Evolution of nuchal glands, unusual defensive organs of Asian natricine snakes (Serpentes: Colubridae), inferred from a molecular phylogeny

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Abstract
A large body of evidence indicates that evolutionary innovations of novel organs have facilitated the subsequent diversification of species. Investigation of the evolutionary history of such organs should provide important clues for understanding the basis for species diversification. An Asian natricine snake, Rhabdophis tigrinus, possesses a series of unusual organs, called nuchal glands, which contain cardiotonic steroid toxins known as bufadienolides. Rhabdophis tigrinus sequesters bufadienolides from its toad prey and stores them in the nuchal glands as a defensive mechanism. Among more than 3,500 species of snakes, only 17 Asian natricine species are known to possess nuchal glands or their homologues. These 17 species belong to three nominal genera, Balanophis, Macropisthodon, and Rhabdophis. In Macropisthodon and Rhabdophis, however, species without nuchal glands also exist. To infer the evolutionary history of the nuchal glands, we investigated the molecular phylogenetic relationships among Asian natricine species with and without nuchal glands, based on variations in partial sequences of Mt-CYB, Cmos, and RAG1 (total 2,767 bp).
Results show that all species with nuchal glands belong to a single clade (NGC). Therefore, we infer that the common ancestor of this clade possessed nuchal glands with no independent origins of the glands within the members. Our results also imply that some species have secondarily lost the glands. Given the estimated divergence time of related species, the ancestor of the nuchal gland clade emerged 19.18 mya. Our study shows that nuchal glands are fruitful subjects for exploring the evolution of novel organs. In addition, our analysis indicates that reevaluation of the taxonomic status of the genera Balanophis and Macropisthodon is required. We propose to assign all species belonging to the NGC to the genus Rhabdophis, pending further study.
1 | INTRODUCTION

In the 20th Century, many biologists were focused on commonalities among taxa, as represented by studies using model organisms (Alberts et al., 2008). On the other hand, appreciating the diversity of life and its evolutionary origins has been another essential pursuit in biology (Rosenzweig, 1995; Whittaker, 1972). Because evolution of novel phenotypic characters, such as wings of birds and mammary glands of mammals, can facilitate the diversification of a lineage (Wagner & Lynch, 2010), investigation of the evolutionary history of such novel characters can provide basic information that clarifies the processes underlying species diversification.

Snakes (Serpentes) comprise a distinct monophyletic taxon within the Squamata (Pyron, Burbink, & Wiens, 2013), including over 3,500 species that are distributed on all continents except Antarctica (Wallach, Williams, & Boundy, 2014). In spite of their seemingly uniform appearance, snakes exhibit prominent morphological and ecological diversity (Greene, 1997; Lillywhite, 2014) and have often evolved novel organs that serve particular ecological functions. A well-known example of a novel defensive structure is the rattle of rattlesnakes, which is used to warn potential predators of the snakes’ venomous bite (Greene, 1997). The rattle evolved once in the ancestor of extant rattlesnakes (Castoe & Parkinson, 2006; Greene, 1997), and it has been lost secondarily in some island populations, where selection for defense is reduced in the absence of mammalian predators (Martins, Arnaud, & Murillo-Quero, 2008; Rowe, Farrell, & May, 2002).

The nuchal gland system is another example of a novel defensive structure that has evolved in snakes (Mori et al., 2012). Nuchal glands were originally described in a Japanese natricine snake, Rhabdophis tigrinus (Figure 1; Nakamura, 1935). The organs, which superficially resemble secretory structures, are embedded in the dermal layer of the dorsal skin of the neck. The nuchal glands of R. tigrinus contain cardiotonic steroid toxins known as bufadienolides (Hutchinson et al., 2007), which are sequestered from toads consumed as prey and can be redeplored as a defensive mechanism (Hutchinson et al., 2007). The glands of some other species also contain bufadienolides (Mori et al., unpublished). Ontogenetically, the nuchal glands are of mesodermal origin (Fukada, 1958; Mori et al., 2012), which is different from any other skin glands of terrestrial vertebrates, all of which arise from ectoderm (Savitzky et al., 2012). The glands lack a secretory epithelium and consist of a homogeneous population of fluid-filled cells surrounding a dense aggregation of capillaries. There is no central lumen or duct, and the glands simply rupture through the skin to expel their fluid contents when the snake is under predatory attack (Mori et al., 2012).

Nuchal glands and the structurally similar nucho-dorsal glands (which extend the full length of the body; Smith, 1938) are currently known in 17 species of Asian Natricinae (Mori et al., 2012; Mori, Jono, Ding, et al., 2016). Hereafter, we refer to all such structures as nuchal glands, for simplicity. No other animals have been reported to possess organs similar in their structural details to the nuchal glands. The 17 species that possess such glands belong to three nominal genera, Balanophis, Macropisthodon, and Rhabdophis. Interestingly, Macropisthodon and Rhabdophis also include species that do not have nuchal glands (Table 1). This distribution might indicate the occurrence of (a) multiple independent origins of these unusual organs, (b) their secondary loss, and/or (c) improper generic assignment of some species.

