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Intraspecific Diversity of Short-legged Horned Toad (*Boulenophrys brachykolos*) in Hong Kong: Identification of a Distinct Conservation Unit on Lantau Island

Yik-Hei SUNG[1,2], Franco Ka-Wah LEUNG[3], Henry Man-Ho CHAN[2], and Jonathan J. FONG[2,*]

[1]*School of Health, Sciences and Society, University of Suffolk, Ipswich, UK*
[2]*Division of Science, Wu Jieh Yee School of Interdisciplinary Studies, Lingnan University, Hong Kong SAR, CHINA*
[3]*College of Science and Engineering, James Cook University, Townsville, Queensland 4814, AUSTRALIA*

Abstract: Delimiting species is crucial, especially for threatened and understudied organisms, because unresolved taxonomy can result in unrecognized species diversity and misallocated conservation efforts. In this study, we used an integrative taxonomic approach, incorporating genetic, morphological, and acoustic analyses, to clarify the phylogenetic relationship of the endangered Short-legged Horned Toad (*Boulenophrys brachykolos*) from South China. We focused on individuals from three geographically separated regions in Hong Kong, one on the mainland (Kowloon) and two on islands (Lantau Island and Hong Kong Island). Our genetic data identified Lantau Island individuals to be an evolutionarily distinct lineage, with a minimum genetic difference of 2.1% from other populations. Acoustic analysis revealed that male *B. brachykolos* from Lantau Island produce calls with similar frequency, but significantly higher pulse and note rate. However, morphological comparisons did not reveal significant differences among the three regions. To conclude, the *B. brachykolos* population from Lantau Island may represent a case of speciation in progress, and in the interim, should be treated as a distinct conservation unit.

Key words: Amphibian; Frog; Megophryidae; Phylogeny; South China

Introduction

Conservation fails when taxonomic units (e.g., species) are improperly delimited (Mace, 2004). This problem is often associated with cryptic species, two or more species classified as a single species due to their similar morphology (Bickford et al., 2007). When species diversity is underestimated, conservation efforts may be misallocated, resulting in irreversible biodiversity loss (Wang et al., 2018; Yan et al., 2018). As such, taxonomic research is crucial for conservation, especially for groups with underestimated diversity such as amphibians (Elmer et al., 2013).

Asian horned toads of the genus

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*Corresponding author.
E-mail address: jonfong@ln.edu.hk

Boulenophrys Fei, Ye, and Jiang, 2016, formerly *Xenophrys* and *Megophrys* (Dubois et al., 2021), represent a cryptic species complex with largely underestimated species diversity (Chen et al., 2017; Mahony et al., 2017). For example, Liu et al. (2018) used a genetic analysis to reveal as many as four undescribed species in China. To date, among the 72 currently recognized *Boulenophrys* species, 30 species were described in the last five years (2020–2024) (Frost, 2024), hinting that more *Boulenophrys* species await discovery and description.

The Short-legged Horned Toad (*B. brachykolos*), the only known *Boulenophrys* species in Hong Kong, is listed as endangered on the IUCN Red List (IUCN, 2024). It is distributed across Hong Kong, including the mainland (Kowloon) and the two largest islands (Lantau and Hong Kong) (Chan et al., 2005; Karsen et al., 1998). This species was once believed to be endemic to Hong Kong, but was recently discovered in the adjacent city of Shenzhen, China (Liu et al., 2018). In recent studies of *Boulenophrys*, many newly described species are considered "micro-endemic", having very small ranges (Liu et al., 2018; Wang et al., 2019). For example, *B. insularis* is endemic to Nao'ao Island, a small (128 km^2) offshore island in Guangdong Province, China (Wang et al., 2017). Compared to Nao'ao Island, Lantau Island is similar in size (147 km^2) and distance to the mainland (both <5km). A comprehensive taxonomic study of *B. brachykolos* is needed to investigate the phylogenetic relationship across different geographically separated populations across Hong Kong and neighboring Guangdong Province, China. In this study, we use an integrative taxonomic approach, analyzing genetic, morphological and acoustic data, to investigate the diversity within the currently recognized *B. brachykolos*. Specifically, we clarify the relationships of individuals among three geographically distinct regions including both Lantau and Hong Kong Islands, and the mainland (Kowloon).

