000	0	-	0	0.018	0.009	0.018	0	0.577
Ishigaki	0.061	**1.000**	**1.000**	**1.000**	-	0	0.991	0.991	0.991	0
Iriomote	**0.362**	**0.586**	0.338	0.326	**0.370**	-	0.036	0.072	0	0
Kuroshima	-0.049	**1.000**	**1.000**	1.000	0	0.285	-	0.991	0.991	0
Taketomi	-0.082	1.000	1.000	1.000	0	0.266	0	-	0.991	0
Lyudao	0.034	**1.000**	**1.000**	**1.000**	0	**0.345**	0	0	-	0
Lanyu	**0.811**	**0.864**	-0.010	-0.025	**0.847**	**0.261**	**0.799**	**0.792**	**0.831**	-

Table 1.7. Population pairwise F_{ST} (lower diagonal) and P (upper diagonal) values for *Laticauda semifasciata*. Bold values represent significant F_{ST} values (Bonferroni-corrected $P < 0.002$). Note that localities with less than 3 specimens were excluded from the population pairwise F_{ST} analyses.

	Kuchinoerabu	Kodakara	Amamioshima	Miyako	Ikema	Ishigaki	Iriomote	Lanyu
Kuchinoerabu	-	0	0	0	0	0	0	0
Kodakara	**0.515**	-	0.505	0	0	0	0	0
Amamioshima	**0.478**	-0.006	-	0	0	0	0	0
Miyako	**0.283**	**0.511**	**0.462**	-	0.387	0.054	0.991	0
Ikema	**0.684**	**0.720**	**0.719**	0.036	-	0.468	0.622	0
Ishigaki	**0.330**	**0.583**	**0.526**	0.122	0.104	-	0.496	0
Iriomote	**0.310**	**0.562**	**0.515**	-0.059	-0.004	-0.023	-	0
Lanyu	**0.962**	**0.938**	**0.947**	**0.883**	**0.991**	**0.786**	**0.916**	-

Table 1.8. Results of the analyses of molecular variance (AMOVA) for *Laticauda laticaudata* and *L. semifasciata*.

	L. laticaudata			***L. semifasciata***		
Sources of Variation	d.f.	Sum of Squares	% Variation	d.f.	Sum of Squares	% Variation
Among subregions	3	15.09	-14.08	3	173.23	75.84
Among samples within subregion	6	28.06	76.29	4	4.05	1.65
Among individuals	122	26.52	37.79	163	72.09	22.51
Total	131	69.67		170	249.37	

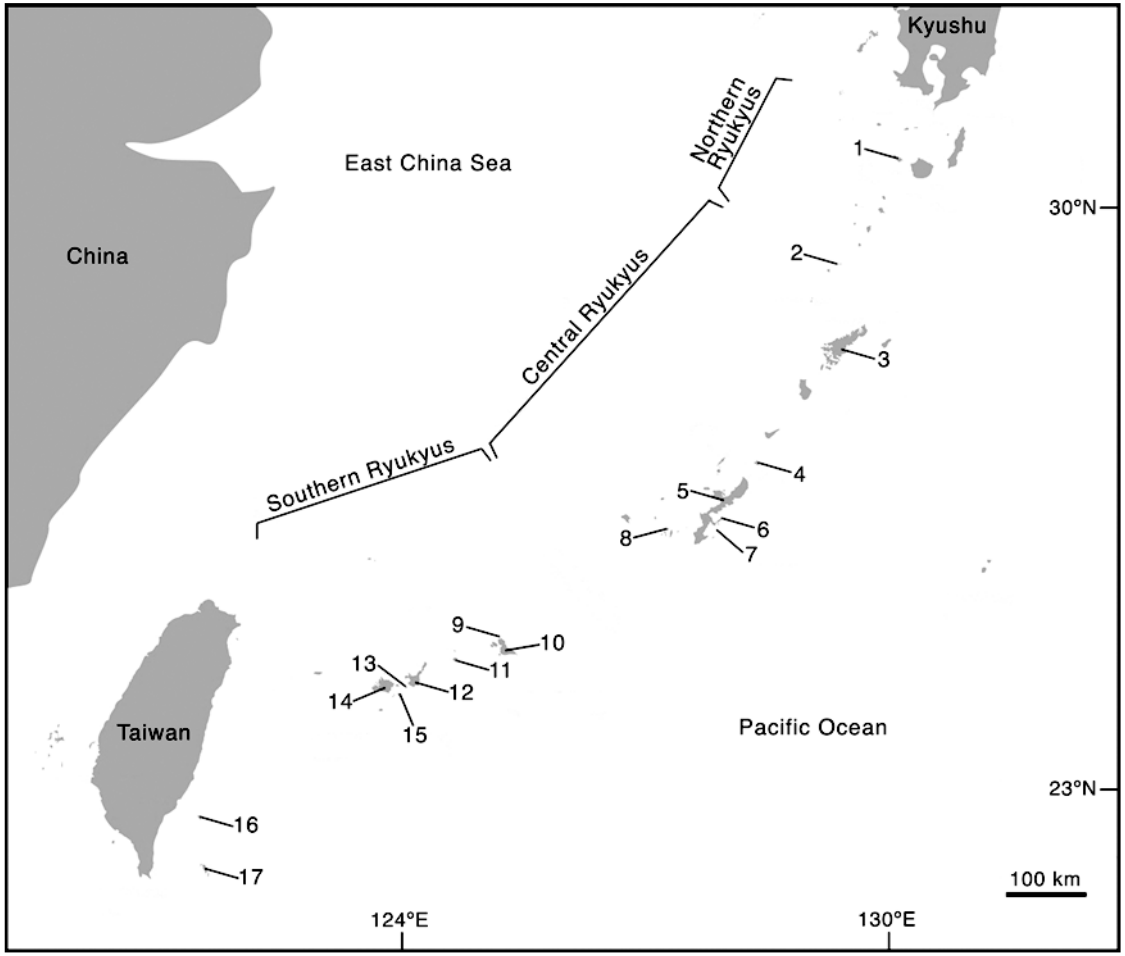


Fig. 1.1. Map of sea kraits sampling localities in the Ryukyu-Taiwan region. The numbers correspond to the names of sampling localities on Table 1.1.

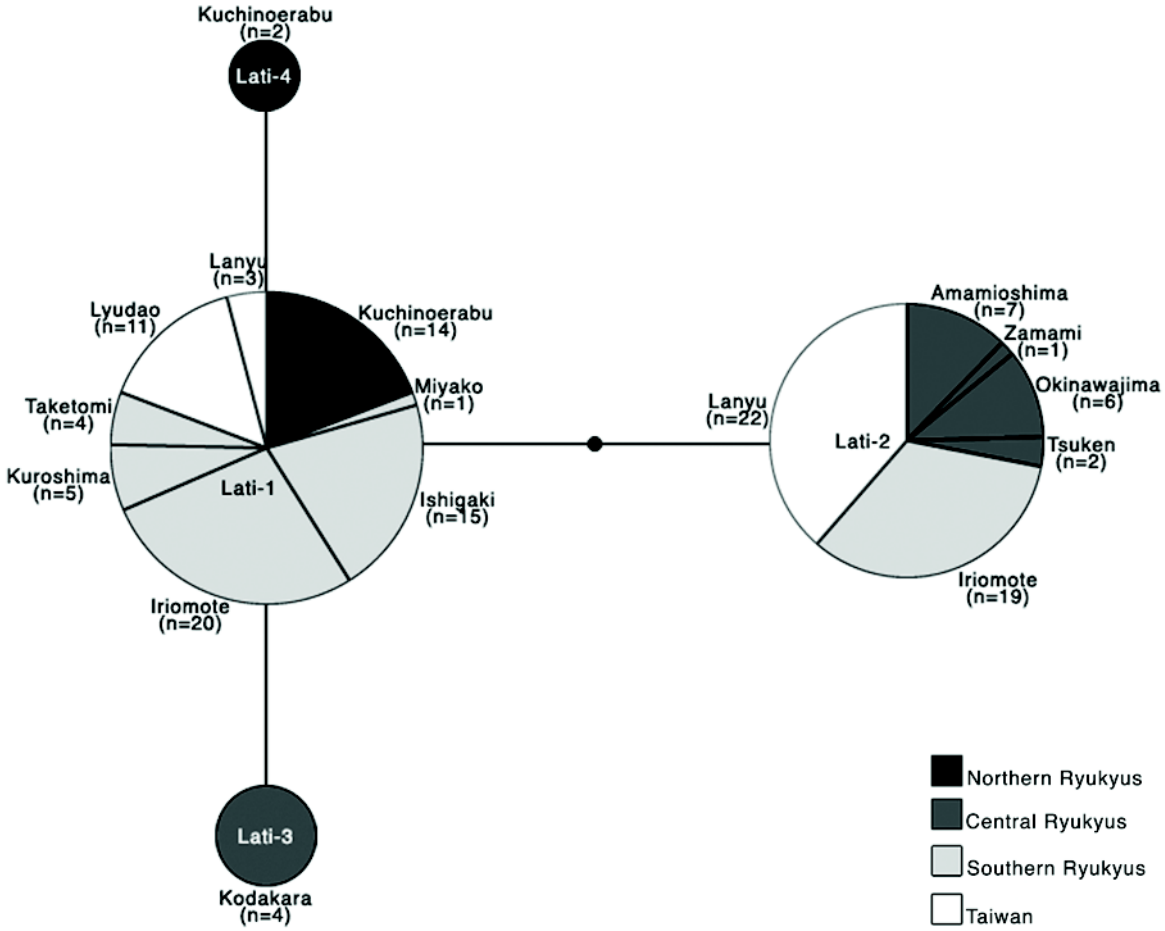


Fig. 1.2. Haplotype network, constructed from mtDNA cytochrome *b* sequence data, for *Laticauda laticaudata* populations from the Ryukyu-Taiwan region. Areas of circles reflect the total numbers of individuals possessing the given haplotypes. Small, black circles represent hypothetical haplotypes, which were not detected from the specimens used in this study.