To infer the evolutionary history of the nuchal glands, we investigated the molecular phylogenetic relationships among Eurasian natricine species, including all but one of the species that have hitherto been reported to possess such glands (Table 1). Our phylogeny is based on partial sequences of the oocyte maturation factor Mos (Cmos) gene, the recombination-activating gene 1 (RAG1), and the mitochondrial cytochrome b (MT-CYB) gene, for a total of 2.7 kbp. Several recent phylogenetic studies of snakes have either focused on or included a number of Asian natricine species (Figueroa, Mickelvy, Grismer, Bell, & Lailvaux, 2016; Guo et al., 2012, 2014; Pyron, Kandambi et al., 2013). However, no previous study has addressed the evolution of the nuchal glands. Furthermore, our sampling of species and populations of Macropisthodon and Rhabdophis is much greater than that of previous studies.

**FIGURE 1** The snake, *Rhabdophis tigrinus*, in a defensive posture is directing the nuchal glands (NG) toward a perceived threat.
Specifically, our main purpose was to answer three questions: (a) Have the nuchal glands originated only once, or have they arisen multiple times independently among natricine snakes? (b) Do the species of *Macropisthodon* and *Rhabdophis* that lack such glands represent the secondary loss of those structures? (c) Are any of the species lacking nuchal glands incorrectly assigned to *Macropisthodon* or *Rhabdophis*?

### Materials and Methods

A total of 122 sequences of natricine snakes and three sequences of outgroup taxa were used for phylogenetic analyses (Appendix 1). Of those, 54 sequences were obtained from GenBank. Because our preliminary analysis suggested that the sequence data for *Rhabdophis adleri* registered in GenBank were incorrectly identified, we did not use the GenBank data for that species. The other 68 sequences were obtained by the following methods.

In each sample, total DNA was extracted from liver, skeletal muscle, or tail tips, which had been preserved in 99.5% ethanol or in freezers, using the DNeasy Tissue Kit (Qiagen). The Cmos, RAG1, and MT-CYB regions were amplified with a PCR System GeneAmp 2700 Thermal Cycler (Applied Biosystems), using an Ex Taq Polymerase Kit (Takara Bio Inc.) and primers S77/S78 for Cmos (Lawson, Sloswinski, Crother, & Burbank, 2005), R13/R18 for RAG1 (Groth & Barrowclough, 1999), and L14910/H16064 for MT-CYB (Burbank, Lawson, & Sloswinski, 2000). The
thermocycling schedule for the polymerase chain reaction (PCR) was identical to that described by these previous studies. Before sequencing, unincorporated primers were removed from the PCR products using polyethylene glycol precipitation. Cycle sequencing reactions were performed with the Big Dye Terminator Cycle Sequence Ready Reaction Kit, version 3.1 (Applied Biosystems), using the same primers as for PCR. The samples purified by ethanol precipitation were sequenced with a 3130xl Genetic Analyzer (Applied Biosystems). All fragments were sequenced for both forward and reverse sense. We assembled them using the GAP 4 program (Staden, 1996).

Using CLUSTAL X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997), 125 sequences were aligned. Identical sequences from different specimens were treated as single units so that 114 sequences were recognized. To infer the phylogeny, we employed Maximum Likelihood (ML) using combined sequences (Cmos + RAG1 + MT-CYB) and Bayesian inference (BI) using the sequence of mitochondrial DNA (MT-CYB). For both data sets, the most appropriate pattern of sequence evolution was selected by applying the Bayesian Information Criterion (BIC; Schwarz, 1978), using MEGAS5 (Tamura et al., 2011). We set the rate categories of discrete gamma rate heterogeneity as eight for ML and BI. Reliability of the ML tree was assessed by calculating bootstrap probability (BP; Felsenstein, 1985), with 1,000 replications. The BI tree was constructed using BEAST version 1.8 (Drummond & Rambaut, 2007), employing a single Markov chain Monte Carlo (MCMC) run for 50 million generations, sampled every 1,000 generations, and excluding the first 5 million generations as burn-in. Convergence of the chains to the stationary distribution was checked by visual inspection, using TRACER version 1.6 (Rambaut, Suchard, Xie, & Drummond, 2007).

To estimate divergence times, we employed Bayesian relaxed-clock dating, using BEAST version 1.8. Because no fossils of Balanophis, Macropisthodon, or Rhabdophis are known, we set the following calibration points: 30 Mya (SD = 0.115) at the crown of natricine snakes, 22 Mya (SD = 0.15) at the crown of the genus Natrix, and 16 Mya (SD = 0.15) at the crown of the genus Thamnophis (Guo et al., 2012).