MATERIALS AND METHODS

Sample collection

We collected 19 specimens of *B. brachykolos* from three major regions in Hong Kong: Lantau Island (n=9), Hong Kong Island (n=6), and Kowloon (n=4) (Fig. 1). Muscle or liver samples were collected from all specimens and preserved in 95% ethanol for genetic analysis. Whole specimens were fixed in 10% formalin and stored in 70% ethanol. These specimens were deposited in the Natural History Collection at Lingnan University (Table 1).

Genetic analysis

We extracted DNA from tissue samples using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol. We amplified and sequenced three mitochondrial DNA loci: 12S ribosomal RNA (12S), 16S ribosomal RNA (16S), and cytochrome c oxidase subunit I (COI). We used the primers and thermocycler conditions described in Lyu et al. (2023). We purified polymerase chain reaction (PCR) products using a commercial kit (Bioneer AccuPrep PCR Purification Kit) and sequenced on an ABI 3730xl System at Macrogen (Seoul, Korea). All sequences were deposited in GenBank (Table 1). New sequences of *B. brachykolos* from this study and sequences of other *Boulenophrys* spp. downloaded from GenBank (in particular, a *B. brachykolos* sample from Shenzhen) were included in the phylogenetic analysis (Table S1 available on Figshare at https://doi.org/10.6084/m9.figshare.30102871.v1). Sequences of *Leptobrachella laui* were used as an outgroup. We used MUSCLE (Edgar, 2004) in Geneious R11 to align the sequences (Kearse et al., 2012). Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). ML analyses were done in RAxML v.8, with 1,000 bootstrap replicates using the GTR+Optimization of substitution rates + GAMMA model of rate heterogeneity (alpha parameter was estimated) (Stamatakis, 2014). Pairwise sequence divergences between clades were calculated using

TABLE 1. Localities, voucher information and GenBank accession number of *Boulenophrys brachykolos* specimens newly used in this study.

Region	Collection locality	Voucher	Genbank accession no.		
			COI	16S	12S
Lantau Isl.	Shek Pik catchwater	LINGU-HERP-247	PQ764746	PQ764764	PQ776237
		LINGU-HERP-952	PQ764748	PQ764766	PQ776239
	Tei Tong Tsai	LINGU-HERP-981	PQ764752	PQ764770	PQ776243
		LINGU-HERP-999	PQ764753	PQ764771	PQ776244
		LINGU-HERP-1000	PQ764754	PQ764772	PQ776245
		LINGU-HERP-1001	PQ764755	PQ764773	PQ776246
	Cheung Sha	LINGU-HERP-1002	PQ764756	PQ764774	PQ776247
		LINGU-HERP-1003	PQ764757	PQ764775	PQ776248
		LINGU-HERP-1004	-	-	-
Hong Kong Isl.	Tai Tam	LINGU-HERP-75	PQ764744	PQ764762	PQ776235
		LINGU-HERP-93	PQ764745	PQ764763	PQ776236
		LINGU-HERP-813	PQ764747	PQ764765	PQ776238
	Pok Fu Lam	LINGU-HERP-963	PQ764749	PQ764767	PQ776240
		LINGU-HERP-967	PQ764750	PQ764768	PQ776241
		LINGU-HERP-971	PQ764751	PQ764769	PQ776242
Kowloon	Kowloon Peak	LINGU-HERP-1118	PQ764758	PQ764776	PQ776249
		LINGU-HERP-1119	PQ764759	PQ764777	PQ776250
		LINGU-HERP-1120	PQ764760	PQ764778	PQ776251
		LINGU-HERP-1121	PQ764761	PQ764779	PQ776252

uncorrected p-distances in MEGA X (Kumar et al., 2018). The analysis was performed searching for best scoring ML tree with a rapid bootstrap analysis. BI analyses were run using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) by running four chains for 500,000 generations, sampling every 1,000 generations through the Markov Chain Monte Carlo (MCMC) method. The first 25% of the sampled values were discarded as burn-in. A consensus tree of the most probable topology was generated by integrating overall possible trees and model parameters.

