Morphological and genetic verification of *Ovophis tonkinensis* (Bourret, 1934) in Hong Kong

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**Abstract.** Hong Kong lies in the putative ranges of two *Ovophis* species: *O. makazayazaya* (Sichuan and Yunnan Provinces, Taiwan, and northern Vietnam) and *O. tonkinensis* (Guangxi, Guangdong, and Hainan Provinces, and northern Vietnam). It is unclear which *Ovophis* species is/are present in Hong Kong. Previous studies identified *O. tonkinensis* using morphology, but without molecular data. In this study, we verified the presence of *O. tonkinensis* in Hong Kong using molecular (four mitochondrial DNA loci) and morphological data of two specimens. The clade that is currently identified as *Ovophis tonkinensis* has a large geographic range and relatively deep divergences, hinting that its taxonomy requires further work.

**Key words.** Hong Kong, *Ovophis tonkinensis*, *Ovophis makazayazaya*, reptile, snake.

**Introduction.** The Asian pitviper genus *Ovophis* has had a complicated taxonomic history. Previously, *Ovophis* was believed to contain three species (*O. chaseni* (Smith, 1931), *O. okinavensis* (Boulenger, 1892), *O. monticola* (Gunther, 1864; 4 subspecies)) (McDiarmid et al. 1999). However, Malhotra and Thorpe (2004) removed the first two species from this genus so that *Ovophis* referred only to the *O. monticola* species complex. Malhotra et al. (2011) delineated five species in *Ovophis* by elevating some subspecies to the species level based on molecular and morphological datasets.

In Hong Kong (and southern China), it is unclear which *Ovophis* species is/are present. Previous morphological work in Hong Kong identified the species in Hong Kong to be *Trimeresurus/Ovophis monticola* (Romer and Bogadek 1983, Zhao et al. 1998, Li et al. 2002), *T. monticola monticola* (Karsen et al. 1998). Malhotra et al. (2011) included four *Ovophis* specimens from Hong Kong (BMNH 1983 281–284) in their morphometric analyses. These four specimens grouped with *O. tonkinensis* specimens from northern Vietnam and southern China. Studies in areas close to Hong Kong also confirmed the presence of *O. tonkinensis*, including Hainan Province (morphology: David (1995), David (2001), Wang et al. (2009); molecular: Malhotra et al. (2011)), western Guangdong Province (morphology: Zhang et al. (2011)), and western Guangxi Province (morphology: Kadoorie Farm & Botanical Garden (2003)). In this study, we use molecular and morphological data from two specimens to clarify the identity of the *Ovophis* species in Hong Kong, and comment on the status of *O. tonkinensis*.

**Materials and Methods**

**Samples.**—Two *Ovophis* specimens were captured on the hillside at Kadoorie Farm and Botanic Garden (KFBG) Tai Po, Hong Kong (22.43121°N, 114.11822°E) by the staff of KFBG in February 2011. General anaesthesia was administered prior to collecting blood and scale clippings. All the procedures were previously approved by the KFBG ethical committee and were performed by the resident veterinary surgeon. Snake 1 (MVP1) was male while snake 2 (MVP2) was female. The total length of each individual was 41 cm and 56 cm, respectively. Upon recovery, the snakes were released back to the site of capture.
Morphology.—Scalation was used to assign the specimens to species. *Ovophis makanayayazaya* and *O. tonkinensis* can be distinguished from other *Ovophis* species based on the relative size of the supralabial scales—these two species have a larger 4th supralabial, while other species have a larger 3rd supralabial (David & Tong 1997). The state of the subcaudal scales and number of ventral scales can be used to distinguish *O. makanayayazaya* and *O. tonkinensis*—*O. makanayayazaya* has paired subcaudal scales and 131–155 ventral scales, while *O. tonkinensis* has predominantly single subcaudal scales and 127–145 ventral scales (Malhotra et al. 2011).

Molecular.—DNA was extracted from blood and/or scales using the Qiagen DNeasy mericon Food Kit. Following the primer sets and thermocycler conditions used in Malhotra et al. (2011), four mitochondrial DNA loci were amplified: 12S rRNA (12S), 16S rRNA (16S), cytochrome b (cytb), and NADH dehydrogenase subunit 4 (ND4). Polymerase chain reactions (PCR) in a final volume of 25 μL containing 2 μL genomic DNA, PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, and 0.01% gelatin), 0.4 mmol/L of each deoxynucleotide triphosphate (dNTPs), 0.5ul forward and reverse primers and 2.0 U DNA Taq polymerase (Applied Biosystems, Foster City, CA). PCR amplifications were carried out in an Applied Biosystems 9700 automated thermal cycler. PCR products were visualised on a 2% agarose gel and purified using the Qiagen QIAquick PCR purification kit. Products were sequenced in both directions using PCR primers on an Applied Biosystems 3130 Genetic Analyzer using BigDye v3.1.

Corresponding sequences for each specimen and locus were visually inspected, assembled in Geneious Pro v5.3.6, and aligned to data from Malhotra et al. (2011) using MUSCLE (Edgar 2004). Alignments for cytb and ND4 were checked for stop codons by translating nucleotides to amino acids. Sequences were submitted to GenBank (KX710068- KX710075).

