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Two Genetically Distinct yet Morphologically Indistinct *Bungarus* Species (Squamata, Elapidae) in Hong Kong

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Abstract: Kraits (genus *Bungarus*) are a group of highly venomous elapid snakes found across tropical Asia. Although they are notorious for causing many, often deadly, envenomations throughout the continent, their taxonomy and species nomenclature is subject to regular revisions. The boundaries demarcating *B. candidus*, *B. multicinctus*, and *B. wanghaotingi*, in Southern China and Northern Myanmar are particularly obscure. Despite the extensively overlapping distributions for the three species, only *B. multicinctus* has been documented in Hong Kong. Following genetic analyses, we suggest that both *B. multincinctus* and *B. wanghaotingi* occur in Hong Kong. However, our morphological examinations are overall inconclusive in distinguishing the two species. With potential interspecific antivenin differences, the new identification of *B. wanghaotingi* in Hong Kong is of important medical interest. We recommend further ecological, taxonomic, and behavioural studies be conducted to properly distinguish *B. multincinctus* and *B. wanghaotingi* in the region.

Key words: *Bungarus*; Hong Kong; Krait; Taxonomy; Genetic; Morphology

INTRODUCTION

Two species of kraits (Genus *Bungarus*) are known to inhabit the Hong Kong Special Administrative Region; the many-banded and banded kraits, *Bungarus multicinctus* and *B. fasciatus*, respectively (Karsen et al., 1998). While *B. fasciatus* is easily identifiable by its black and yellow bands, the white and black bands exhibited by *B. multicinctus* closely resemble those of other congeners with overlapping geographic distributions (Xie et al.,

2018; Chen et al., 2021). Beyond Hong Kong, *B. multicinctus* has been believed to be widely distributed across Myanmar, Laos, Thailand, Vietnam, as well as Southern China (Shan et al., 2016; Uetz and Hošek, 2017). Overlapping in distribution is a closely related congener (Tan and Tan, 2015), *B. candidus*, which is mainly restricted to Southeast Asia (Nguyen et al., 2017; Uetz and Hošek, 2017).

In a recent study, Xie et al. (2018) genetically and morphologically re-identified several specimens of *B. multicinctus* collected from Southern and Southwestern China as *B. candidus*, including one from Hong Kong. Since then, however, Chen et al. (2021) suggested that *B. candidus* records in Southern and

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Southwestern China (Xie et al., 2018) and *B. multicinctus* records in Thailand, Vietnam, and Laos (Uetz and Hošek, 2017) be revised to represent *B. wanghaotingi*. This latter species is known to occur in Myanmar and Southwestern China, and is closely related to *B. candidus* and *B. multicinctus* (Leviton et al., 2003; Uetz and Hošek, 2017; Chen et al., 2021). Together, the three species are suggested to form a complex distinct from other congeners (Kuch, 2007; Chen et al., 2021).

Debates surrounding the discrimination of *B. multicinctus* as distinct from *B. candidus* and other congeners, such as India's *B. caeruleus*, are at least as old as the 19th century (Blyth, 1860; Boulenger, 1896; Stejneger, 1907). The identification of black-and-white kraits in Hong Kong as *B. multicinctus* was accordingly founded on assumed differences in geographic distributions and the number of bands displayed by these congeners (Stejneger, 1907; Herklots, 1933). Yet this assumption pre-dates and potentially disregards the initial description of *B. wanghaotingi* as a species (Pope, 1928). Therefore, with no molecular work conducted on Hong Kong's *B. multicinctus* prior to the inclusion of a single specimen in the study by Xie et al. (2018) initially identifying it as *B. candidus*, the tenability of this distinction is worth exploring through phylogenetic and further morphological analyses. In this study we apply such methods to (1) determine whether *B. wanghaotingi* or *B. candidus* occur in Hong Kong, and (2) if *B. multicinctus* is present as well.