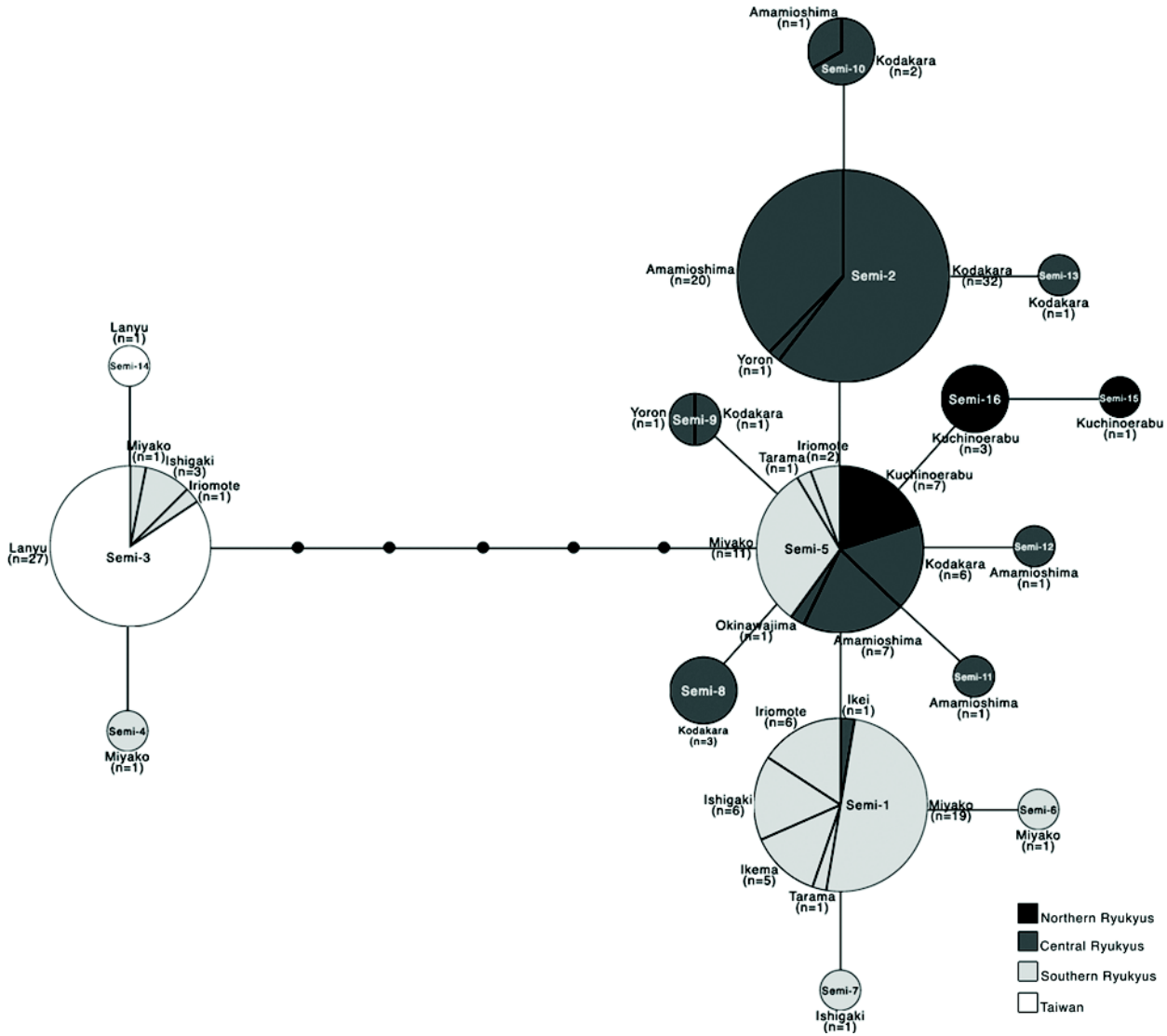


Fig. 1.3. Haplotype network, constructed from mtDNA cytochrome *b* sequence data, for *Laticauda semifasciata* populations from the Ryukyu-Taiwan region. Areas of circles reflect the total numbers of individuals possessing the given haplotypes. Small, black circles represent hypothetical haplotypes, which were not detected from the specimens used in this study.

Chapter II

Origins of *Laticauda laticaudata* and *L. semifasciata* (Elapidae: Laticaudinae) individuals collected from the main islands of Japan as inferred from molecular data

2.1. Introduction

Three species of sea kraits, *Laticauda laticaudata*, *L. semifasciata*, and *L. colubrina*, are known from Japan, of which the former two are distributed throughout the entire region of the Ryukyu Archipelago (Nakamura and Ueno, 1963), an island arc located in the southwestern part of Japan (Fig. 2.1). In the south, the ranges of *L. laticaudata* and *L. semifasciata* extend to the southwestern Pacific and Indonesia, respectively, covering Taiwan and China (Heatwole, 1999; Ota and Masunaga, 2005). In the north, the Northern Ryukyus is considered the northernmost limit of the breeding populations of these two species (Ota, 2003; Ota and Masunaga, 2005). Nonetheless, there have been several reports of sea krait occurrences along the coasts of the main islands of Japan. *Laticauda laticaudata* specimens have been found in Mie (Tomida, 1994) and Wakayama (Kubota et al., 2010) Prefectures. *Laticauda semifasciata* specimens have been found in Niigata (Honma and Kitami, 1976), Kanagawa (Nakamura, 1980), Mie (Tomida, 1994), and Wakayama (Yamato et al., 1996) Prefectures. The number of the occurrences along coasts of Japan's main islands is possibly higher, considering that there are some unreported cases, including those from a single locality, i.e., live and stranded specimens of *L. semifasciata* from Shima, Mie Prefecture, collected in 1989, 1993, and 2000 (S. Okubo, personal communication). Since these regions are located further away from the known breeding sites of these species, it is generally believed that such individuals were accidental migrants or drifters, which were transported by the Kuroshio Current (Honma and Kitami, 1976; Yamato et al., 1996). The Kuroshio, which flows from the northern coast of the Philippines to the eastern coast of Japan's main islands, has been considered an effective transport mechanism for marine organisms (e.g., Mukai et al., 2009; Yasuda et al., 2009, see also Kojima, 2006; Aoki et al., 2008; Nishikawa, 2008; Iwamoto et al., 2012 for counter examples).

Tandavanitj et al. (2013) studied the geographic genetic variations among the populations of *L. laticaudata* and *L. semifasciata* in the Ryukyu-Taiwan region and detected significant genetic differentiations among samples from different island groups, which are separated by deep straits. They argued that deep waters serve as barriers to dispersals in these species. Furthermore, in *L. laticaudata*, genetic differentiations were detected even between samples from neighboring islands within the same lagoon system. The latter result suggested that the limited gene flow was caused by a high degree of site fidelity. Such a high site

fidelity, or strong philopatry, has also been reported in other laticaudine sea snakes, *L. colubrina* (Shetty and Shine, 2002) and *L. saintgironsi* (Brischoux et al., 2009).

Considering that the Kuroshio flows along the Ryukyu-Taiwan region, the limited gene flows of the sea kraits among island groups within the region appear to contradict the idea that they are occasionally transported northward to the main islands of Japan by the Kuroshio. If the specimens found along the coasts of Japan's main islands are, indeed, accidental drifters, their haplotypes would resemble those of the Northern Ryukyus because there are no deep ocean expanses between the Northern Ryukyus and the main islands (Quaternary Study Society, 1987). Another possibility, though less likely, is that local populations of the two species actually exist along the coasts of the main islands of Japan. If this is the case, we can expect that they would possess unique haplotypes different from those of the Ryukyu-Taiwan populations, or otherwise, their haplotypes would resemble those of the Northern Ryukyus because of certain degrees of gene flow are expected due to the absence of deep bodies of waters between the two regions. The aim of this study is to determine the origins of *L. laticaudata* and *L. semifasciata* specimens found along the coasts of Japan's main islands by comparing their genetic data with those previously detected from in the Ryukyu-Taiwan region.

2.2. Materials and Methods

One specimen of *L. laticaudata* was collected from Yamaguchi Prefecture (western Honshu) and two specimens of *L. semifasciata* were collected from Oita Prefecture (northeastern Kyushu) and Mie Prefecture (southwestern Honshu)(Fig. 2-1). The Mie specimen was a female and 843 mm in snout-vent length. This was considered a sexually matured or nearly matured female on the basis of its body size (Bacolod, 1983; Tu et al, 1990). Unfortunately, only tissue samples were available for the other two specimens and there were no data regarding the sexes and measurements.

Total genomic DNA was extracted using Qiagen's DNeasy Blood and Tissue Kit, following the given instructions from the manufacturer. A part of the mitochondrial cytochrome *b* gene was amplified and sequenced using the protocols and procedures provided by Tandavanitj et al. (2013), using the forward primer GLU-5'eeg (5'-

TGATATGAAAAACCACCGTTG-3') (Suzuki and Hikida, 2010) and the reverse primer H16064 (5'-CTTTGGTTTACAAGAACAATGCTTTA-3') (Burbrink et al., 2000). The edited sequences were compared with those detected from *L. laticaudata* and *L. semifasciata* in the Ryukyu-Taiwan region (Tandavanitj et al., 2013). In order to assess the affinities of the former sequences among the latter, haplotype networks were constructed based on the combined data set analyzed by Arlequin 3.5 software (Excoffier and Lischer, 2010) and drawn by TreeView software (Page, 1996).

2.3. Results

Cytochrome *b* sequences obtained from the three specimens were 962 bp and 1,029 bp for *Laticauda laticaudata* and *L. semifasciata*, respectively. The haplotype detected from the *L. laticaudata* specimen from Yamaguchi Prefecture was identical to Haplotype Lati-1 from the previous study (Chapter I). In Tandavanitj et al. (2013), a total of four haplotypes (Lati-1 to Lati-4) were detected in *L. laticaudata*. Haplotype Lati-1 was one of the two main haplotypes, which was shared among samples from the Northern and Southern Ryukyus and Taiwan, but absent in the Central Ryukyus. The other common haplotype, Lati-2, were shared among samples the Central and Southern Ryukyus and Taiwan. In addition, there were two additional less-common haplotypes (Lati-3 and Lati-4), both of which were differentiated from haplotype Lati-1 by a single substitution (Fig. 2-2a).

In *L. semifasciata*, the haplotype detected from the specimen from Mie Prefecture was identical to Haplotype Semi-10 in the previous study (Fig. 2-2b). Among the 16 haplotypes previously detected, Semi-10 was exclusive to the Central Ryukyus (Tandavanitj et al., 2013). The specimen from Oita Prefecture possessed a unique haplotype, Semi-17, which was not previously detected. Haplotype Semi-17 belonged to one of the two distinct haplotype groups. This haplotype group consisted of four haplotypes detected from samples from Taiwan's Lanyu, the Southern Ryukyus, and Oita, and the other group was consisted of haplotypes detected from samples from all subregions of the Ryukyus. In the former group, Haplotype Semi-3, the major haplotype detected mainly from the Taiwan's Lanyu sample, but also from Southern Ryukyu samples, was located in the central position in the haplotype network. The other, minor haplotypes, including Semi-17 from Oita, were located at the derived position in the network with single nucleotide substitutions from the central

haplotype, Semi-3 (Fig. 2-2b). These haplotypes, Semi-4, Semi-14, and Semi-17, were detected from single specimens from Miyako of the Southern Ryukyus, Lanyu (Taiwan), and Oita Prefecture, respectively.

2.4. Discussion

The *Laticauda laticaudata* specimen from Yamaguchi Prefecture possessed haplotype Lati-1, which was shared among samples from all subregions of the Ryukyu-Taiwan region except for the Central Ryukyus. Particularly, the result that this specimen shared an identical haplotype with the majority of the individuals from the Northern Ryukyus did not allow us to choose one of the two hypotheses, whether it was a drifter or a part of the presumptive local population of the main islands of Japan. Assuming the latter case, it was predicted that an individual from the presumptive populations would show certain level of genetic similarity with the Northern Ryukyu populations due to the absence of deep straits between the two regions, which is compatible with the present results. It is also clear that the present results do not negate the possibility that the individual was a drifter from anywhere in the Ryukyu-Taiwan region excluding the Central Ryukyus. Evidently, more detailed surveys are needed, e.g., examinations of a longer mtDNA sequence or highly variable nuclear DNA markers, to arrive at the conclusion regarding the possible origin of the Yamaguchi specimen.

In *L. semifasciata*, the Mie specimen possessed an identical haplotype as samples from the Central Ryukyus (Semi-10), which was not detected from any other subregions including the Northern Ryukyus. Thus, it is almost certain that the Mie specimen was an accidental drifter and its geographic origin was the Central Ryukyus.

The Oita specimen possessed a unique haplotype (Semi-17), which was not previously detected from any of the Ryukyu-Taiwan samples. This haplotype belonged to the haplotype group detected from Taiwan samples and a few Southern Ryukyu samples and it was much diverged from the remaining Ryukyu haplotypes. Although it was unique, Haplotype Semi-17 differed from the major haplotype of this group by only one nucleotide substitution and its closely related haplotypes were not detected from the Northern and Central Ryukyus. This strongly suggests that the Oita specimen was an accidental drifter and

had drifted to the coast of Japan main islands from either the Southern Ryukyu-Taiwan area or even further south.