3 | RESULTS

The final alignment of three gene fragments consisted of 2,767 aligned base pairs. Of those, 787–1,149 bp were from MT-CYB (114 taxa), 259–689 bp were from Cmos (86 taxa), and 855–929 bp were from RAG1 (21 taxa). The most appropriate model under the BIC was the GTR + G + I model for the data sets of both the ML and BI trees. The ML and BI trees were almost identical in topology. The ML tree (−ln L = −35078.3994) is shown in Figure 2. A consensus tree from the ML and BI analyses is shown in Figure 3, along with the BP values from ML and the posterior probability (PP) value from BI at each node (shown only for BP ≥ 70% in ML and PP ≥ 0.90 in BI). The main difference between the ML and BI trees is the status of Rhabdophis chrysargus. Unlike the ML tree, the BI tree supported monophyly of R. chrysargus + R. conspicillatus + 3 species of Xenochrophis (Figure 3a).

Monophyly of Natricinae was strongly supported by the PP value. Within this subfamily, monophyly of the New World taxa (the Thamnophiini), and the Old World taxa Natrix, Sinonatrix, Hebius, and Amphiesma + Xenochrophis + Atretil + Rhabdophis + Macropisthodon (except M. rudis) + Balanophis clades were highly supported. Of the latter clade, a subclade of Rhabdophis (except R. chrysargus and R. conspicillatus) + Macropisthodon (except M. rudis) + Balanophis was separated from the remainder with strong support (Figure 2b). The average estimated divergence time of this subclade was 19.18 Mya (16.28–22.16 in 95% credible ranges). Hereafter, we refer to this subclade as the nuchal gland clade (NGC). Within this clade, Macropisthodon plumibicolor first diverged from the other species. The latter include Rhabdophis subminutus, R. murudensis + Macropisthodon flaviceps, R. himalayanus + Balanophis ceylonensis, R. tigrinus + R. lateralis + R. formosanus, and a large group including R. adleri + R. callichromus + R. nigrocinctus + R. swinhonis + R. guangdongensis + R. nuchalis + R. leonardi + R. pentasupralabialis (with >90% support in BP and/or 0.9 in PP). The latter clade comprises two subclades: R. adleri + R. callichromus + R. nigrocinctus and R. swinhonis + R. guangdongensis + R. nuchalis + R. leonardi + R. pentasupralabialis. Several nominal species exhibit substantial population structuring. Rhabdophis subminutus exhibits strong differentiation between Laos/Vietnam and Thailand samples, and R. nuchalis consists of a number of population segments and is paraphyletic with respect to both R. leonardi and R. pentasupralabialis.

4 | DISCUSSION

Although differing in some details, recent molecular phylogenetic analyses of the Natricinae (Figueroa et al., 2016; Guo et al., 2012, 2014; Pyron, Burbriik et al., 2013; Pyron, Kandambi et al., 2013), including ours, agree on the general pattern of relationships among the major lineages. A basal dichotomy separates the subfamily into two major clades. One includes the entire North American natricine fauna (the Thamnophiini) and its sister group, the Eurasian genus Natrix. Those two, in turn, are sister to a clade containing the Asian genera Opisthotropis and Sinonatrix. A clade containing two endemic Sri Lankan genera, Aspidura and Haplocerus, is variously recovered as

**FIGURE 2** Maximum likelihood tree (−ln L = −35078.3994) based on the combined sequence data of the MT-CYB, Cmos, and RAG1 genes under GTR + G + I. Bootstrap probabilities are provided at each node. Numerals following scientific names indicate individual codes (see Appendix 1). Status of nuchal or nucho-dorsal glands of our three focal genera (Rhabdophis, Macropisthodon, and Balanophis) is indicated by blue (present), red (absent), purple (present/absent), and green (unknown; see also Table 1). The photographs have been digitally modified for clarity. Photograph of Balanophis ceylonensis by Udaya Chanaka
FIGURE 2 Continued

(b)
sister to this North American–Eurasian clade (Pyron, Burbrink et al., 2013; Pyron, Kandambi et al., 2013) or as the most basal branch of the natricine clade (our study, but with weak support).

The other major clade of natricines is almost entirely Asian, the sole exception being a monophyletic group of three African genera (Afronatrix, Natriciteres, and Lycognathophis, the latter not included in our analysis). The African clade is variously recovered as sister to, or embedded within, the much larger Asian radiation. The relationships among the Asian taxa display varying topologies among recent analyses, as taxon sampling within this group has improved. Consistent with other recent studies (Guo et al., 2014), we recover a monophyletic genus Hebius, distant from Amphisema stolatum, as well as a polyphyletic Xenochrophis, some related to Atretium and others close to Rhabdophis and Macropisthodon. These results engender confidence in our analysis of the relationships within the NGC.