Phylogenetic analyses were performed on five datasets: 12S, 16S, cytb, ND4, and the concatenated dataset. Phylogenetic trees were inferred using Maximum Likelihood (ML) in RAxML v8.2.4 (Stamatakis 2014) and Bayesian Inference (BI) in MrBayes v3.2.5 (Ronquist et al. 2012). The loci coding for gene products (cytb and ND4) were partitioned by codon position, while the concatenated dataset was partitioned by locus and codon position. For ML, we performed the analysis that combines the ML search with bootstrapping. The GTR+G model of nucleotide substitution was used for each partition in the 100 ML tree searches and 1000 bootstrap replicates. For BI analyses, models of nucleotide substitution were chosen within MrBayes using the reversible jumping model choice (nst=mixed) with both rate variation and invariable sites (rates=invgamma). Two BI searches with random starting trees were run and compared using four Markov Chain Monte Carlo (MCMC) chains, each chain run for 10 million generations, and sampled every 1000th generation. Stationarity was evaluated by plotting the log-likelihood scores in Tracer v.1.5 (Rambaut et al., 2013), and data from the first 2 million generations were discarded before combining to building a consensus tree. We evaluated sequence divergence for each locus by calculating the uncorrected p-distances using MEGA v7.0.16 (Kumar et al. 2016). Calculations of uncorrected p-distances were made with partial deletion of missing data and a site coverage cutoff of 95%.

![Figure 1](image-url) Scale characters of the *Ovophis* specimen (MPV1) collected from Kadoorie Farm and Botanic Garden, Hong Kong. Both specimens in this study had the same characters, which are unique to identify *O. tonkinensis*. A) 4th supralabial larger than 3rd supralabial, and B) unpaired subcaudal scales.
Figure 2 Maximum Likelihood (ML) phylogeny of the concatenated *Ovophis* dataset—12S rRNA (12S), 16S rRNA (16S), cytochrome b (*cytb*), and NADH dehydrogenase subunit 4 (*ND4*). Support values of major nodes are indicated (ML bootstrap/ Bayesian Inference posterior probability). Asterisks (*) indicate ML bootstrap >90 and Bayesian Inference posterior probability >0.9. Both *Ovophis* specimens from Hong Kong (MPV1, MPV2), indicated by arrows and larger, bold font, were nested within the *O. tonkinensis* clade. Clade names and taxonomy follow Malhotra et al. (2011).
Results and Discussion

Our study is the first to include DNA sequence data from Hong Kong Ovophis specimens. Both morphological and molecular data confer, suggesting that the two Ovophis specimens in this study are O. tonkinensis. The deep divergences within O. tonkinensis, combined with the large geographic range (China including Hong Kong to central Vietnam), suggests that O. tonkinensis is a species complex that requires further study.

Scale characters matched those of O. tonkinensis—both specimens had 4th supralabials larger than 3rd supralabials (Figure 1A) and unpaired subcaudal scales (Figure 1B). Ventral scale counts largely overlap between O. makazayazay (131–155 scales) and O. tonkinensis (127–145 scales) and were not diagnostic for Hong Kong specimens (MPV1=136, MPV2=131).

Phylogenetic analyses of individual loci (not shown) had poor resolution and did not resemble the consensus tree of Malhotra et al. (2011). Analysing the concatenated dataset, the topologies of the inferred phylogenetic trees were identical when using ML and BI (only slight differences in branch lengths and support values) (Figure 2). This tree was identical to that of Malhotra et al. (2011). Both Hong Kong specimens are genetically highly similar (sequence divergence for the concatenated dataset: 0–1.2%) and were nested in O. tonkinensis (Clade B of Malhotra et al. (2011)) with strong support (ML bootstrap=82, BI posterior probability=1.0). The O. tonkinensis clade (including the Hong Kong specimens) had medium to high levels of intraspecific sequence divergence (maximum sequence divergence: ND4=12.3%, cyt b=18.5%, 12S=4.3%, 16S=2.0%) compared to other species/clades (maximum sequence divergence: ND4=0–14.8%, cyt b=0–9.9%, 12S=0–5.6%, 16S=0–4.2%). The sequence divergences between O. tonkinensis individuals are of the same magnitude as between individuals of different species.

Based on the available sequences, our phylogenetic analyses show that Ovophis tonkinensis contains three deeply diverged lineages: (1) twelve individuals from China and Vietnam, (2) two individuals from Hong Kong, and (3) one individual from northern Vietnam. The relatively deep divergences and large geographic range of O. tonkinensis indicate that what we currently identify as O. tonkinensis may be a species complex. The availability of Ovophis specimens from southern China is currently limited. Unfortunately, the Chinese specimens in the main clade (previously part of the private collection of Mr. Andreas Gumprecht) do not have any additional locality information beyond “China” (Malhotra et al. 2011, M.-O. Rödel and F. Tillack Pers. Comm. 2 Dec 2016). Based on scant records with morphological data (Zhang et al. 2011, Kadoorie Farm & Botanical Garden 2003) we expect O. tonkinensis is present in Guangdong and Guangxi Provinces. Future studies should focus on southern China to determine species presence based on both morphological and molecular data. In addition to delineating the ranges of O. makazayazay and O. tonkinensis in the region, these data will help us understand whether O. tonkinensis is a single genetically diverse species, or a species complex.

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References


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