Both of the two *L. semifasciata* individuals collected from Japan main islands are considered accidental drifters, originating from the Central Ryukyus and an area further south. This is in accordance with the previous belief of passive transportation of the sea kraits by the Kuroshio (Honma and Kitami, 1976; Yamato et al., 1996; Ota and Yamadashima, 2012) and it is enough to raise questions regarding the effects of these drifters on the maintenance of the existing population genetic structures in the Ryukyus (Tandavanitj et al., 2013). The Kuroshio is a relatively fast-flowing current (Douglass et al., 2012). Therefore, it is highly plausible that, once caught in the flow of the current, the putative drifters are unable to exit at will in such a manner that they are not able to intermix with local populations along the path of the Kuroshio. As such, the population genetic structure in the Ryukyu Archipelago is maintained and is unaffected by the presence of the occasional, accidentally drifting individuals. Lastly, it can be inferred that the Kuroshio poses threats, even for a vagile marine species without planktonic larval stages, such as the sea kraits, as the current may carry them out of their distributional range and suitable habitats.

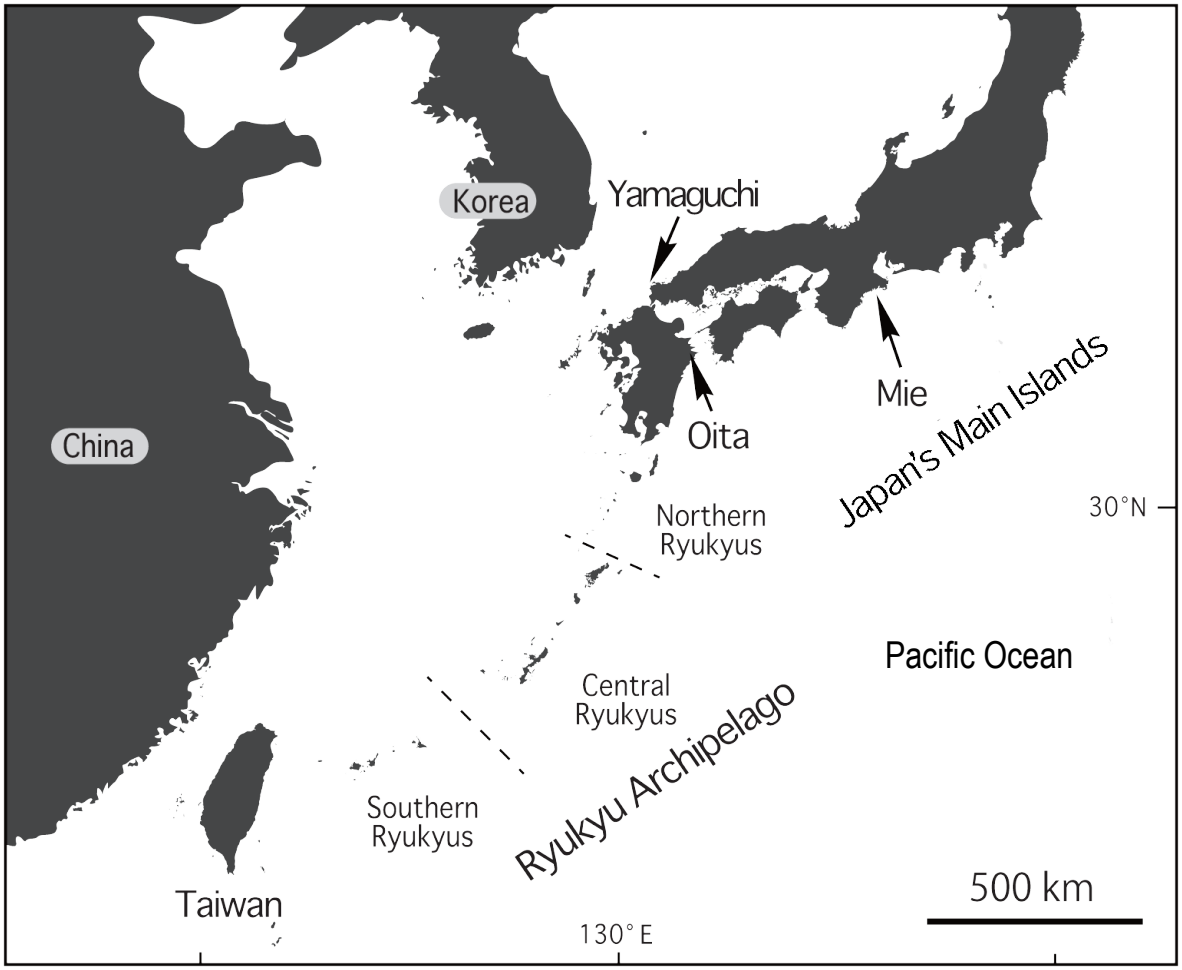
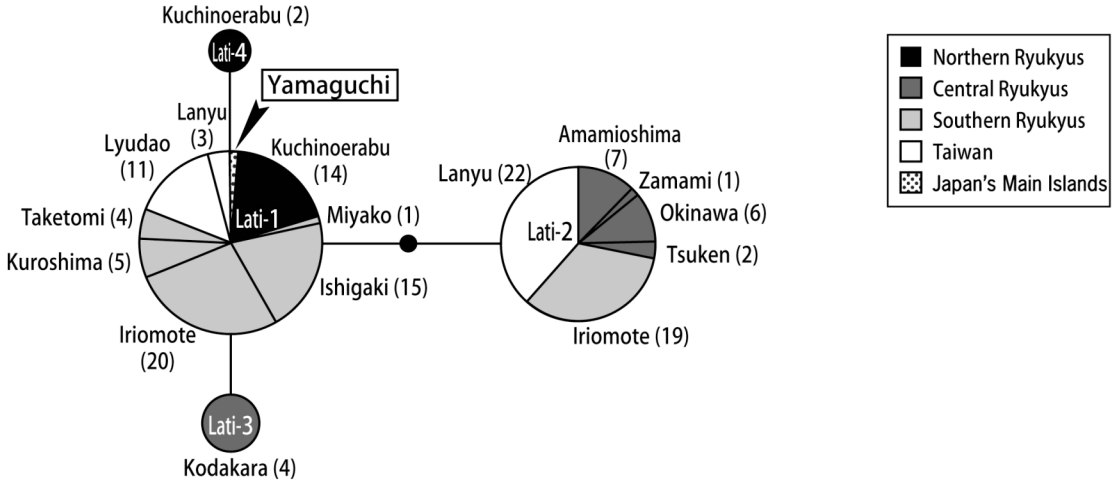
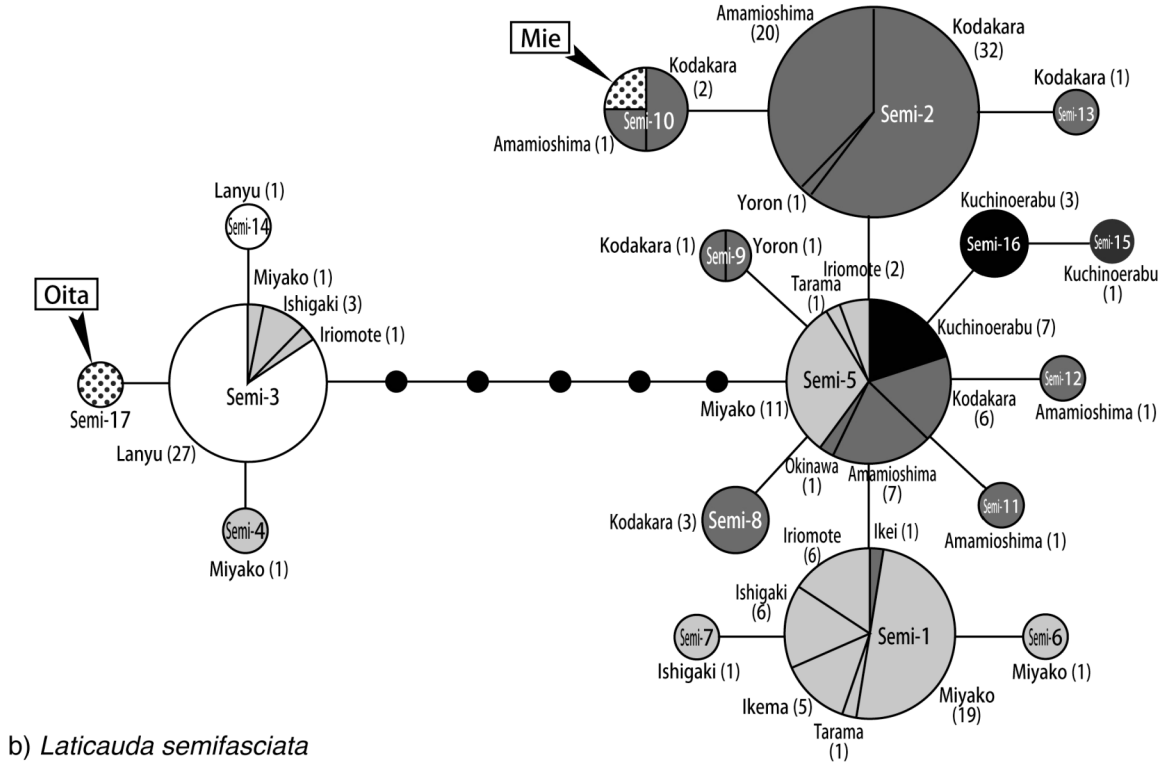


Fig. 2.1. Map of the Ryukyu-Taiwan region and the western part of Japan's main islands, showing localities of the sea krait specimens used in the study.



a) *Laticauda laticaudata*



b) *Laticauda semifasciata*

Fig. 2.2. Haplotype networks, constructed from mtDNA cytochrome *b* sequence data, for (a) *Laticauda laticaudata* samples from the Ryukyu-Taiwan region with additional sample from Yamaguchi and (b) *Laticauda semifasciata* samples from the Ryukyu-Taiwan region with samples from Mie and Oita.

Chapter III

Taxonomic reevaluation of two hydrophiine sea snakes, *Hydrophis melanocephalus* and *Hydrophis cyanocinctus* (Elapidae: Hydrophiinae), in the Ryukyu Archipelago: A molecular approach

3.1. Introduction

Sea snakes of the subfamily Hydrophiinae (Squamata: Elapidae), or true sea snakes, are a group of marine reptiles characterized by a number of unique features, which reflect their adaptation to the marine environment including vertically compressed, paddle-shaped tail, valvular nostrils, and viviparity (Heatwole and Cogger, 1993). Although the highest diversity is found in the Indo-Australian region, hydrophiine sea snakes are generally found in various coastal habitats throughout the Indian and western Pacific Oceans, from the Persian Gulf to the tropical waters of Asia and Australia, islands of southwestern Pacific, Japan, and northwestern China (Heatwole, 1999). At present, there are 61 recognized species of hydrophiine sea snakes, including one recently described species (Rasmussen et al., 2011; Ukuwela et al., 2012), among which, the genus *Hydrophis* Sonnini and Latreille, 1801 is the largest, consisting of 36 currently recognized species (Rasmussen 2001, Rasmussen et al. 2001, 2007; but see Sanders et al. (2013a) for a different view). In Japan, hydrophiines, as well as the other group of elapid sea snakes (i.e., laticaudines), are mainly found in the subtropical waters of the Ryukyu Archipelago, although some apparently drifting individuals have been reported from coastal waters in lower temperature of the main island of Japan, as far north as those surrounding Hokkaido (Nakamura and Uéno, 1963). So far, five species of hydrophiine sea snakes have been reported from the Japanese waters, three of which belong to the genus *Hydrophis*, namely *Hydrophis ornatus* (Gray, 1842), *H. melanocephalus* Gray 1849, and *H. cyanocinctus* Daudin 1803 (Toriba, 1994; Uchiyama et al., 2002).