Morphological analysis

Following Yan et al. (2018), we measured and compared 17 morphological measurements between the three regions (Lantau and Hong Kong Islands, and Kowloon). We used digital calipers to measure to the nearest 0.1 mm, and we followed the terminology and definitions in Lyu et al. (2023): snout-vent length (SVL); head length from the tip of the snout to rear of jaws (HDL); head width at commissure of jaws (HDW); snout length from the tip of the snout to anterior corner of eye (SNT); horizontal diameter of exposed portion of eyeball (ED); interorbital distance, closest distance between the upper eyelids (IOD); internasal distance, distance between center of the nares (IND); upper eyelid width measured as greatest width of the upper eyelid (UEW); nostril-eye distance, measured from the posterior margin of the nares to the anterior corner of the eye (EN); horizontal diameter of tympanum (TD); distance from anterior edge of tympanum to posterior corner of eye (TED); tibia length (TIB); manus length from the tip of third digit to proximal edge of outer palmar tubercle (ML); length of lower arm and hand (LAHL); foot length from the tip of fourth toe to proximal edge of the inner metatarsal tubercle (FL); and hindlimb length from the tip of fourth toe to vent (HLL). We compared the difference in morphological measurements among the three populations of toads (Lantau and Hong Kong Islands, and Kowloon) using Kruskal-Wallis test (Zar, 1999) with separate tests for each sex to account for any sexually dimorphic characters.

Additionally, we recorded ten morphological characteristics that are distinctive features of other *Boulenophrys* species, including the presence of the following characters: tubercles on upper eyelids; vomerine teeth and ridge; notched tongue; lateral fringe on toes; canthus rostralis; conical spine on temporal region; subarticular tubercles at each finger and toe base. The last two characters are if the heels overlap when limbs flexed and degree of webbing on toes.

Acoustic analysis

We recorded the advertisement call of male *B. brachykolos* from the three regions (Lantau and Hong Kong Islands, and Kowloon) between April 2018 and May 2024. All recordings were made between 19:00–23:00 h. When a calling male was detected, a directional audio recorder (DR-40, Tascam) was placed approximately 0.1–1 m from the individual to record the call with a sampling frequency of 44,100 Hz and 16-bit resolution. After each recording, we measured air temperature using a thermometer.

All recordings were saved in WAV format and then analyzed using Raven Pro 1.5 (Cornell Lab of Ornithology, Ithaca, USA). We followed the definition of call properties described in Messenger et al. (2019): “call” is a continuous vocalization with a pause less than one second; “note” is the smallest non-split syllable. For each recording, we measured temporal acoustic parameters, including note duration, note rate, pulse rate and call duration using Raven’s waveform display (Köhler et al., 2017). We also measured the dominant frequency (DF/Hz) using the spectrogram slice view (1,024 points fast-Fourier transform [FFT], Hann’s sampling window, 50% overlap; 63.1 Hz grid-spacing; a frequency resolution, 56 Hz). Advertisement calls were detected and selected artificially using Raven’s Band Limited Energy Detector (Max Frequency: 5,000 Hz, Min Frequency: 1,500 Hz, Max Duration: 0.2 s, Min Duration: 0.01 s, Min Separation: 0.01 s). Background noise was estimated and used to find sections of signal that exceeded a user-specified signal-to-noise ratio threshold in a specific frequency band, during a specific time. Selections of ‘disturbed’ call note with extreme background noise were unselected manually to avoid disturbance in spectral and temporal analysis. We compared the differences in acoustic parameters between the calls of the three regions (Lantau and Hong Kong Islands, and Kowloon) using ANCOVA with temperature as a covariate (Zar, 1999). If a significant difference was found, pairwise comparisons were done using Tukey HSD tests.

RESULTS

Genetic analysis

We focus on the analyses of the concatenated dataset of mtDNA loci (12S, 16S, and COI) because they show the highest resolution. For the BI analysis, *B. brachykolos* is monophyletic, with the existence of two major clades—Hong Kong Island/Kowloon/Shenzhen and Lantau (Clades L and M, respectively in Fig. 2). In contrast, the ML analysis did not support the monophyly of *B. brachykolos* with full support, but with the same two major clades of Hong Kong Island/Kowloon/Shenzhen and Lantau (Clade L and M, respectively in Fig. S1 available on Figshare at https://doi.org/10.6084/m9.figshare.30102871.v1). The differences between the topologies of BI and ML trees are likely due to their different evolutionary models and sampling approaches adopted. BI integrates over all possible trees and model parameters to produce a consensus tree, while ML identifies a single best tree whose topology represents the highest likelihood. Despite these differences, both methods reveal a clear