4.1 | Evolution of the nuchal glands

Our results show that all species that possess nuchal glands belong to a single, strongly supported clade (NGC). Therefore, based on the principle of parsimony, we infer that the common ancestor of this clade possessed nuchal glands. We find no evidence of multiple, independent origins of the glands. Thus, interspecific differences in the distribution and morphology of the glands, such as the occurrence of nucho-dorsal glands along the entire length of the body in M. plumbicolor and several species of Rhabdophis (Mori, Jono, Ding et al., 2016; Mori, Jono, Takeuchi, & Das, 2016; Smith, 1938) and the presence of elongate, nonsacculated glands accompanied by scaleless areas of skin in M. rhodomelas (not included in our analysis), M. flavipecs, and B. ceylonensis (Smith, 1938), are considered to represent alternative morphologies that arose after a single evolutionary origin of the nuchal gland system. Further study of the morphological details is needed to clarify the process of glandular diversification within this clade.

Among species currently included in Rhabdophis and Macropisthodon, R. chrysargos, R. conspicillatus, and M. rudis have been reported to lack nuchal glands (Table 1; Mori et al., 2012; Mori, Jono, Takeuchi, & Das, 2016). Macropisthodon rudis is only distantly related to the NGC (see below), and R. conspicillatus and R. chrysargos also belong to clades outside the NGC. Thus, the absence of the
nuchal glands in these species does not constitute secondary loss. Rather, it appears that they have simply retained the ancestral condition of the absence of integumentary defensive glands.

*Rhabdophis swinhonis* has been reported to lack nuchal glands (Table 1; Mao & Chang, 1999). However, in contrast to *R. conspicillatus* and *R. chrysargos*, our analysis shows that this species occupies a position within the NGC. This strongly suggests that *R. swinhonis* has secondarily lost the nuchal glands. However, Hsiang, Li, and Yang (2009) noted the presence of nuchal glands in this species. If both observations are correct, there are two possible interpretations: either the occurrence of intraspecific variation or the presence of two distinct but cryptic species. Whichever is true, the deeply nested position of *R. swinhonis* within the NGC implies the recent or ongoing secondary loss of the glands in at least some populations.

Intraspecific variation in the presence of the nuchal glands also has been described in *R. murudensis* and *M. flaviceps* (Table 1; Smith, 1938; Mori et al., 2012). In our analysis, both species are recovered within the NGC. Therefore, as with *R. swinhonis*, the nuchal glands of *R. murudensis* and *M. flaviceps*, if accurately described in the literature, might be in a transitional stage of secondary loss or these nominal species may contain closely related cryptic species.

We estimate that the common ancestor of the NGC arose 19.18 Mya. This is only slightly later than the date of 23–24 Mya shown by Guo et al. (2012, Figure 2) for the origin of *Rhabdophis*, suggesting that nuchal glands arose at or soon after the origin of this genus.

### 4.2 Taxonomy

Our analysis requires a reevaluation of the taxonomic status of the genera *Balanophis* and *Macropisthodon*. The validity of the monotypic genus *Balanophis* (Smith, 1938) has been controversial. Malnate (1960) recognized the species as *Rhabdophis ceylonensis*, and McDowell (1961) supported his position. Figueroa et al. (2016) found the species nested within *Rhabdophis*, as sister to *R. himalayanus*, and despite stating in the text (p. 21) that they declined to synonymize the genera, they recognized the species as *R. ceylonensis* in their figure 7a. Our analysis also strongly supports a sister relationship between *B. ceylonensis* and *R. himalayanus*, and thus, we formally propose that *Balanophis* be synonymized with *Rhabdophis*.

Our analysis includes three of the four currently recognized species of *Macropisthodon* (Wallach et al., 2014), no two of which are recovered as each other’s closest relative. When the genus was described by Boulenger (1893), most other natricine snakes were treated as members of the genus *Tropidonotus*. Stejneger (1907) placed *Tropidonotus* in the genus *Natrix*, where it remained until Malnate (1960) divided *Natrix* sensu lato into six genera, resurrecting *Rhabdophis Fitzinger, 1843*. Malnate suggested that *Macropisthodon* might later prove not to be distinct from *Rhabdophis*, but the overreliance on characters of the maxillary dentition had precluded its earlier inclusion in *Natrix* and presumably influenced Malnate’s decision to retain the genus.

In our analysis, the type species of *Macropisthodon*, *M. flaviceps*, is strongly supported as sister to *R. murudensis*. Figueroa et al. (2016) show the fourth species, *M. rhodomelas*, nested well within *Rhabdophis*. Therefore, we synonymize *Macropisthodon* with *Rhabdophis*. Thus, it is presently reasonable to include all species belonging to the NGC within *Rhabdophis*, the type species of which is *R. subminiatus*. However, partitioning of this morphologically diverse clade should be considered in the future.