MATERIALS AND METHODS

We obtained specimens previously assumed to be *B. multicinctus* from across Hong Kong through donations by Kadoorie Farm and Botanic Garden (KFBG) and the Hong Kong Agriculture, Fisheries, and Conservation Department (AFCD). These included tissue samples as well as whole individuals that were caught locally, had arrived at KFBG via the Wild Snake Rescue Project (www.kfbg.org/en/wild-snake-rescue-project), and which would

have been dead on arrival or euthanized. We also collected dead specimens opportunistically throughout Hong Kong. We received 11 tissue samples, 10 of which were donated by KFBG, and one collected opportunistically. We received seven whole individuals, of which four were donated by KFBG, one by AFCD, and two collected opportunistically. The whole individuals were mostly collected from across Hong Kong's New Territories, including Hoi Ha, Shing Mun, Tai Po Kau, Kau Lung Hang Village, and Pat Sin Leng, with the exception of one collected in Shek O, Hong Kong Island. These 18 total specimens range in their initial date of collection from September 1998 to June 2021 (see Appendix). We deposited all whole individual specimens at Lingnan University's Natural History Collection.

Morphological examinations

We measured morphological traits potentially distinguishing *B. multicinctus*, *B. candidus* and *B. wanghaotingi* according to Chen et al. (2021; Table 1). These included number of ventral scales, number of white bands on the body, head and neck patterns in adults, head and neck patterns in juveniles, ventral body surface pattern, ventral tail surface pattern, and snout-vent length.

DNA extractions and phylogenetic analyses

We extracted DNA from the samples using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols. We measured DNA yields using a Nanodrop One Microvolume UV-Vis Spectrophotometer. We amplified a 355 bp region of Cytochrome B (*Cyt b*) using primers H15149B (5'-CCCCTCAGAAATGATA TTTGTCTCA-3') and L14841 (5'-ATCCAAC ATCTCAGCATGATGAAA-3') designed by Kocher et al. (1989), and previously applied by Wong et al. (2004) to identify confiscated snake meat samples in Hong Kong. We performed polymerase chain reaction in 20 µl reactions containing 1 ng DNA, 1× Biotechrabbit™ Lyo Hot Start PCR Master Mix and 1 mM of each of the primers. We

TABLE 1. Morphological traits suggested to identify three, black-and-white, closely related *Bungarus* species overlapping in geographic distributions.

Species	Ventral scales	White bands on body	Head and necks in adults	Head and necks in juveniles	Ventral surface of body	Ventral surface of tail	Reference
<i>B. candidus</i>	209–224	18–26	Temporal area and lateral neck stained white	Temporal area and lateral neck creamily white	Immaculate white	Broad dark cross bands	Chen et al. 2021
<i>B. wanghaotingi</i>	209–259	18–33	Uniform black	Light brown	Immaculate white	A row of small light brown dots on middle	Chen et al. 2021; Yang and Rao 2008; Pope 1928
<i>B. multicinctus</i>	196–236	31–50	Uniform black	Scales on lateral neck dim white edged	White, with dense brown pigments	Dense black bands and patches	Chen et al. 2021

programmed initial template denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 30 s; 50°C for 20 s and 72°C for 45 s, and then a final elongation step at 72°C for 5 min. We visually confirmed PCR amplifications on a 2% agarose gel using 5 µl of PCR product. We purified PCR products using a QIAGEN QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and submitted purified reactions to The Centre for PanorOmic Sciences (CPOS) at the Li Ka Shing Faculty of Medicine, The University of Hong Kong, for sequencing.

We visually assessed sequence chromatograms, generated consensus sequences for all our samples, and used the global alignment algorithm to align all consensus sequences in Geneious 10.0.2 (Kearse et al., 2012; Biomatters Ltd., Auckland, New Zealand). We obtained *Cyt b* consensus sequence entries for *B. candidus*, *B. wanghaotingi*, *B. multicinctus* and *B. suzhenae* from the GenBank database. We chose to include *B. suzhenae* because it was recently described as a species, its distribution spanning from Northern Myanmar to Southwestern China overlaps with that of *B. multicinctus* and *B. wanghaotingi*, and it forms a group sister to the complex formed by the other three species (Chen et al., 2021).