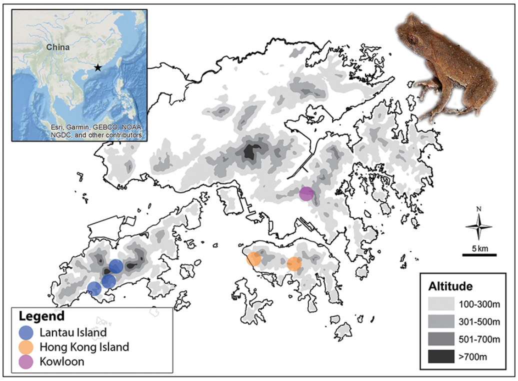

FIG. 1. Collection localities of the *Boulenophrys brachykolos* from three regions in Hong Kong (Lantau Island, Hong Kong Island, Kowloon).

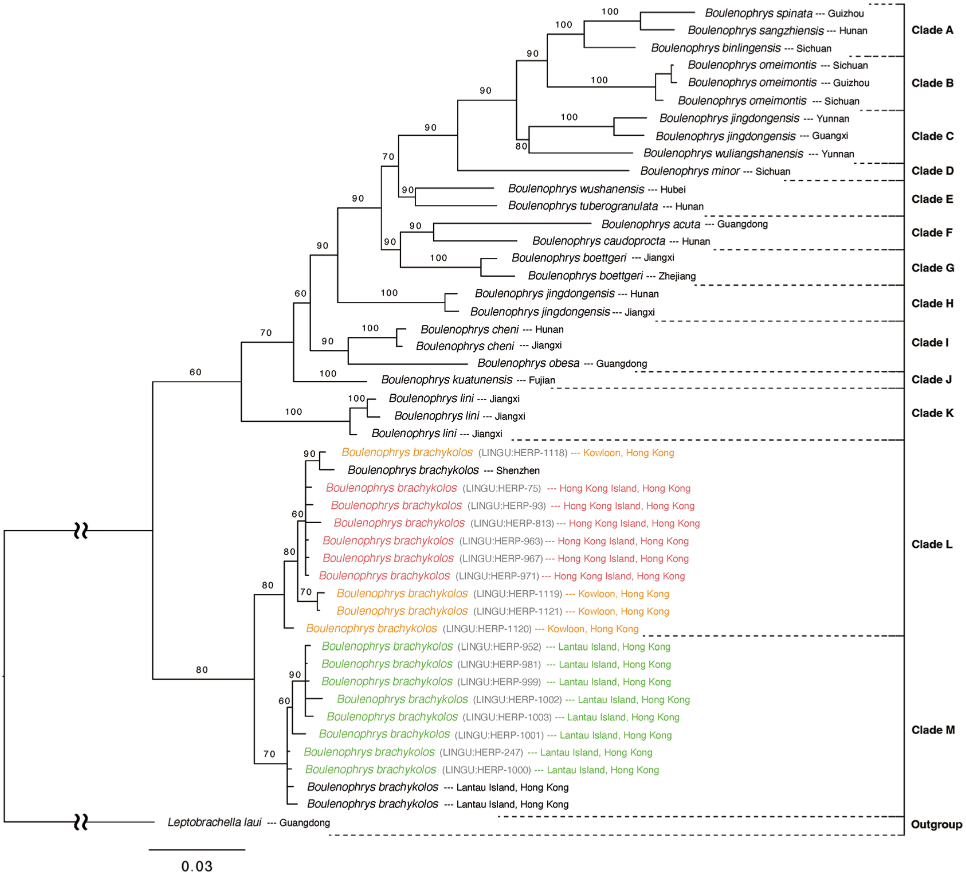

FIG. 2. Phylogenetic tree of *Boulenophrys* spp. inferred from the concatenated dataset of mitochondrial loci (12S, 16S, and COI) constructed using Bayesian inference. Clade L represents the populations of *B. brachykolos* from Hong Kong Island, Kowloon, and Shenzhen, and Clade M represents the *B. brachykolos* populations from Lantau Island. Colored sequences indicate the new sequences generated in this study while sequences in black were obtained from GenBank.

genetic disparity between the Lantau population of *B. brachykolos* compared to populations found in the rest of its range. The mean pairwise distances between the two major clades of *B. brachykolos* are 2.1% for both BI (Table 2) and ML analyses (Table S2 available on Figshare at https://doi.org/10.6084/m9.figshare.30102871.v1).