The divergent position of *Macropisthodon rudis*, which lacks nuchal glands and is recovered as distant from the NGC, supports the resurrection of the monotypic genus *Pseudokistron* (Van Denburgh 1909), as suggested by Wallach et al. (2014). Although recent studies have differed in the exact placement of this species (Guo et al., 2012, 2014), no analysis with sufficient taxon sampling of Asian natricines has placed it close to *Rhabdophis*. The taxonomic status of “*R*. conspicillatus” and “*R*. chrysargos”, which lie just outside the NGC, remains to be determined.

Our analysis suggests that *Rhabdophis* contains several undescribed species. Substantial genetic divergence occurs within *R. nigrocinclus*, *R. swinhonis*, *R. nuchalis*, and especially *R. subminiatus*. A comprehensive analysis of this complex subclade, including both morphological and molecular studies, will be necessary before this group can be reliably partitioned.

### 5 CONCLUSIONS

Our analysis indicates that the nuchal and nucho-dorsal glands, as a group, have evolved only once among Asian natricine snakes. The absence of the nuchal glands in some nominally congenic species, such as *M. rudis*, *R. conspicillatus*, and *R. chrysargos*, reflects old classifications based on phenetic analysis of morphological characters. All of those species lie outside the single clade that possesses the defensive glands. However, a few species within the nuchal gland clade (*M. flaviceps*, *R. murudensis*, and *R. swinhonis*) may represent a transitional stage in the secondary loss of the glands. Clarification of the developmental origin of these unique organs is likely to provide insight into how these neomorphic structures have arisen, diversified, and may subsequently be disappearing in a few species. The nuchal glands are fruitful subjects for investigating the evolution of novel biological systems that involve the complex interplay of morphology, physiology, ecology, and behavior.

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DATA ACCESSIBILITY

DDBJ accessions LC325298–LC325357, LC325746–LC325803, and LC326011–LC326031 (DNA sequences).

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## APPENDIX 1

Accession numbers and their localities (countries) for all DNA sequence data used in the phylogenetic analyses in this study. Individuals with an asterisk indicate identical sequences within the species, and thus have the same accession number. Names (and No.) in the species column correspond to those shown in Figure 1

| Species                          | Individual No. | Country   | Accession no. of GenBank | References                                                                 |
|----------------------------------|----------------|-----------|--------------------------|-----------------------------------------------------------------------------|
|                                  |                |           | Cyt.b                  | C-mos                        | Rag-1                      | References                                                                 |
| Afronatrix anoscopus             | ROM19842       | Liberia   | AF420073                | AF471123                     | EU402832                   | Lawson et al., 2005, de Queiroz, Lawson, and Lemos-Espinal 2002, and Wiens et al. (2008) |
| Amphiesma stolatum_1             | HT0548         | China     | LC325319                | LC325765                     | –                          | This study                                                                 |
| Amphiesma stolatum_2             | HT0798         | Sri Lanka | LC325347                | LC325793                     | LC326030                   | This study                                                                 |
| Amphiesma stolatum_3             | GP2213         | China     | KJ685693                | KJ685643                     | KJ685585                   | Guo et al. (2014)                                                         |
| Aspidura guentheri               | RAP0437        | Sri Lanka | KC347472                | KC347380                     | KC347418                   | Pyron, Kandambi et al. (2013)                                              |
| Atretium schistosum_1            | HT0799         | Sri Lanka | LC325348                | LC325794                     | –                          | This study                                                                 |
| Atretium schistosum_2            | –              | Sri Lanka | KC347487                | KC347383                     | KC347421                   | Pyron Kandambi et al. (2013)                                              |
| Atretium yunnanensis             | GP842          | China     | JQ678448                | JQ281787                     | KJ685602                   | Guo et al. (2014)                                                         |
| Balanophis ceylonensis_1         | HT0785         | Sri Lanka | LC325339                | LC325785                     | LC326026                   | This study                                                                 |
| Balanophis ceylonensis*_2        | HT0786         | Sri Lanka | LC325339                | –                            | –                          | This study                                                                 |
| Balanophis ceylonensis_3         | HT0787         | Sri Lanka | LC325340                | LC325786                     | –                          | This study                                                                 |
| Haplocercus ceylonensis          | RS145          | Sri Lanka | KC347478                | KC347401                     | KC347438                   | Pyron, Kandambi et al. (2013)                                              |
| Hebius atemporale                | HT0550         | China     | LC325320                | LC325766                     | –                          | This study                                                                 |
| Hebius craspedogaster            | HT0801         | China     | LC325350                | LC325796                     | –                          | This study                                                                 |
| Hebius ishigakiensis             | HT0800         | Japan     | LC325349                | LC325795                     | –                          | This study                                                                 |
| Hebius khasiense                 | HT0679         | Vietnam   | LC325327                | LC325773                     | –                          | This study                                                                 |
| Hebius octolineatus              | HT0586         | China     | LC325321                | LC325767                     | –                          | This study                                                                 |
| Hebius pryeri                    | HT0340         | Japan     | LC325312                | LC325758                     | –                          | This study                                                                 |
| Hebius vibakari_1                | HT0274         | Japan     | LC325309                | LC325755                     | –                          | This study                                                                 |
| Hebius vibakari_2                | HT0277         | Japan     | LC325310                | LC325756                     | –                          | This study                                                                 |
| Macropisthodon flaviceps         | HT0809         | Malaysia  | LC325355                | LC325801                     | –                          | This study                                                                 |
| Macropisthodon plum bicolor_1    | HT0782         | Sri Lanka | LC325336                | LC325782                     | LC326025                   | This study                                                                 |
| Macropisthodon plum bicolor_2    | HT0783         | Sri Lanka | LC325337                | LC325783                     | –                          | This study                                                                 |
| Macropisthodon plum bicolor_3    | HT0784         | Sri Lanka | LC325338                | LC325784                     | –                          | This study                                                                 |
| Macropisthodon rudis_1           | HT0339         | China     | LC325311                | LC325757                     | LC326016                   | This study                                                                 |
| Macropisthodon rudis_2           | GP1266         | China     | JQ687452                | JQ687434                     | KJ685566                   | Guo et al. (2014)                                                         |
| Natriciteres olivacea            | –              | Congo     | AF471058                | AF471146                     | –                          | Lawson et al. (2005)                                                      |
| Natrix maura_1                   | –              | Spain     | AY866530                | –                            | –                          | Guicking, Lawson, Joger and Wink (2006)                                    |
| Natrix maura_2                   | –              | Tunisia   | AY487682                | –                            | –                          | Guicking, Joger and Wink (2008)                                            |
| Natrix maura_3                   | –              | Italy     | AY487683                | –                            | –                          | Guicking et al. (2008)                                                    |
| Natrix natrix_1                  | –              | Spain     | AY866536                | –                            | –                          | Guicking et al. (2006)                                                    |
| Natrix natrix_2                  | –              | France    | AY866537                | –                            | –                          | Guicking et al. (2006)                                                    |
| Natrix tessellata_1              | –              | Iran      | AY487574                | –                            | –                          | Guicking et al. (2006)                                                    |
| Natrix tessellata_2              | –              | Iran      | AY487575                | –                            | –                          | Guicking, Joger and Wink (2009)                                            |