We used the Neighbour-Joining method (Saitou and Nei, 1987; Dantas et al., 2021) to

construct a phylogenetic tree including our sequences obtained in Hong Kong together with the Genbank entries, as well as a voucher specimen of *Naja atra* as the outgroup. We generated a consensus tree inferred from 1000 replicates (Felsenstein, 1985) using the Maximum Likelihood method together with the Tamura 3-parameter model (Tamura, 1992) to obtain bootstrap values above a 70% cutoff as support for the tree. We estimated evolutionary distances between sequences using the *p*-distance method, which estimates the proportion of nucleotide sites differing between sequences, and conducted all phylogenetic analyses in MEGA X (Kumar et al., 2018).

RESULTS

Phylogenetic analyses

We successfully obtained consensus sequences from 16 specimens, while two of our samples (KFBG23 and AFCD01) from whole individuals failed to yield adequate sequences. Phylogenetically, two samples grouped with *B. multicinctus*, and 14 with *B. wanghaotingi* (Fig. 1). None of our samples grouped with either *B. candidus*, or the recently described *B. suzhenae* (Fig. 1). The evolutionary distances between our two *B. multicinctus* samples and 14 *B. wanghaotingi* samples ranged from 2.6% to 2.9% for KFBG12, and from 3.3% to 3.6%

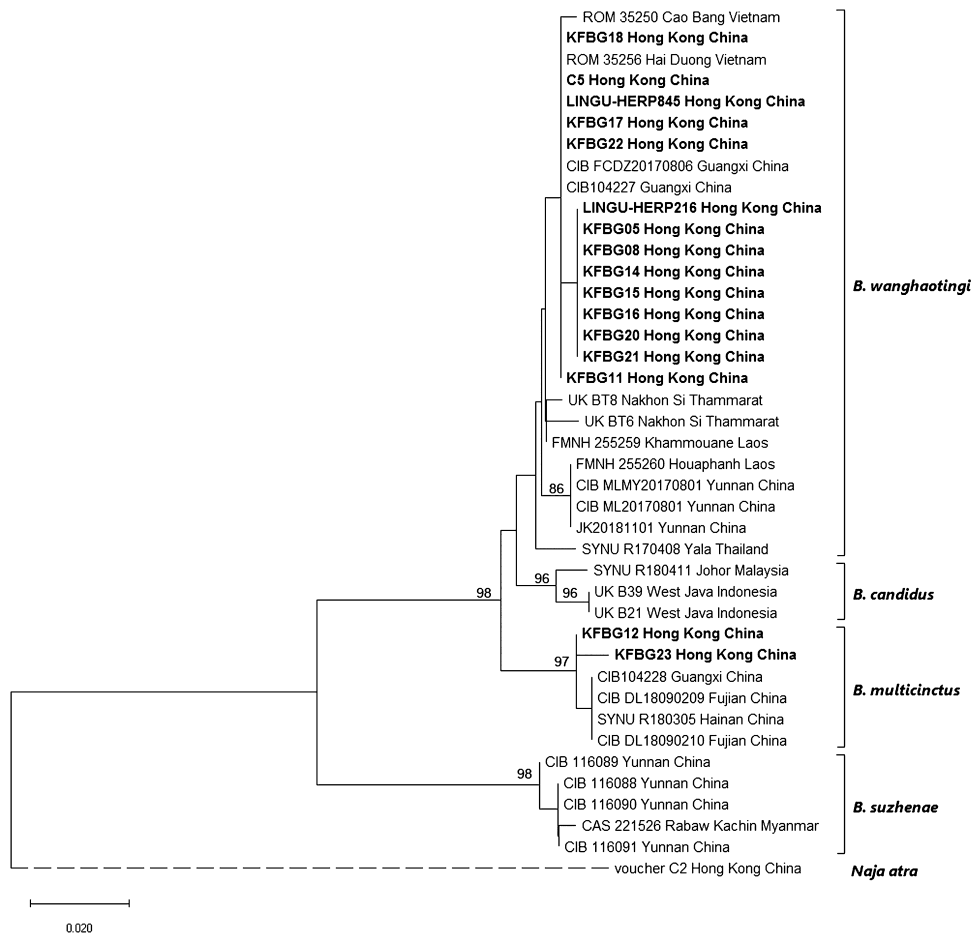


FIG. 1. Phylogenetic tree generated with the neighbor-joining method for black-and-white krait specimens collected in Hong Kong (bolded), together with genetic sequences for four potentially matching *Bungarus* species obtained from the GenBank database. Numbers at nodes are bootstrap values above a cutoff value of 70% and extracted from the maximum likelihood consensus tree. A voucher specimen of *Naja atra* collected in Hong Kong serves as the outgroup (dashed line), while the scale represents branch length.