Morphological analysis

We found significant differences among the three regions in IOD and ML; these measurements, however, overlapped among populations (Table 3). All other measurements were statistically similar with overlapping ranges. Morphological characteristics were identical among *B. brachykolos* specimens from the three regions (Table 4).

Acoustic analysis

We recorded and analyzed a total of 38 advertisement calls (10 Lantau Island, 15 Hong Kong Island, and 13 Kowloon). We found that the calls were generally distinct between Lantau Island and Hong Kong Island, with Kowloon occupying an intermediate position (Fig. 3). The dominant frequency of calls was similar among the three regions ($F_{2,39}$=3.09, P=0.057), while the call duration significantly differed among the three regions ($F_{2,39}$=5.54, P=0.008). Toads from Lantau Island have a shorter call duration than those from Hong Kong Island (P=0.005), while the calls from Kowloon were intermediate to the other two regions (P>0.25). For pulse rate, there was a significant difference among the three regions ($F_{2,39}$=12.86, P<0.001), with Lantau Island toads having higher pulse rates compared to Hong Kong Island (P=0.006) or Kowloon (P<0.001) toads. For note duration, there was a significant difference among the three regions ($F_{2,39}$=7.43, P=0.002); Lantau Island toads having shorter note duration than those from Hong Kong Island (P<0.001); Kowloon toads were similar to those from the other two regions (P>0.19). For note rate, there was a significant difference among the three regions ($F_{2,39}$=10.79, P<0.001), with Lantau Island toads having higher note rates than those from Hong Kong Island (P<0.001) or Kowloon (P<0.001).

DISCUSSION

Identifying species boundaries is particularly challenging within cryptic species complexes (Balakrishnan, 2005), especially in amphibians, where some groups exhibit conservative morphological evolution (Cherty et al., 1978). While closely related species may appear morphologically similar, molecular and acoustic analyses often prove more effective in identifying cryptic species (Stuart et al., 2006). In this study, we employed an integrative taxonomic approach, incorporating genetic, morphological, and acoustic data, to elucidate the taxonomic relationships among three geographically distinct regions where *Boulenophrys brachykolos* is found (Hong Kong: Lantau and Hong Kong Islands, and Kowloon).

Our findings reveal that the Lantau individuals represent a genetically distinct evolutionary lineage, exhibiting a 2.1% genetic divergence from those on Hong Kong Island, Kowloon, and Shenzhen. This level of divergence is noteworthy given Lantau Island's relatively recent isolation from other landmasses during the early Holocene (Fyfe et al., 1999). Studies have shown that genetic divergence of 0.5–2% is the problematic range in species delimitation (Roux et al., 2016). The 2.1% divergence observed in our study suggests a considerable degree of reproductive isolation, representing a distinct evolutionary unit. It should be noted that ML and BI analyses of the data recovered different topologies (Fig. 2 and S1), which is likely due to the differences in evolutionary models and parameters used in the analyses, as well as a long-branch attraction. Although this raises some questions about the relationship within what is presently recognized as *B. brachykolos*, it does not affect the discovery of two evolutionarily distinct lineages in this species.

Furthermore, the Lantau individuals exhibit quantitative differences in acoustic traits com-

TABLE 2. Mean (bottom left of the table) and standard deviation (top right) of pairwise distance of concatenated mitochondrial loci (16S, 12S, and COI) between *Boulenophrys* spp. estimated using Bayesian inference. Clade M represents *B. brachykolos* individuals from Lantau Island and Clade L represents the *B. brachykolos* individuals from Hong Kong Island, Kowloon, and Shenzhen. The mean pairwise distance between the clade of *B. brachykolos* individuals from Lantau Island and *B. brachykolos* individuals from elsewhere is in bold font (comparison of Clades M and L).