In the subtropical Ryukyu Archipelago, where sea snakes occur in high abundance, *H. ornatus* is easily distinguished from its congeners through its physical appearance. However, *H. melanocephalus* and *H. cyanocinctus* are often confused with each other (Toriba, 1994). Several previous authors argued that the two species can be distinguished from each other by several morphological characteristics, namely head size, relative eye size, and scale counts including the numbers of preoculars, postoculars, anterior temporals, scale rows around the neck and body, and ventral scales, as well as in coloration (Stejneger, 1901, 1907; Toriba, 1994) (Table 3.1). In general, *H. cyanocinctus* and *H. melanocephalus* have been considered to exhibit certain degree of morphological differences in the color of the head (yellow in *H. cyanocinctus* vs. black in *H. melanocephalus*) and the number of anterior

temporal scales (one in *H. melanocephalus* vs. more than one in *H. cyanocinctus*) (Stejneger, 1907; Toriba, 1994). The majority of the character states, however, overlap and consequently cause confusion and misidentification. In the Ryukyus, *H. melanocephalus* is relatively common and abundant compared to other sea snake species (Takahashi, 1984). In contrast, *H. cyanocinctus* is relatively rare. The only representative of the species is a specimen collected from Aja, Okinawajima and is deposited in the Zoological Collection of Kyoto University Museum (KUZ), the photographic evidence of which can be found in the Photographic Guide; Amphibians and Reptiles of Japan (Uchiyama et al., 2002).

Recently, Sanders et al. (2013b) examined morphological, ecological, and genetic variations in putative representatives of four species of the genus *Hydrophis*, including *H. cyanocinctus* and *H. melanochephalus*, and demonstrated that they can be categorized into two ecomorphs: 'macrocephalic' and 'microcephalic' types. The 'macrocephalic' type, represented by *H. cyanocinctus,* is characterized by a large body, larger relative head size, and preys mainly on moray and conger eels. In comparison, the 'microcephalic' type, represented by the other three mutually allopatric species in the analyses, *H. melanocephalus*, *H. coggeri*, and *H. parviceps*, is characterized by a smaller body, higher ratio of hind-body to neck girth, and preys on snake eels in burrows on sandy substrates. Since the two ecomorphs can be distinguished by molecular analyses using microsatellite markers, regardless of sympatry or allopatry of samples representing them, Sanders et al. (2013b) argued for rather recent commencement of limitation in gene flow between the two ectomorphs, driving them towards speciation. On the other hand, preliminary observations of the *Hydrophis* speciemens from the Ryukyus suggested no obvious bimodal pattern in relative head size.

Due to its medical and ecological importance, it is critical to ascertain the numbers of *Hydrophis* species occurring in the subtropical water of Japan (e.g., Mishima, 1983; Higa et al., 1990). Thus, the objectives of this study are to perform a molecular reassessment of *H. melanocephalus* and *H. cyanocinctus* in the Ryukyu Archipelago and to reexamine the validity of some external, morphological characteristics of both species.

3.2. Materials and Methods

3.2.1. Molecular assessment

Sampling localities

Hydrophis melanocephalus and *H. cyanocinctus* specimens used in this study were collected from three localities (i.e. Amamioshima, Okinawajima, and Tsuken Islands) in the Central Ryukyus and two localities (i.e. Ishigakijima and Iriomotejima Islands) in the Southern Ryukyus. In addition to the Ryukyu specimens, one specimen, which was supposedly transported by ocean current to as far north as Mie Prefecture in central Japan, was also used. Furthermore, four specimens obtained from Phuket, Thailand, and identified as *H. cyanocinctus* following Rasmussen (2011) were also included. Tissue sample obtained from the preserved specimen from Aja, Okinawajima, which is the only known voucher of *H. cyanocinctus* (Higa et al., 1990, Uchiyama et al., 2002) from the Ryukyu Archipelago after Mishima (1965), was subjected to molecular analyses despite the fact that it had been fixed in 10% formalin and long preserved in 70% ethanol. One *Lapemis curtus* specimen from Hong Kong was also included as an outgroup (Fig. 3.1, Table 3.2).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from tissue samples using Qiagen's DNeasy Blood and Tissue Kit, following standard methods. The target fragment of mitochondrial cytochrome *b* gene (cyt *b*) was amplified in polymerase chain reactions (PCR) (see Tables 3.3 and 3.4 for primer details). PCRs were performed using a cycling profile consisting of an initial denaturation step at 94°C for 90 s followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min. PCR products were verified using 1% agarose gel electrophoresis and purified with either PEG Precipitation methods or ExoSAP-IT (USB). Purified PCR products were cycle sequenced with BIGDYE v 3.1(Applied Biosystems) using a cycling profile of initial denaturation at 96°C for 60 s followed by 35 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. Direct sequencing was performed

by a commercial firm (Invitrogen) using automated capillary sequencers: ABI 3130x1 Genetic Analyzer and ABI 3730x1 DNA Analyzer (Applied Biosystems).

Formalin-fixed, ethanol-preserved tissue samples of the Aja specimen were thoroughly washed with MilliQ water and soaked overnight prior to washing with phosphate buffered saline solution (PBS). Total genomic DNA was extracted following the same procedures as those preformed on frozen tissue samples. A total of 16 primers (8 forward-reverse pairs), each of which was designed to target short (100-200 bp) fragments of the cyt *b* gene, were used in the amplification and sequencing processes (see Table 4 for primer details). The target fragments were amplified in PCRs using a different cycling profile consisting of an initial denaturation step at 94°C for 90 s followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min. PCR products were verified using 3% agarose gel electrophoresis and purified with either PEG Precipitation methods or ExoSAP-IT (USB). Lastly, sequencing reactions and direct sequencing were performed following the same procedures as frozen tissue samples. In order to minimize the possibility of contamination due to the expected low quality of DNA in the formalin-fixed samples, the entire process (from the removal of the tissues) was independently performed twice; first, in a normal laboratory setting, and in another contaminant-free laboratory setting, where all the chemicals, including the PBS solution and primers, were prepared especially for this particular purpose.

3.2.2. Phylogenetic analyses

Prior to analyses, sequenced fragments were manually screened and edited using 4Peaks software (Griekspoor and Groothuis, 2005). Edited sequences were assembled and aligned using Geneious v5.5 (Drummond et al., 2010). Sequence data of *H. melanocephalus* (from Vietnam and Indonesia), *H. cyanocinctus* (from Sri Lanka, Thailand, Vietnam, Indonesia, and Australia), *H. coggeri* (from Indonesia and Australia), *H. parviceps* (from Vietnam), *H. pacificus* (from Australia), and *H. spiralis* (from Thailand) were obtained from DDBJ/EMBL/GenBank (Table 3.5). Sequence of *H. ornatus* (from Australia) was also obtained from DDBJ and incorporated into the analyses as an outgroup. *Hydrophis ornatus*

had been demonstrated to be rather distantly related to other *Hydrophis* species (Lukoschek and Keogh, 2006).

Phylogenetic trees were obtained using neighbor-joining (NJ) and maximum likelihood (ML) analyses. In the NJ analysis I used full data set with total maximum length of 1091 bp. NJ tree was constructed on the basis of the Kimura's two parameter distance and reliability of each node was tested by 1,000 times bootstrap pseudoreplications. The sites possessing undetermined nucleotides were deleted by pairwise comparison method. The analyses were made using MEGA 4 (Tamura et al., 2007). In the ML analysis, short sequences or sequences with many data missing sites were excluded from the data set. For tree constructions by ML analysis, the nucleotide substitution models were estimated, by use of Kakusan 4 (Tanabe, 2011), based on the Akaike information criterion with corrections for finite sample sizes (AICc). As a result, invariant-codon proportional model, with partitioning of codon positions, was selected as the optimal model. The nucleotide substitution models for the first, second, and third codon positions were HKY85, HKY85+I, and J2, respectively. The best ML tree was searched by likelihood ratchet method and branch support was assessed with 100 bootstrap replicates by Treefinder ver. Mar. 2011 (Jobb, 2011) and Phylogear 2 (Tanabe, 2008). In both the NJ and ML analyses, nodes with bootstrap values of >70% were considered well supported following Huelsenbeck and Hillis (1993).

3.2.3. Morphological examination

The morphological characters examined in this study are those commonly used in the identification of *Hydrophis* in Japan, which included snout-vent length (SVL), maximum body circumference (C_{max}), circumference at the neck (C_{neck}), and head width (HW), relative eye size (ratio between eye diameter and distance from the bottom orbit to the oral labial margin), numbers of preoculars (left and right), postoculars (left and right), anterior temporals (left and right), supralabials, as well as neck, mid-body, and ventral scales. Number of black to dark brown colored transverse band on body (from neck to dorsal part of cloaca) was also counted. Relative head size was evaluated by HW/SVL and C_{max}/C_{neck}. Neck and mid-body scales were counted three times. In addition, based on Sanders et al. (2013b) relative girths were calculated using the ratio of hind-body girth to the girth of the neck. The

minimum count and the maximum count were taken for the neck and mid-body scales, respectively.

3.3. Results

3.3.1. Molecular assessment

The sequencing results of the preserved Aja specimen revealed clear amplification signals for three out of eight primer pairs, (Hydro-Cytb-L1 and H1, Hydro-Cytb-L6 and H6, and Hydro-Cytb-L8 and H8) in the first and the second trials and one additional pair (Hydro-Cytb-L4 and H4) in the latter. DNA sequences obtained for the former three pairs (109 bp for Hydro-Cytb-L1 and H1, 111 bp for Hydro-Cytb-L6 and H6, and 113 bp for Hydro-Cytb-L8 and H8) were completely identical between the two trials, and were confirmed as parts of the *Hydrohis* mitochondrial cyt b gene by the BLAST search. Although the forth fragment (Hydro-Cytb-L4 and H4) yielded 133 bp of sequence, which was also confirmed as a part of the *Hydrohis* mitochondrial cyt *b* gene, it was discarded because it was obtained only in the second trial. Thus, the former three fragments of sequences (a combined length of 333 bp) were used as the representing sequence of the Aja specimen in the following data analyses.

Longer sequence fragments (981 bp), containing the segments which were amplified for the Aja specimen, were amplified and sequenced for the remaining specimens with frozen tissue samples using the primers Glu-5'eeg (Suzuki and Hikida, 2010) and H16064 (Burbrink et al., 2000). Prior to alignment, amino-acid translations were performed for all sequence fragments and no stop codons were detected, thus, confirming that the sequences were indeed part of the cyt *b* gene.

The fragment of the Aja specimen (333 bp) was identical with those of nine of the Okinawajima specimens, as well as of the unique Tsuken and Amamioshima specimens. On the other hand, the Aja specimen differed from other ingroup specimens by 1 nucleotide (0.3%, from three Thai *H. cyanocinctus* specimens including DQ233946) to 15 nucleotides (3.9% from the other three Thai *H. cyanocinctus* specimens including DQ233945).

Sequence comparisons of the longer portion of the mitochondrial cyt *b* gene (981 bp), thus excluding the Aja sequence, supported the presence of two sequence groups in the Central Ryukyu sample. One of the groups was composed of nine Okinawajima, one Tsuken, and one Amamioshima specimens, all of which shared identical sequence fragment of 333 bp with the Aja specimen (see above). The other group was composed of the four remaining Central Ryukyu specimens, all from Okinawajima, and the Southern Ryukyu specimens from Ishigakijima and Iriomotejima. The two groups differed from each other by 15 (1.5%) nucleotides.