for KFBG23. Distances between our two *B. multicinctus* samples and the *B. candidus* sequences from GenBank was 2.9% for KFBG12 and 3.6% for KFBG23. Distances between our 14 *B. wanghaotingi* samples and the GenBank *B. candidus* sequences ranged from 2.3% to 2.6%.

Morphological examinations

We examined the morphological traits of seven individuals, including two females, one male, and four juveniles for which we could

not determine the sex (Table 2). Overall, specimens displayed more traits unique to *B. multicinctus*, as described by Chen et al. (2021), despite three individuals phylogenetically grouping with *B. wanghaotingi*. Of those latter three, all morphological traits measured for KFBG11 and LINGU-HERP216, and all but one for LINGU-HERP845, matched those attributed to *B. multicinctus* (Tables 1 and 2). Of the specimens phylogenetically grouping with *B. multicinctus*, four and three morphological traits matched those of this species for

TABLE 2. Morphological traits and genetic identification results for whole black-and-white krait specimens collected in Hong Kong. Traits closely matching those described for *B. candidus*, *B. wanghaotingi* and *B. multicinctus* are respectively indicated by an asterisk (*), dagger (†), and double dagger (‡).

Specimen	Genetic identification	Sex	SVL	Ventral scales	White bands on body	Head and necks	Ventral surface of body	Ventral surface of tail
KFBG13	—	F	45.4	207‡	35‡	Scales on lateral neck dim white edged‡	Immaculate white*†	Black bands and patches‡
KFBG12	<i>B. multicinctus</i>	—	31.8	214*†‡	34‡	Temporal area and lateral neck creamily white*	Immaculate white*†	Black bands and patches‡
KFBG11	<i>B. wanghaotingi</i>	M	73	218*†‡	34‡	Uniform black†‡	White with scattered brown pigments‡	Black bands and patches‡
LINGU-HERP216	<i>B. wanghaotingi</i>	F	82	206‡	39‡	Uniform black†‡	White with scattered brown pigments‡	Black bands and patches‡
LINGU-HERP845	<i>B. wanghaotingi</i>	—	37.5	213*†‡	46‡	Scales on lateral neck dim white edged‡	Immaculate white*†	Black bands and patches‡
KFBG23	<i>B. multicinctus</i>	—	47.2	206‡	36‡	Light brown†	White with scattered brown pigments‡	Black bands and patches‡
AFCD01	—	—	39.4	211*†‡	41‡	Light brown†	Immaculate white*†	Black bands and patches†

KFBG23 and KFBG12, respectively. KFBG11 and LINGU-HERP216, our only adult-sized whole individual specimens, displayed similar morphological traits, all consistent with *B. multicinctus*, yet both phylogenetically grouped with *B. wanghaotingi*. The number of ventral scales for KFBG11 matches all three species, and the uniform black head and necks in both KFBG11 and LINGU-HERP216 agrees with *B. wanghaotingi* and *B. multicinctus*.

All specimens displayed a number of white bands on the body exceeding the maximum of 33 previously described for *B. wanghaotingi*, and within the range given for *B. multicinctus*. The ventral surface on the tails of all specimens were covered in black bands and patches (Fig. 2); another trait attributed to *B. multicinctus*. The number of ventral scales matched those of *B. multicinctus* alone for three specimens (KFBG13, KFBG23 and LINGU-HERP216). Head neck patterns exhibited by specimens were varied (Fig. 2). Two speci-

mens phylogenetically grouping with *B. wanghaotingi* displayed uniform black head and necks, a trait attributed to adults of both *B. wanghaotingi* and *B. multicinctus*. The ventral surface of the body was white with scattered brown pigments, a trait attributed to *B. multicinctus*, for two specimens phylogenetically grouping with *B. wanghaotingi* (KFBG11 and LINGU-HERP216), and one grouping with *B. multicinctus* (KFBG23). Yet the ventral surface of the body was immaculate white, a trait attributed to *B. candidus* and *B. wanghaotingi*, for the other four specimens, one of which phylogenetically grouped with *B. multicinctus* (KFBG 12), and another with *B. wanghaotingi* (LINGU-HERP845; Fig. 2).