(Continues)
| Species                        | Individual No. | Country  | Accession no. of GenBank | Cyt.b   | C-mos | Rag-1 | References                  |
|-------------------------------|----------------|----------|--------------------------|---------|-------|-------|----------------------------|
| *Natrix tessellata_3*         |                | Bulgaria | AY866533 - -             | -       | -     | -     | Guicking et al. (2006)      |
| *Nerodia cyclopion*           |                | USA      | AF402909 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Nerodia erythrogaster*       |                | USA      | AF402912 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Nerodia fasciata*            |                | USA      | AF402910 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Nerodia floridana*           |                | USA      | AF402911 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Nerodia rhombifer*           |                | USA      | AF402915 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Nerodia sipedon*             |                | USA      | AF402913 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Nerodia taxispilota*         |                | USA      | AF402914 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Opisthotropis cheni*         | GP383          | China    | GQ281779 JQ687441 KJ685595 | -       | -     | -     | Guo et al. (2012)          |
| *Opisthotropis guangxiensis*  | GP746          | China    | GQ281776 JQ687447 -      | -       | -     | -     | Guo et al. (2012)          |
| *Opisthotropis lateralis*     | GP646          | China    | GQ281782 JQ687445 -      | -       | -     | -     | Guo et al. (2012)          |
| *Opisthotropis latouchii*     | GP647          | China    | GQ281783 JQ687446 -      | -       | -     | -     | Guo et al. (2012)          |
| *Opisthotropis typica*        | HT0794         | Malaysia | LC325343 LC325789 LC326028 | -       | -     | -     | This study                 |
| *Pseudoxenodon macrops* (Out group)_1 | HT0646 | China | LC325323 LC325769 - | -       | -     | -     | This study                 |
| *Pseudoxenodon macrops* (Out group)_2 | HT0802 | Malaysia | LC325351 LC325797 - | -       | -     | -     | This study                 |
| *Regina grahami*              |                | USA      | AF402918 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Regina rigida_1*             |                | USA      | AF402919 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Regina rigida_2*             | CAS:HERP:165994 USA | AF471052 AF471120 | - | - | - | Lawson et al. (2005) |
| *Regina septemvittata*        |                | USA      | AF402917 - -             | -       | -     | -     | Alfaro and Arnold (2001)    |
| *Rhabdophis adleri_1*         | HT0831         | China    | LC325356 LC325802 -      | -       | -     | -     | This study                 |
| *Rhabdophis adleri_2*         | HT0832         | China    | LC325357 LC325803 -      | -       | -     | -     | This study                 |
| *Rhabdophis callichromus_1*   | HT0654         | Vietnam  | LC325324 LC325770 -      | -       | -     | -     | This study                 |
| *Rhabdophis callichromus_2*   | HT0674         | Vietnam  | LC325325 LC325771 LC326020 | -     | -     | -     | This study                 |
| *Rhabdophis chrysargos*       | HT0342         | Malaysia | LC325313 LC325759 LC326017 | -       | -     | -     | This study                 |
| *Rhabdophis consplicatus*     | HT0791         | Malaysia | LC325342 LC325788 LC326027 | -       | -     | -     | This study                 |
| *Rhabdophis formosanus_1*     | HT0033         | Taiwan   | LC325304 LC325750 -      | -       | -     | -     | This study                 |
| *Rhabdophis formosanus*_2     | HT0031         | Taiwan   | LC325304 - -             | -       | -     | -     | This study                 |
| *Rhabdophis formosanus*_3     | HT0030         | Taiwan   | LC325304 - -             | -       | -     | -     | This study                 |
| *Rhabdophis guangdongensis*   | SY5r000018     | China    | KF800930 KF800920 -      | -       | -     | -     | Zhu et al. (2014)          |
| *Rhabdophis himalayanus_1*    | HT0847         | China    | LC325299 LC325746 LC326011 | -       | -     | -     | This study                 |
| *Rhabdophis himalayanus*_2    | HT0848         | China    | LC325299 - -             | -       | -     | -     | This study                 |
| *Rhabdophis himalayanus*_3    | HT0849         | China    | LC325299 - -             | -       | -     | -     | This study                 |
| *Rhabdophis himalayanus*_4    | CAS224420      | Myanmar  | KF800929 KF800919 -      | -       | -     | -     | Zhu et al. (2014)          |
| *Rhabdophis lateralis_1*      | HT0855         | China    | LC325302 - -             | -       | -     | -     | This study                 |
| *Rhabdophis lateralis_2*      | GP613          | China    | JQ687444 GQ281785 KJ685600 | -       | -     | -     | Guo et al. (2014)          |
| *Rhabdophis leonardi_1*       | HT0851         | China    | LC325300 LC325747 LC326012 | -       | -     | -     | This study                 |
| *Rhabdophis leonardi*_2       | HT0852         | China    | LC325300 - -             | -       | -     | -     | This study                 |
| *Rhabdophis leonardi*_3       | HT0853         | China    | LC325300 - -             | -       | -     | -     | This study                 |
| *Rhabdophis leonardi*_4       | RDQ200905367   | China    | KF800932 KF800922 -      | -       | -     | -     | Zhu et al. (2014)          |