The NJ and ML trees based on the partial cyt *b* data obtained from frozen tissues and DDBJ/EMBL/GenBank were mostly compatible with each other in the major topology (Figs. 3.2 and 3.3). In both analyses, the ingroup was divided into three, well-supported clades (hereafter referred to as Clades 1, 2, and 3). Clade 1 (bootstrap values in NJ and ML analyses were 99/92) was composed of *H. spiralis* from Thailand and *H. cyanocinctus* from Thailand and Sri Lanka. Clade 2 (79/91) was composed of a part of the Okinawajima sample, all 16 specimens from the Southern Ryukyus (i.e. Ishigakijima and Iriomotejima), a unique Mie specimen, along with the Southeast Asian *cyanocinctus*, Australian *cyanocinctus*, *H. pacificus*, *H. parviceps*, and Southeast Asian *melanocephalus*. Cyt *b* sequences of all 16 Southern Ryukyu specimens were identical, but differed from the sequences of the four Okinawajima specimens by 1 nucleotide substitution (0.1%). These two haplotypes were unique to the Ryukyus. Clade 3 (91/95) was sister to Clade 2 and was composed of the remaining 11 Central Ryukyu specimens (i.e. Okinawajima, Amamioshima, and Tsuken), which shared identical 333 bp fragment with the Aja specimen (see above), and Thailand *cyanocinctus*, Sulawesian *melanocephalus*, and *H. coggeri* from Australia and Sulawesi, of which the *H. coggeri* sequences were relatively well supported subclade.

3.3.2. Morphological examination

The examinations of external morphological characters revealed that the Ryukyu specimens exhibited considerably wide variations in relative head size and that high degree of overlaps between the two mitochondrial clades in nearly all examined features. The relative head size, expressed in terms of the ratio between SVL or C_{max} and head width, clearly overlapped for all three clades (Fig. 3.4). The relative eye size, expressed in terms of the ratio

between the eye diameter and distance to the oral margin, also overlapped between the two clades from the Ryukyus. Most specimens had relatively smaller eyes (Fig. 3.5). The results of the scale count analyses revealed that *Hydrophis* specimens from both Clade 2 and 3 possessed one anterior temporal scale or two anterior temporal scales while specimens from Clade 1 possessed 2 or 3 anterior temporals (Fig. 3.6).

The examinations of body scale counts and the number of transverse bands also showed that the Ryukyu specimens were highly variable (Fig. 3.7). Shapes of the histograms were apparently unimodal as a whole and there were no clear differences in these characters between the mitochondrial clades. Moreover, the numbers of scales around the neck and mid-body overlapped between Clades 2 and 3, although the former tended to be slightly smaller in these scale counts. The numbers of neck scales ranged from 24-27 and 24-32, and the numbers of mid-body scales ranged from 30-37 and 30-41 in Clade 2 and Clade 3, respectively. The ventral scale exhibited a relatively greater overlap between Clades 2 and 3: the numbers of ventral scales ranged from 292-360 and 284-339 in Clade 2 and Clade 3, respectively. Likewise, the number of transverse bands on body also much overlapped between the clades (Fig. 3.7).

3.4. Discussion

It can be inferred from the phylogenetic trees that, besides *Hydrophis ornatus*, *Hydrophis* spp. in the Ryukyu Archipelago are divided into two separate mitochondrial lineages. Each of these lineages, however, contains sequences of both *H. cyanocinctus* and *H. melanocephalus* from Southeast Asia reported in Sanders et al. (2013b) (Figs. 3.2 and 3.3). Sanders et al. (2013b) demonstrated that there were considerable discordance between the groupings obtained by the analyses of nuclear microsatellite and mitochondrial datasets and that the result with the former was much more concordant with the prevailing species delimitation. Thus, they assumed that the observed cytonuclear discordance was the consequence of the past mitochondrial introgression, especially between *H. cyanocinctus* and *H. melanocephalus*. Therefore, it is possible that the existence of the two distinctive mitochondrial lineages in the Ryukyu specimens also reflects introgression of mitochondrial genome of one species into the other species. Even so, however, it is unlikely that only one species has persisted in the Ryukyu region and it had received mitochondrial genes from

another species outside of the Ryukyus. In both clades, the Ryukyu specimens possessed haplotypes apparently endemic to this region, suggesting that the non-*ornatus* assemblage of the Ryukyu *Hydrophis* has lacked gene flow with the Southeast Asian relatives for considerably long period of time. In this case, and assuming that the only one species exists in the Ryukyu region, one of the two mitochondrial lineages will most likely disappear through lineage sorting. Thus, the present results favor the possibility that there are two different taxonomic entities in the Ryukyus, even if the possessed mitochondrial type does not necessarily reflect the species correctly.

The results of the morphological examination are supportive of the above view. Sanders et al. (2013b) demonstrated remarkable morphological difference between *H. cyanocinctus* and *H. melanocephalus*, where all *H. cyanocinctus* samples from Vietnam, Java, and Australia exhibited relative girths of neck against hind-body of less than 1.8. In the samples of this study, except for several young specimens with SVL < 600 mm, this score was larger than 1.8 in the Ryukyu specimens (Fig. 3.8). Therefore, if the Ryukyu sample consists of a single species, and the observed two mitochondrial lineages were the results of introgression, it could possibly be *H. melanocephalus*. On the other hand, all of the specimens of *H. melanocephalus* examined by Sanders et al. (2013b) exhibited relative girths of over 2.0. In contrast, this score was smaller than 2.0 in a few specimens from the Ryukyus. Similarly, Sanders et al.'s *H. melanocephalus* was smaller than 1000 mm in SVL, but several specimens from the Ryukyus were much larger than 1000 mm. Thus, it is certain that the Ryukyu sample exhibited a wider range of morphological variation, suggesting that more than one taxonomic entity was included. Nonetheless, morphological variations in the Ryukyu sample as a whole are mostly unimodal and it is almost impossible to recognize the two groups by any of the morphological characters.

An alternative explanation is that the Ryukyu sample was actually represented by *H. melanocephalus* only and the Ryukyu population exhibits wider morphological variation and grows much larger than the Southeast Asian conspecifics because of the absence of *H. cyanocinctus*. However, there is no evidence to either support or rule out this possibility at the moment. Further analyses using microsatellite markers are indispensable in order to make any robust conclusions regarding the taxonomic status and character evolution in the non-*ornatus Hydrophis* spp. of the Ryukyu assemblage.

Within Clade 2, the specimen from Mie Prefecture shared a haplotype exclusively with the Southern Ryukyus sample: the same haplotype was not found at all in the Central Ryukyu sample. It is, therefore, likely that the Mie specimen is a migrant from the Southern Ryukyus or from even more south. There are some previous records of *H. melanocephalus* and *H. cyanocinctus* from several localities on the main island of Japan, i.e. Shikoku, Honshu, and Hokkaido (Nakamura and Uéno, 1963). Nonetheless, records accompanying explicit vouchers, such as specimens and photographs, are rare. Nakamura and Kawase (1976) and Masunaga et al. (2005) reported occurrences of *H. melanocephalus* from Kanagawa and Wakayama Prefectures, respectively, with voucher specimens, but most other mainland Japanese records of the species lacked such objective evidence. The scarcity of records suggests that there are no stable populations of this and related species in Honshu and the distribution is restricted to the Ryukyu-sourthern Kyushu regions (Masunaga et al. 2005). Since only a single non-Ryukyu specimen was available to this study, future molecular and morphological examinations of additional specimens are strongly desired to confirm the absence of stable *Hydrophis* populations along the coasts of the main islands of Japan.

Table 3.1. Summary of some diagnostic features of *Hydrophis melanocephalus* and *H. cyanocinctus* from Japan (Stejneger 1901 and 1907, Toriba 1994).

Features	***H. melanocephalus***		***H. cyanocinctus***	
	Stejneger 1901 and 1907	Toriba 1994	Stejneger 1901 and 1907	Toriba 1994
Head size		Small	Small, not larger than preocular	Not small
Head coloration	Black with irregular yellow marks on anterior half and behind eyes	Black with few yellowish spots		Black with many yellow spots
Eye size		Large, diameter equal to or greater than distance from oral margin		Not large, diameter less than distance from oral margin
Number of preoculars	1	1	1	
Number of postoculars	1 or 2	1 or 2	2	
Number of anterior temporals	Single and large	Single and large	2	2 or 3
Number of supralabials	7, second in contact with prefrontal, third and forth entering eye, sixth very small	7 or 8, third to forth, or fifth, touching eye, second to last very small	7-8	7-8, last few small
Number of neck scales	23-25	23-27	33	27-31
Number of body scales	32-35	33-41	41	35-43
Number of ventral scales	326-341	289-358	329	290-390

Table 3.2. Specimens' names, localities, and museum reference numbers.

Name	Country	Locality	Reference No.
Hydrophis 01	Japan	Okinawajima Is	KUZ R67678
Hydrophis 02		Amamioshima Is	20100626
Hydrophis 07		Okinawajima Is (Aja)	-
Hydrophis 08		Tsuken Is	KUZ R65808
Hydrophis 09		Ishigaki Is	KUZ R65822
Hydrophis 10		Iriomote Is	KUZ R67679
Hydrophis 11		Okinawajima Is	KUZ R67240
Hydrophis 12		Mie Prefecture	-
Hydrophis 13		Ishigaki Is	KUZ R47896
Hydrophis 14		Okinawajima Is	KUZ R56623
Hydrophis 15		Okinawajima Is	KUZ R57258
Hydrophis 16		Okinawajima Is	KUZ R57424
Hydrophis 17		Okinawajima Is	KUZ R57580
Hydrophis 18		Iriomote Is	KUZ R57592
Hydrophis 19		Okinawajima Is	KUZ R57812
Hydrophis 20		Okinawajima Is	KUZ R57820
Hydrophis 21		Ishigaki Is	KUZ R57935
Hydrophis 22		Ishigaki Is	KUZ R57936
Hydrophis 23		Ishigaki Is	KUZ R57937
Hydrophis 24		Ishigaki Is	KUZ R57938
Hydrophis 25		Okinawajima Is	KUZ R57948
Hydrophis 26		Okinawajima Is	KUZ R57949
Hydrophis 27		Okinawajima Is	KUZ R57950
Hydrophis 28		Okinawajima Is	KUZ R67956
Hydrophis 29		Okinawajima Is	KUZ R67958
Hydrophis 30		Ishigaki Is	KUZ R57939
Hydrophis 31		Ishigaki Is	KUZ R57940
Hydrophis 32		Ishigaki Is	KUZ R57941
Hydrophis 33		Ishigaki Is	KUZ R57942
Hydrophis 34		Ishigaki Is	KUZ R57943
Hydrophis 35		Ishigaki Is	KUZ R57944
Hydrophis 36		Ishigaki Is	KUZ R57945
Hydrophis 37		Ishigaki Is	KUZ R57946
Hydrophis 03	Thailand	Phuket (South Thailand)	KUZ R62347
Hydrophis 04		Phuket (South Thailand)	KUZ R62348
Hydrophis 05		Phuket (South Thailand)	KUZ R62349
Hydrophis 06		Phuket (South Thailand)	KUZ R62350

Table 3.3. Primers used to generate PCR products and DNA sequences.

Region	Name	Sequence (5' – 3')
Cytochrome *b*	Glu-5'eeg (Suzuki and Hikida, 2010)	TGATATGAAAAACCACCGTTG
	H16064 (Burbrink et al., 2000)	CTTTGGTTTACAAGAACAATG CTTTA

Table 3.4. Primer used to generate PCR products and DNA sequences for formalin-preserved specimen from Aja, Okinawajima, Japan.