DISCUSSION

We genetically identified both *B. multicinctus* and *B. wanghaotingi* among specimens collected in Hong Kong. We further found

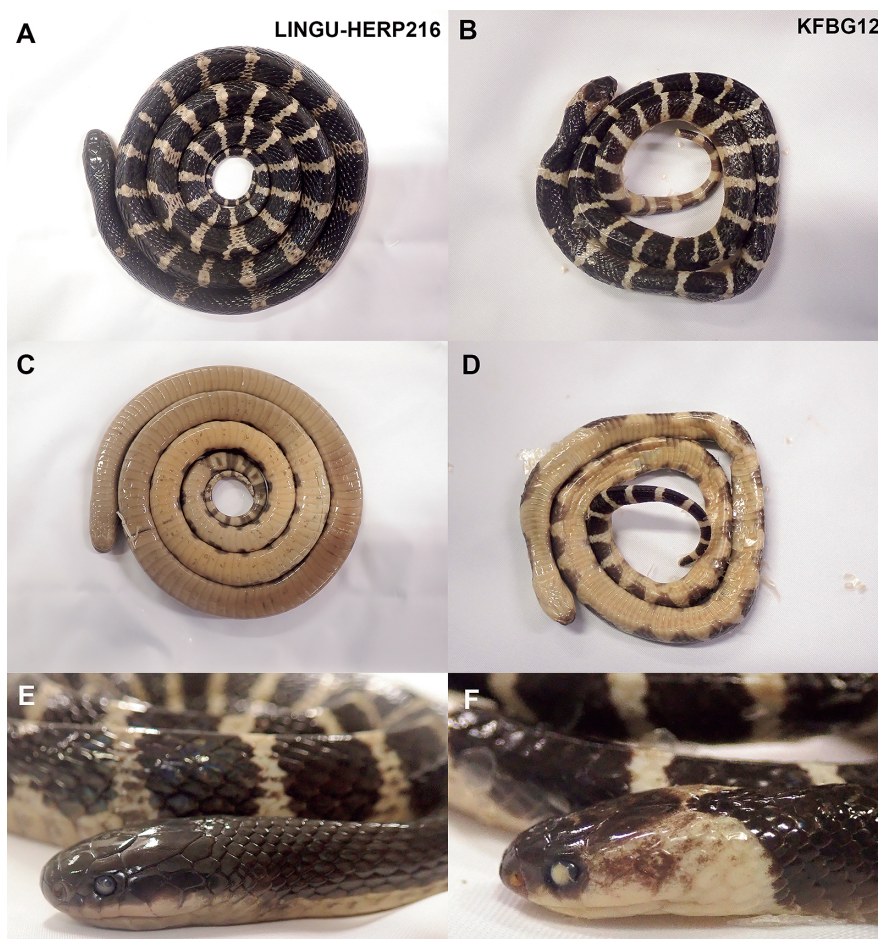


FIG. 2. Morphology of two *Bungarus* specimens, LINGU-HERP216 (left column) and KFBG12 (right column) collected from Hong Kong, that phylogenetically grouped with *B. wanghaotingi* and *B. multicinctus*, respectively. A and B: dorsal view. C and D: ventral view. E and F: lateral view of head and neck.

these specimens to display morphological traits mostly matching those described for *B. multicinctus* and, to a lesser extent, *B. wanghaotingi* (Chen et al., 2021). Following the recent re-classification of Chinese *B. candidus* specimens as *B. wanghaotingi* (Xie et al., 2018; Chen et al., 2021), we found no genetic and little morphological evidence of *B. candidus* occurring in Hong Kong.