(Continues)
| Species                     | Individual No. | Country      | Accession no. of GenBank | Cyt.b  | C-mos   | Rag-1 | References                        |
|-----------------------------|----------------|--------------|--------------------------|--------|---------|-------|-----------------------------------|
| Rhabdophis murudensis       | HT0788         | Malaysia     | LC325341                 | LC325787 | –     |       | This study                        |
| Rhabdophis nigrocinctus_1   | HT0253         | Thailand     | LC325307                 | LC325753 | LC326015 | –     | This study                        |
| Rhabdophis nigrocinctus_2   | HT0343         | Thailand     | LC325314                 | LC325760 | –     |       | This study                        |
| Rhabdophis nigrocinctus_3   | HT0845         | China        | LC325298                 | –        | –     |       | This study                        |
| Rhabdophis nuchalis_1       | HT0701         | China        | LC325333                 | LC325779 | LC326022 | –     | This study                        |
| Rhabdophis nuchalis_2       | HT0803         | Thailand     | LC325352                 | LC325798 | –     |       | This study                        |
| Rhabdophis nuchalis_3       | HT0807         | China        | LC325353                 | LC325799 | LC326031 | –     | This study                        |
| Rhabdophis nuchalis_4       | HT0854         | China        | LC325301                 | LC325748 | –     |       | This study                        |
| Rhabdophis nuchalis_5       | SICAU090001    | China        | KF800925                 | KF800935 | –     |       | Zhu et al. (2014)                 |
| Rhabdophis pentasupralabialis_1 | HT0699     | China        | LC325331                 | LC325777 | –     |       | This study                        |
| Rhabdophis pentasupralabialis_2 | HT0700    | China        | LC325332                 | LC325778 | LC326021 | –     | This study                        |
| Rhabdophis pentasupralabialis_3 | HT0808    | China        | LC325354                 | LC325800 | –     |       | This study                        |
| Rhabdophis subminiatus_1    | HT0267         | Laos         | LC325308                 | LC325754 | –     |       | This study                        |
| Rhabdophis subminiatus_2    | HT0344         | Thailand     | LC325315                 | LC325761 | –     |       | This study                        |
| Rhabdophis subminiatus_3    | HT0345         | Thailand     | LC325316                 | LC325762 | –     |       | This study                        |
| Rhabdophis swinhonis_1      | HT0021         | Taiwan       | LC325303                 | LC325749 | –     |       | This study                        |
| Rhabdophis swinhonis_2      | HT0717         | Taiwan       | LC325334                 | LC325780 | LC326023 | –     | This study                        |
| Rhabdophis swinhonis*_3     | HT0716         | Taiwan       | LC325334                 | –        | –     |       | This study                        |
| Rhabdophis swinhonis*_4     | HT0718         | Taiwan       | LC325334                 | –        | –     |       | This study                        |
| Rhabdophis swinhonis*_5     | HT0719         | Taiwan       | LC325334                 | –        | –     |       | This study                        |
| Rhabdophis tigrinus_1       | HT0098         | Japan        | LC325305                 | LC325751 | LC326013 | –     | This study                        |
| Rhabdophis tigrinus_2       | HT0177         | Japan        | LC325306                 | LC325752 | LC326014 | –     | This study                        |
| Sibynophis subpunctatus     | RAP0491        | Sri Lanka    | KC347471                 | KC347411 | KC347449 |       | Pyron, Kandambi et al. (2013)    |
| (Out group)                 |                |              |                          |         |        |       |                                   |
| Sinonatrix aequifasciata_1  | HT0678         | Vietnam      | LC325326                 | LC325772 | –     |       | This study                        |
| Sinonatrix aequifasciata_2  | HT0681         | Vietnam      | LC325329                 | LC325775 | –     |       | This study                        |
| Sinonatrix aequifasciata_3  | GP357          | China        | JQ687430                 | JQ687440 | –     |       | Guo et al. (2012)                 |
| Sinonatrix annularis        | GP889          | China        | JQ687431                 | JQ687449 | KJ685604 |       | Guo et al. (2012, 2014)          |
| Sinonatrix percarinata      | GP956          | China        | JQ687433                 | JQ687451 | KJ685607 |       | Guo et al. (2012, 2014)          |
| Storeria dekayi             | CAS:HERP:196039 | USA        | AF471050                 | AF471154 | –     |       | Lawson et al. (2005)             |
| Thamnophis butleri          | –              | USA          | AF402923                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis couchii          | –              | USA          | AF402936                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis cyrtopsis        | –              | USA          | AF402924                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis elegans          | –              | USA          | AF402925                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis godmani          | –              | Mexico       | AF420135                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis marcianus        | –              | USA          | AF402926                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis ordinoides       | –              | USA          | AF402927                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis proximus         | –              | –            | AF402928                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis radix            | –              | USA          | AF402934                 | –        | –     |       | Alfaro and Arnold (2001)          |
| Thamnophis sirtalis_1       | –              | –            | AF402929                 | –        | –     |       | Alfaro and Arnold (2001)          |