Region	Name	Sequence (5' – 3')
Cytochrome *b*	Hydro-Cytb-L1	GCCCTACAAACATCAACAGG
	Hydro-Cytb-H1	ATGAAAAATATAGATGCGCCAAT
	Hydro-Cytb-L2	ATCCTACGAGATGTGCCTAA
	Hydro-Cytb-H2	TTTAAATATGAGCCGTAGTAGA
	Hydro-Cytb-L3	CATCTACACCCATATTGCAC
	Hydro-Cytb-H3	ATAACCAAAGAAGGATGTGG
	Hydro-Cytb-L4	CATAGCCACATCCTTCTTTGG
	Hydro-Cytb-H4	AGAGCAAAGAATCGGGTCAG
	Hydro-Cytb-L5	CTGACCCGATTCTTTGCTCT
	Hydro-Cytb-H5	CTTTATATGAGTGGTATGGGTGGA
	Hydro-Cytb-L6	ACCCAGACACCGACAAAATC
	Hydro-Cytb-H6	AGAGGGTTTGCTTTGGAGAA
	Hydro-Cytb-L7	GAAAATTTCTCCAAAGCAAACC
	Hydro-Cytb-H7	AGGTGTGGGTAAATGGTGCT
	Hydro-Cytb-L8	CACCATTTACCCACACCTCTT
	Hydro-Cytb-H8	TGAGGCTGTTTGGCTAATAAA

Table 3.5. List of species, localities, and DDBJ/EMBL/GenBank accession numbers for the downloaded sequences used in the phylogenetic analyses.

Species	Country	Locality	Accession No.
Hydrophis melanocephalus	Vietnam	Ham Tân (South Vietnam)	KC572617
			KC572618
			KC572619
			KC572620
			KC572621
			KC572622
			KC572623
	Indonesia	Makassar (South Sulawesi)	JQ217207
			KC572587
			KC572599
			KC572601
			KC572602
			KC572607
			KC572608
			KC572609
			KC572613
			KC572614
			KC572615
Hydrophis cyanocinctus	Sri Lanka	Trincomalee	KC572597
			KC572598
	Thailand	Phuket (South Thailand)	DQ233945
			DQ233946
			KC572583
	Vietnam	Ham Tân (South Vietnam)	KC572584
			KC572585
			KC572624
	Indonesia	Pasuruan (East Java)	KC014409
		Pelabuhan Ratu Bay (West Java)	KC014410
			KC014411
			KC014412
			KC572610
			KC572611
			KC572612
			KC572616
	Australia	Gulf of Carpentaria, Queensland	KC572590
			KC572593
			KC572594
			KC572595
			KC572596
Hydrophis coggeri	Indonesia	Makassar, South Sulawesi	KC014406
			KC014407
			KC014408
			KC572600
			KC572603
			KC572604
			KC572605
			KC572606
	Australia	Fraser Is, Queensland	KC572591
Hydrophis parviceps	Vietnam	Ham Tân (South Vietnam)	KC014441
			KC014442
Hydrophis pacificus	Australia	Mornington Is	DQ233963
			DQ233964
		Groote Eylandt	DQ233965
Hydrophis spiralis	Thailand		DQ233966
Hydrophis ornatus	Australia	Groote Eylandt	DQ233958

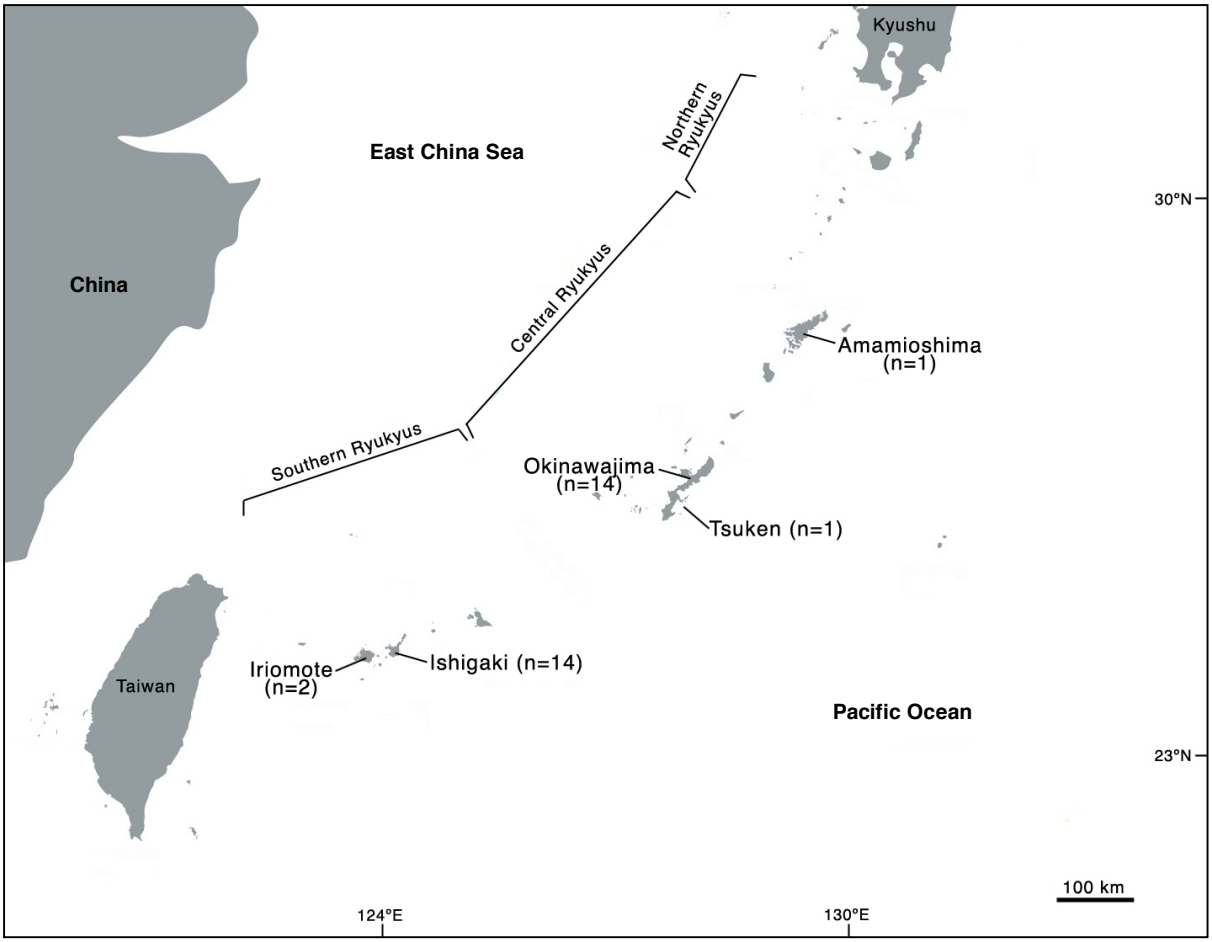


Fig. 3.1. Map of *Hydrophis* spp. sampling localities in the Ryukyu Archipelago.

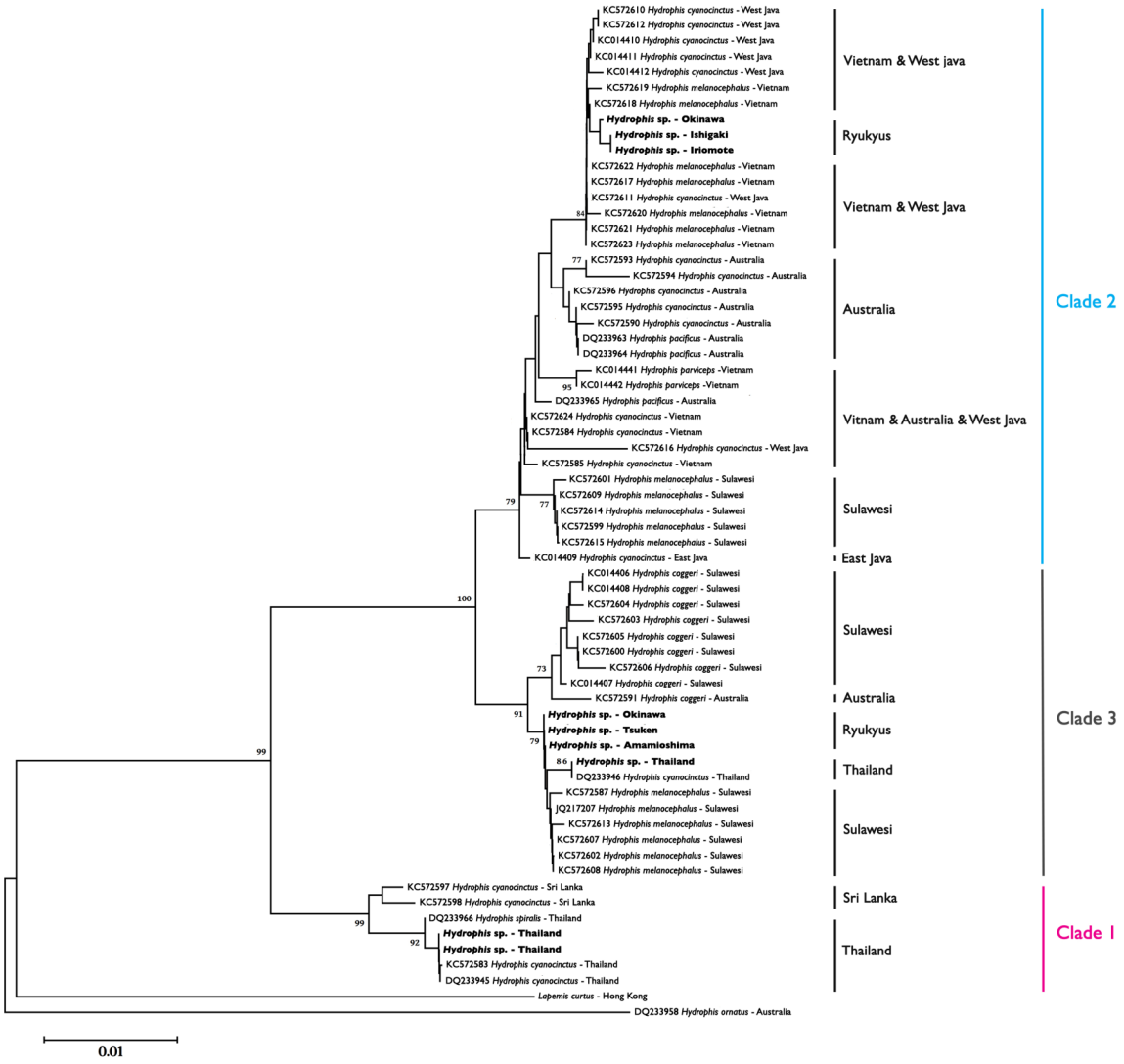


Fig. 3.2. Neighbor-joining tree constructed from cytochrome *b* sequence data showing the three major *Hydrophis* clades.