While *B. multicinctus* was already known to occur in Hong Kong, the new identification of *B. wanghaotingi* in the region we present in this study is of potential medical significance.

As members of the elapid family, *Bungarus* species carry extremely potent venom with the capacity to be lethal to humans (Tan and Ponnudurai, 1990; Yanoshita et al., 2006; Chanhom et al., 2009). Envenomation by snake bite from these snakes is a serious public health concern in Asia, and often occur at night when victims are sleeping due to the nocturnal hunting behaviour of these snakes (Alirol et al., 2010; Warrell, 2010). In Hong Kong, victims of bites by *B. multicinctus*, which produces some of the most potent snake venoms in the world (Gopalkrishnakone and

Chou, 1992; Yin et al., 2020), are typically administered an anti-venom specifically designed for the species (Mong et al., 2017). Likewise, patients bitten by *B. candidus*, which also involves severe and potentially lethal consequences (Trinh et al., 2010; Chaisakul et al., 2017; Charoenpitakchai et al., 2018), are treated with species-specific anti-venoms in Thailand (Leeprasert and Kaojarern, 2007).

While venoms within this genus do share similarities, those of *B. multicinctus* and *B. candidus* differ in some biological properties (Kuch et al., 2003; Yanoshita et al., 2006; Tan and Tan, 2015), such as their respective contents of alkaline phosphomonoesterase, L-amino acid oxidase, acetylcholinesterase and hyaluronidase (Tan and Ponnudurai, 1990). As a lesser known and seldom studied species, the properties of *B. wanghaotingi* venom should be further studied to understand possible differences with those of its congeners. Given the shorter evolutionary distance between *B. wanghaotingi* and *B. candidus* (Figure 1; Chen et al., 2021), anti-venom designed for *B. candidus* venom could be more effective for *B. wanghaotingi* bites than for those by *B. multicinctus*. In the case of Hong Kong, an awareness of nuances specifically between *B. wanghaotingi* and *B. multicinctus* venoms could provide better medical support for envenomed victims (Laxme et al., 2019). Of critical importance, treating patients bitten by one species using the anti-venom for another, despite being closely related, could prove to be ineffective (Laxme et al., 2019). This has been documented for one *B. candidus* bite victim in Thailand unsuccessfully being administered anti-venom derived from a Taiwanese *B. multicinctus* (Warrell et al., 1983; Tan and Tan, 2015). Further complicating the issue are potential intraspecific differences in the effects of *Bungarus* venoms depending on geographic locality (Chaisakul et al., 2017; Charoenpitakchai et al., 2018; Laxme et al., 2019; Hia et al., 2020; Oh et al., 2021).

These issues frame venomics research as a priority from the perspective of public health, which will be instrumental for ensuring

adequate treatment of future victims of bites by these two species. However, our genetic evidence for the occurrence of both *B. wanghaotingi* and *B. multicinctus* in Hong Kong, as well as the possible shortcomings for morphological distinctions (Chen et al., 2021), require further work to be substantiated. Although we received only one adult male specimen, hemipenial morphology is suggested to be a distinctive feature for *B. wanghaotingi* and *B. multicinctus* (Chen et al., 2021), and should be a focal point for future studies. Accordingly, the lack of distinctive morphological traits among our specimens could be due to the fact that many were juveniles.

Beyond its medical importance, *B. multicinctus* carries economic value in Asia, where it is often traded for consumption (Wong et al., 2009; Ji et al., 2020), and is known to have been heavily exported from mainland China (Zhou and Jiang, 2005). Distinguishing *B. wanghaotingi* and *B. multicinctus* in Hong Kong, as well as in mainland China, would be crucial for adequately monitoring the trade and conservation statuses of these two species. The consumption of snakes in Hong Kong sees many additional non-native species regularly imported (Wong et al., 2009), raising the possibility of accidental releases. This could have resulted in a relatively recent arrival of *B. wanghaotingi* in Hong Kong, and explain the lapse in early descriptions of the species alongside *B. multicinctus* records (Stejneger, 1907; Herklots, 1933). Conversely, the opposite scenario is also possible, with *B. wanghaotingi* already present and *B. multicinctus* introduced.