(Continues)
| Species                        | Individual No. | Country     | Accession no. of GenBank | References                  |
|-------------------------------|----------------|-------------|--------------------------|-----------------------------|
| *Thamnophis sirtalis* _2      | –              | –           | AF402930                 | Alfaro and Arnold (2001)    |
| *Trachischium monticola*      | GP1487         | China       | JQ687428, JQ687435, KJ685570 | Guo et al. (2012, 2014)    |
| *Xenochrophis aspermimus* _1  | HT0797         | Sri Lanka   | LC325346, LC325792, –    | This study                  |
| *Xenochrophis aspermimus* _2  | –              | Sri Lanka   | KC347480, KC347414, KC347451 | Pyron, Kandambi et al. (2013) |
| *Xenochrophis flavipunctatus* | HT0682         | Vietnam     | LC325330, LC325776, –    | This study                  |
| *Xenochrophis maculatus*      | HT0720         | Malaysia    | LC325335, LC325781, LC326024 | This study                  |
| *Xenochrophis piscator* _1    | HT0347         | Thailand    | LC325317, LC325763, LC326018 | This study                  |
| *Xenochrophis piscator* _2    | HT0371         | Vietnam     | LC325318, LC325764, –    | This study                  |
| *Xenochrophis piscator* _3    | HT0796         | Sri Lanka   | LC325345, LC325791, –    | This study                  |
| *Xenochrophis trianguligerus* | HT0795         | Malaysia    | LC325344, LC325790, LC326029 | This study                  |
| *Xenochrophis vittatus* _1    | HT0615         | Indonesia   | LC325322, LC325768, LC326019 | This study                  |
| *Xenochrophis vittatus* _2    | HT0527         | Indonesia   | LC325322, –, –           | This study                  |