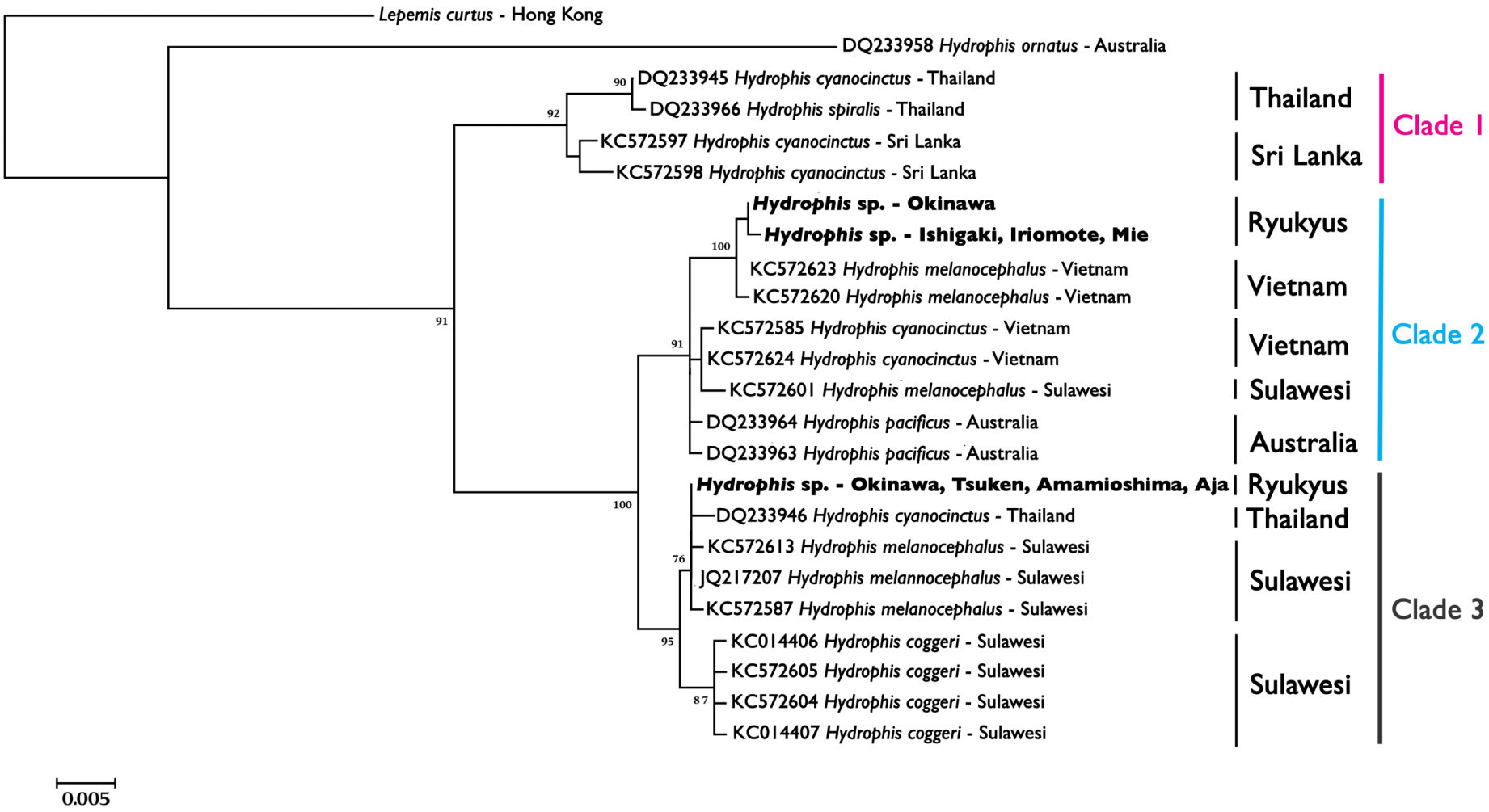
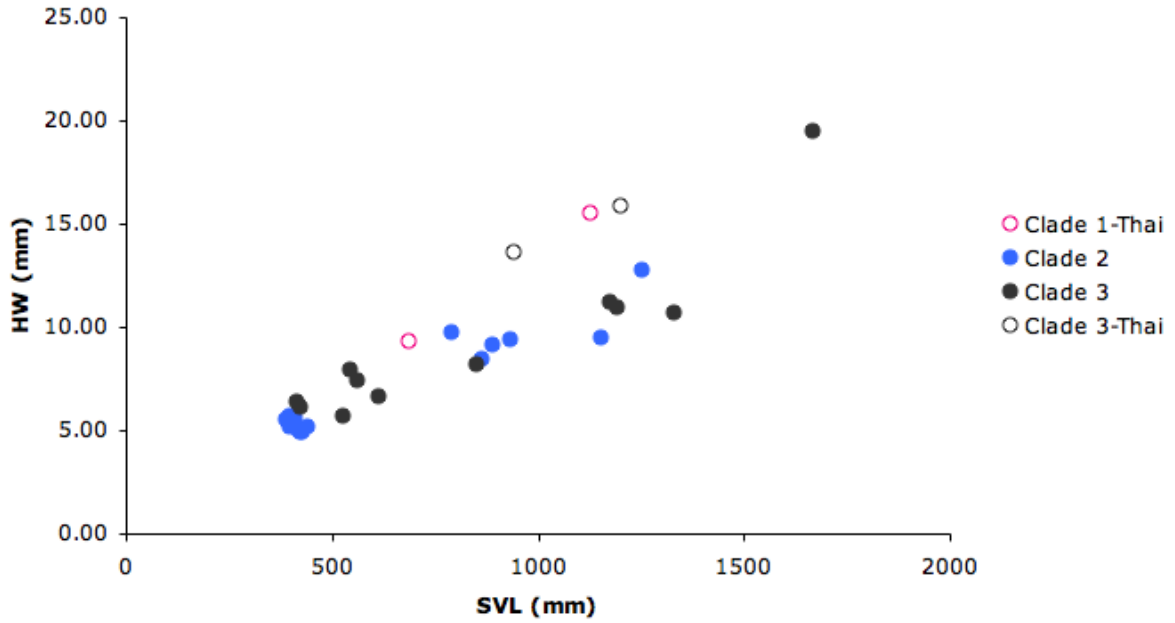


Fig. 3.3. Maximum likelihood tree constructed from cytochrome *b* sequence data showing the three major *Hydrophis* clades.

(a)



(b)

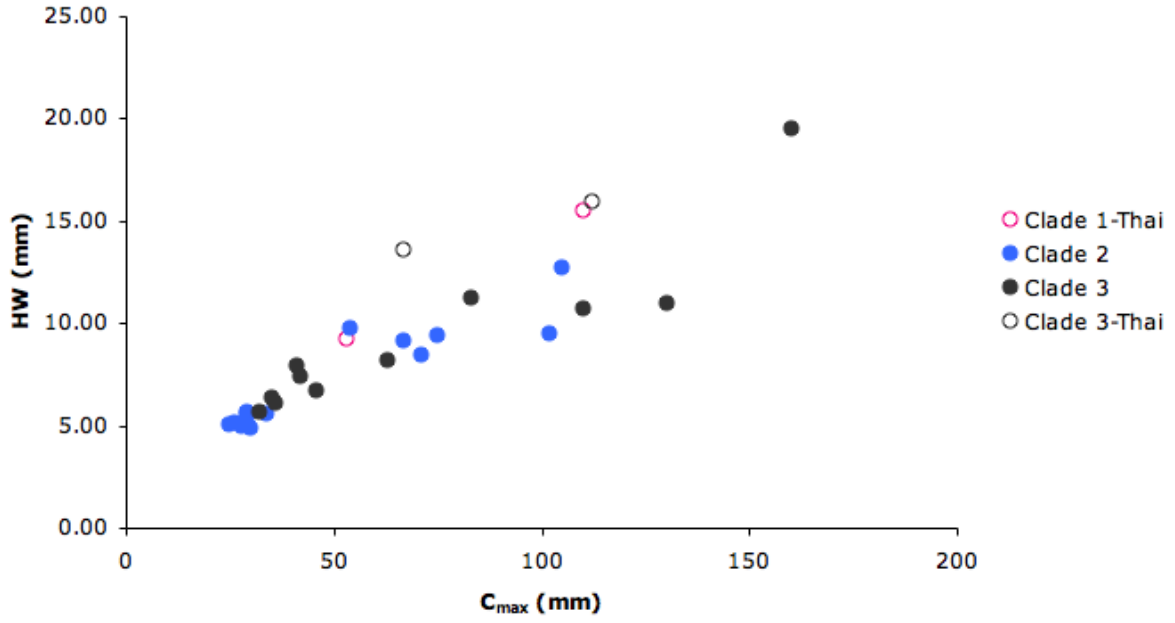


Fig. 3.4. Comparison of relative head sizes, expressed in terms of the ratios between SVL and HW (a) and C_{max} and HW (b), between the three *Hydrophis* clades. Open circles represent Thai specimens.

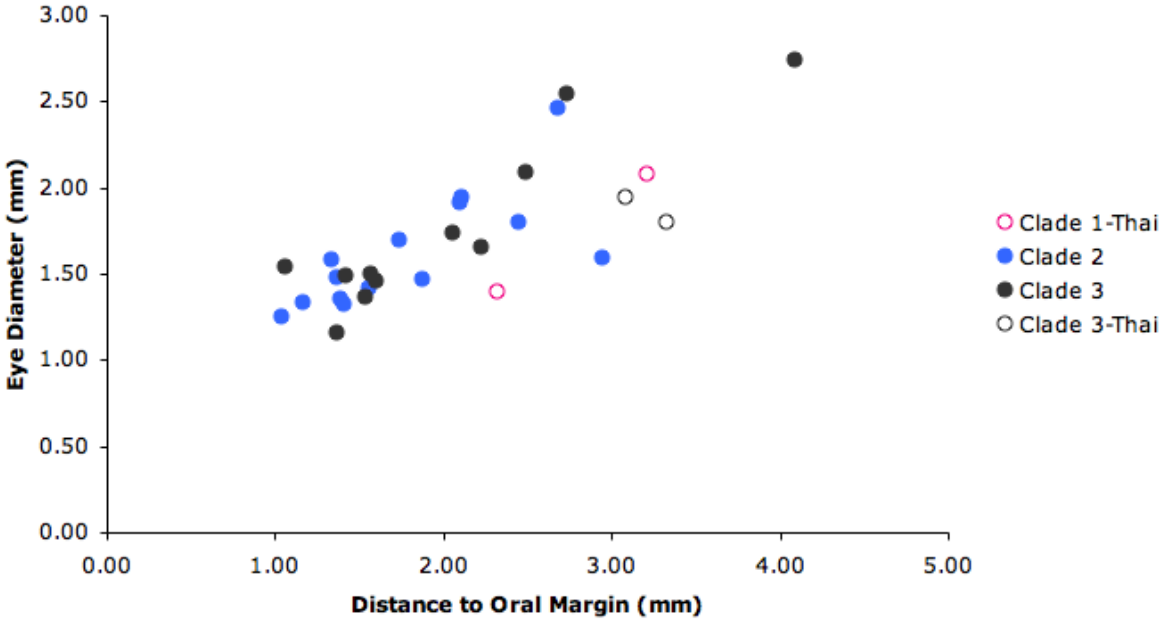


Fig. 3.5. Relative eye sizes, expressed in terms of eye diameter and distance to oral margin, among the members of the three *Hydrophis* clades. Open circles represent Thai specimens.