Yet evolutionary processes resulting in the distributional overlap of black-and-white krait species deserve further exploration (Kuch et al., 2005). Identifying the geological time and geographic location of speciation would be a critical step in elucidating points of divergence within the *Bungarus candidus/multicinctus/wanghaotingi* complex. Currently our study provides foundational groundwork for future molecular, morphological and ecological investigations on a larger regional scale. The inconsistency in morphological features distin-

guishing the two species in our results highlight the need for larger sample sizes and additional methods for resolving the taxonomy of this species complex. Given the limited number of specimens from Southern China examined by Chen et al. (2021), future work identifying and differentiating the morphology, evolutionary history and distribution of *Bungarus* species across the region is necessary. This would be instrumental in decisively identifying each species, potentially supporting our current phylogenetic results. Ultimately, distinguishing *B. wanghaotingi* and *B. multicinctus* within Hong Kong should be prioritized for economic, public health, and conservation reasons.

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APPENDIX

Genetic species identification, collection dates, locality information, sample types and GenBank Accession numbers of Hong Kong specimens used in this study.

Voucher	Genetic species ID	Locality	District	Region	Collection date	Sample type	GenBank Accession No
C2	<i>Naja atra</i>	Unknown	Unknown	New Territories	2010/10/19	Head	OL963577
C5	<i>Bungarus wanghaotingi</i>	Kam Tin	Yuen Long	New Territories	2004/11/19	Tissue	OL963578
LINGU-HERP216	<i>Bungarus wanghaotingi</i>	Shing Mun	Tsuen Wan	New Territories	21/05/2020	Whole snake	OL963592
LINGU-HERP845	<i>Bungarus wanghaotingi</i>	Pat Sin Leng	Tai Po	New Territories	10/11/2020	Whole snake	OL963593
KFBG05	<i>Bungarus wanghaotingi</i>	Chai Wan	Eastern	Hong Kong Island	23/03/2019	Tissue	OL963579
KFBG08	<i>Bungarus wanghaotingi</i>	Chai Wan	Eastern	Hong Kong Island	18/07/2019	Tissue	OL963580
KFBG11	<i>Bungarus wanghaotingi</i>	KFBG	Tai Po	New Territories	09/09/2010	Whole snake	OL963581
KFBG12	<i>Bungarus multicinctus</i>	Tai Po Kau Headland	Tai Po	New Territories	09/09/2011	Whole snake	OL963582
KFBG13	No sequence data	Kau Lung Hang Village	Tai Po District	New Territories	21/09/1998	Whole snake	No sequence data
KFBG14	<i>Bungarus wanghaotingi</i>	Cheung Sha Wan	Sham Shui Po	Kowloon	03/08/2012	Tissue	OL963583
KFBG15	<i>Bungarus wanghaotingi</i>	Happy Valley	Wan Chai	Hong Kong Island	30/10/2018	Tissue	OL963584
KFBG16	<i>Bungarus wanghaotingi</i>	Tseung Kwan O	Sai Kung	New Territories	21/06/2018	Tissue	OL963585
KFBG17	<i>Bungarus wanghaotingi</i>	Tai Po	Tai Po	New Territories	18/06/2012	Tissue	OL963586
KFBG18	<i>Bungarus wanghaotingi</i>	Lok Ma Chau	North	New Territories	04/01/2018	Tissue	OL963587
KFBG20	<i>Bungarus wanghaotingi</i>	Tseung Kwan O	Sai Kung	New Territories	15/08/2019	Tissue	OL963588
KFBG21	<i>Bungarus wanghaotingi</i>	Tsing Yi	Kwai Tsing	New Territories	27/09/2013	Tissue	OL963589
KFBG22	<i>Bungarus wanghaotingi</i>	Tai Po	Tai Po	New Territories	30/10/2018	Tissue	OL963590
KFBG23	<i>Bungarus multicinctus</i>	Hoi Ha	Sai Kung	New Territories	06/2021	Whole snake	OL963591
AFCD01	No sequence data	Shek O	Southern	Hong Kong Island	12/11/2003	Whole snake	No sequence data