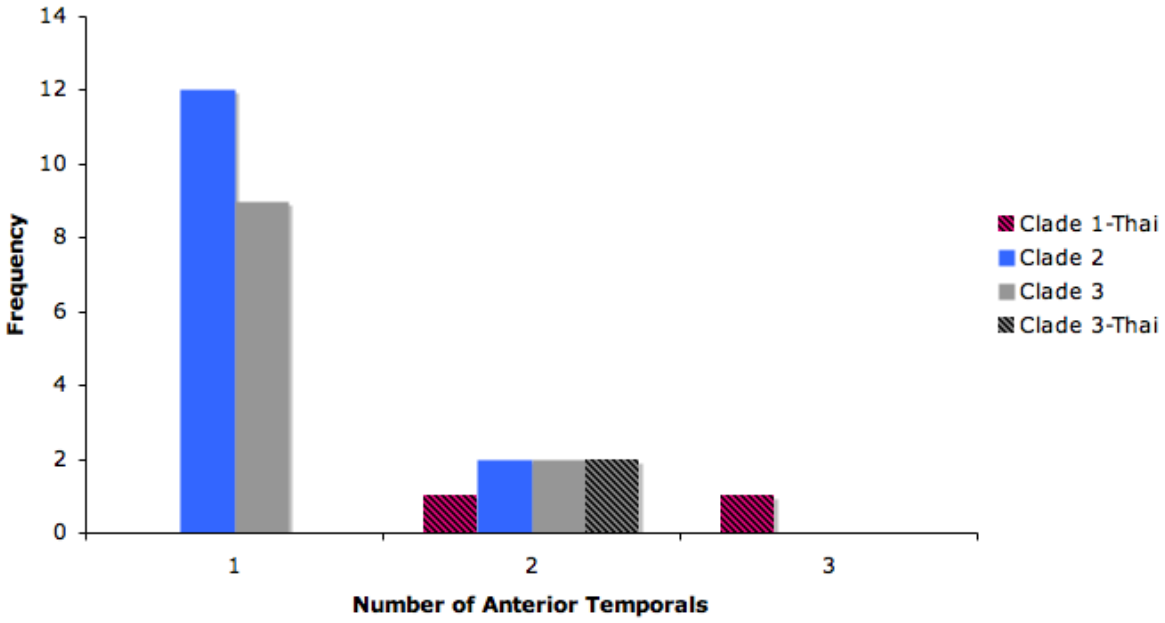


Fig. 3.6. Comparison of the number of anterior temporal scales between the three *Hydrophis* clades.

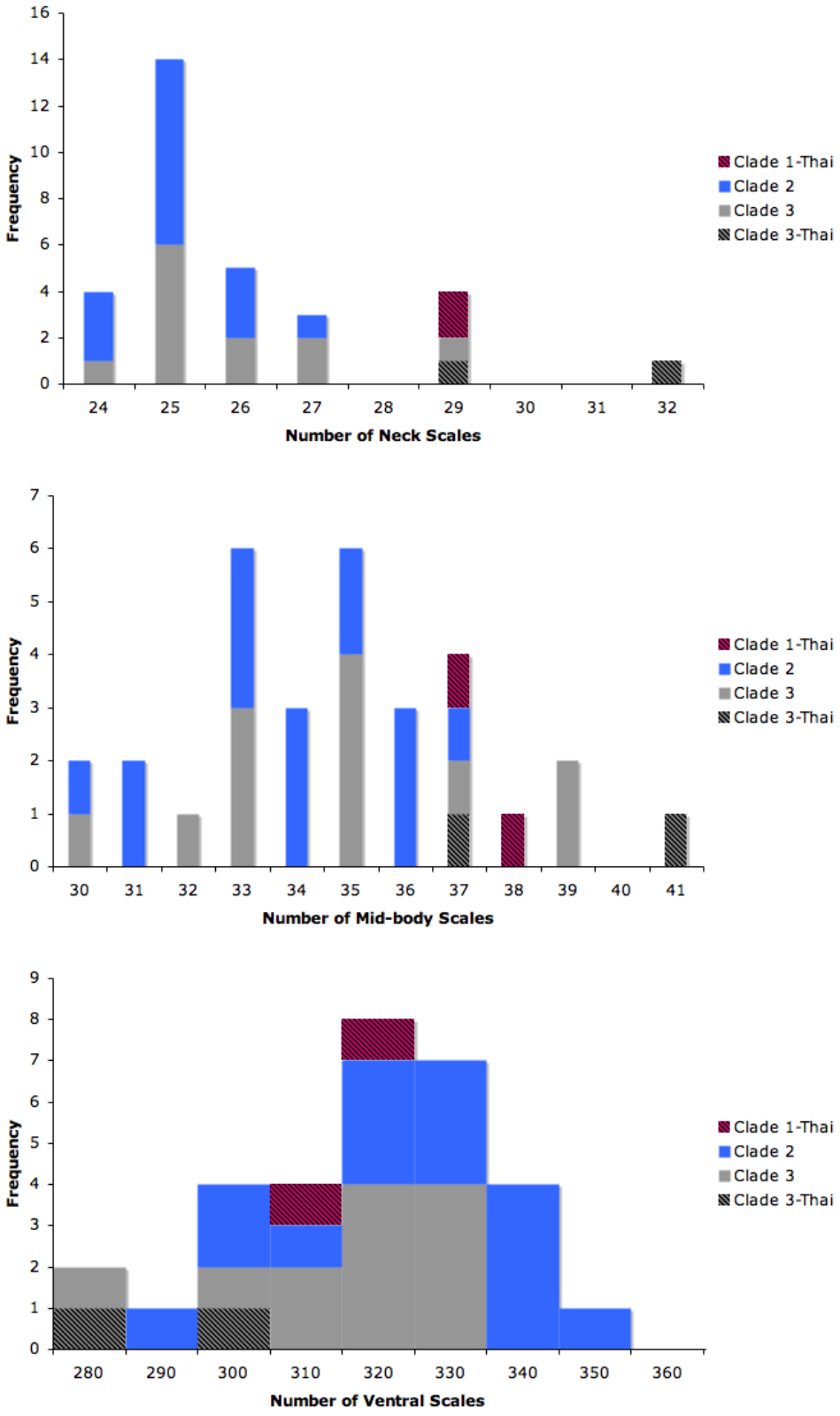


Fig. 3.7. Histograms of scale counts and number of bands in *Hydrophis* spp. samples from the Ryukyu Islands and Thailand. From top to bottom, the scale rows at neck, the scale rows at mid-body, ventrals, and number of transverse bands on body.

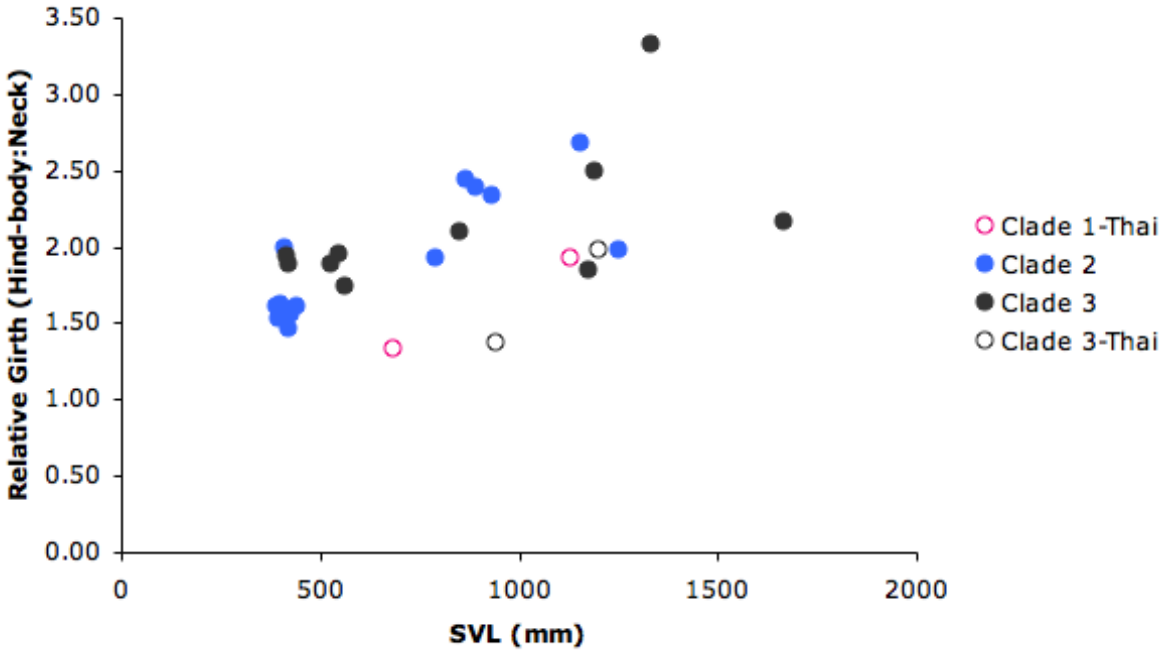


Fig. 3.8. Relative girths, expressed in terms of the ratio between hind-body and neck girths, with respect to the SVL (mm), among the members of the three *Hydrophis* clades. Open circles represent Thai specimens.

General Discussion

Here, the study on the geographic genetic structures of the laticaudine sea kraits, *Laticauda laticaudata* and *L. semifasciata*, revealed distinct population genetic structures among all Ryukyu-Taiwan subregions, which are separated by deep straits (Chapter 1). Interestingly, these deep straits (i.e., the Tokara Tectonic Strait, the Kerama Gap, and the Yonaguni Strait) serve as barriers to dispersal and play important roles in the formation and maintenance of the current population structures of these amphibious sea kraits despite the fact that they are morphologically adapted for aquatic locomotion. Moreover, in *L. laticaudata*, significant genetic differentiations were observed among island samples within the same subregion, suggesting that this particular species does not disperse even between nearby islands. The results further suggested that *L. laticaudata* may exhibit higher degree of philopatry, or site fidelity, compared to *L. semifasciata*. In addition to ocean depth and philopatry, other factors are worth exploring in futures studies, i.e. bottom topography of the ocean and habitat availability.

The geographic patterns observed are similar and comparable to the geographic faunal pattern of amphibians and terrestrial reptiles, which were previously reported (Ota, 2000). However, based on the analyses, the divergences of sea kraits were the results of relatively recent isolations, most probably at the end of the last glacial maximum, during the late Pleistocene (0.02-0.015 MYA), when sea level declines were accompanied by exposures and extensions of continental coastlines. The relatively shallow waters and exposed coastlines would have facilitated range expansions and explorations of new habitats.

Besides the unique geological features, which resulted from series complex geohistorical events mentioned earlier, the oceanographic features of the Ryukyu-Taiwan region play important roles in the distribution of marine organisms. The Kuroshio Current, which flows from the Philippines, pass the eastern coast of Taiwan, the western coast of the Central and Southern Ryukyus, and the eastern coast of mainland Japan, serves as a marine transport system northward dispersals of marine larvae as well as adults. In Chapter 2, the study on the origins of *L. laticaudata* and *L. semifasciata* individuals collected from the coasts of the main islands of Japan verified the presence of accidental, long-distance drifters, most probably transported by the Kuroshio. Based on the results of the molecular analyses, *L.*

semifasciata individuals collected from Mie and Oita Prefectures were drifters from the Central Ryukyus and the Southern Ryukyus (or further south), respectively. Nonetheless, it was concluded that the Kuroshio doesn't contribute to gene flow and the presence of drifting individuals does not affect the population genetic structures in the region. In fact, the Kuroshio poses threats to the amphibious sea kraits as the strong current may carry them to unsuitable habitats.

Although the unique geographical and oceanographic features of the Ryukyu-Taiwan region have attracted various scientific interests, issues regarding the taxonomic confusions and species diversity of sea snakes still exist. In Chapter 3, genetic assessments were conducted to verify the taxonomic status of 2 species of hydrophiine sea snakes, namely *Hydrophis melanocephalus* and *H. cyanocinctus*, which are sympatrically distributed in the Ryukyu-Taiwan region. The results of the mtDNA analyses revealed the presence of two separate taxonomic entities in the Ryukyu Archipelago. However, due to the possibility of mitochondrial introgression, coupled with overlapping morphological characteristics between the two entities, further studies are needed to confirm the taxonomic status and determine the diagnostic features of each species. Due to its medical importance, accurate identification is very crucial for the administration of species-specific anti-venom and treatments.

Lastly, the present studies on population genetics and taxonomy of laticaudine and hydrophiine sea snakes can be considered one of the first studies conducted in the islands of East Asia. The findings obtained from this study serve as valuable information for the conservation and preservation of biodiversity in the Ryukyu-Taiwan region. Moreover, the northern Ryukyus is the northernmost limit of the breeding populations of sea snakes. It is, therefore, important to take into account the possible connectivity with the areas south of the Ryukyu-Taiwan region in future studies.

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