	A	B	C	D	E	F	G	H	I	J	K	L	M	Outgroup
Clade A		0.015	0.015	0.022	0.020	0.023	0.021	0.024	0.024	0.024	0.024	0.027	0.027	0.117
Clade B	0.069		0.016	0.022	0.020	0.023	0.023	0.022	0.023	0.024	0.025	0.028	0.027	0.102
Clade C	0.074	0.074		0.021	0.019	0.022	0.021	0.023	0.022	0.024	0.023	0.026	0.025	0.102
Clade D	0.106	0.104	0.100		0.021	0.022	0.022	0.024	0.023	0.024	0.027	0.025	0.024	0.104
Clade E	0.096	0.095	0.093	0.099		0.015	0.014	0.014	0.015	0.015	0.017	0.021	0.020	0.096
Clade F	0.108	0.109	0.108	0.105	0.072		0.016	0.017	0.019	0.019	0.020	0.023	0.023	0.104
Clade G	0.101	0.108	0.103	0.101	0.064	0.075		0.017	0.016	0.017	0.019	0.023	0.023	0.102
Clade H	0.112	0.105	0.111	0.111	0.067	0.083	0.078		0.015	0.014	0.018	0.022	0.020	0.106
Clade I	0.114	0.111	0.109	0.110	0.073	0.092	0.078	0.069		0.013	0.015	0.021	0.020	0.098
Clade J	0.116	0.114	0.114	0.111	0.072	0.091	0.079	0.065	0.059		0.015	0.020	0.020	0.102
Clade K	0.113	0.117	0.109	0.127	0.079	0.093	0.086	0.082	0.072	0.067		0.020	0.021	0.097
Clade L	0.129	0.135	0.125	0.115	0.100	0.111	0.107	0.101	0.099	0.093	0.095		0.006	0.094
Clade M	0.129	0.130	0.123	0.111	0.096	0.112	0.107	0.096	0.097	0.093	0.100	**0.021**		0.095
Outgroup	0.376	0.357	0.358	0.360	0.355	0.368	0.367	0.369	0.361	0.361	0.356	0.353	0.355	

TABLE 3. Morphological measurements [mean±SD (min–max)] (in mm) and results (P-value) of Kruskal-Wallis tests for *Boulenophrys brachykolos* from Lantau Island, Hong Kong Island, and Kowloon. Descriptions of measurements are provided in the Methods. Significant differences (P<0.05) are highlighted in bold.

Measurements	Lantau Isl.	Hong Kong Isl.	Kowloon	P (male)	P (female)
SVL	40.2±5.3 (33.3–48.8)	34.7±2.4 (31.1–37.9)	37.4±4.9 (33.6–44.1)	0.288	0.108
HDL	11.9±1.4 (9.8–14.1)	11.6±0.7 (10.9–12.7)	12±1.1 (10.5–12.9)	0.450	0.128
HDW	14.8±1.8 (12.8–18.6)	13.5±0.6 (12.5–14.1)	14.2±1.2 (13.2–15.8)	0.734	0.105
SNT	4.6±0.4 (4.2–5.3)	4.9±0.4 (4.3–5.4)	5.5±0.7 (4.9–6.2)	0.090	0.127
ED	4.8±0.6 (3.8–5.6)	4.4±0.1 (4.2–4.6)	4.3±0.6 (4–5.2)	0.278	0.201
IOD	6.2±1.2 (4.3–7.8)	3.9±0.2 (3.6–4.1)	4.5±0.2 (4.2–4.7)	**0.031**	**0.049**
IND	4.4±0.8 (3.5–5.7)	4.6±0.2 (4.4–4.8)	4.5±0.3 (4.2–4.9)	0.072	0.368
UEW	4.1±0.6 (3–5.1)	3.9±0.5 (3.1–4.3)	4±0.7 (3.5–5)	0.580	0.288
EN	2.2±0.5 (1.2–2.6)	2.1±0.2 (1.9–2.5)	2.3±0.3 (2–2.5)	0.753	0.105
TD	3.3±0.5 (2.6–4)	2.7±0.2 (2.6–3)	3±0.4 (2.6–3.4)	0.391	0.105
TED	2.7±0.3 (2.3–3.2)	2.5±0.3 (2.2–3)	2.8±0.3 (2.5–3.3)	0.753	0.135
TIB	16.1±1.2 (14.2–17.6)	14.9±0.6 (14.2–15.5)	15.3±1.4 (14.4–17.4)	0.293	0.105
ML	9.3±0.6 (8.6–10.5)	8.6±0.3 (8.3–8.9)	10±0.5 (9.7–10.8)	**0.039**	0.105
LAHL	18.9±2.1 (16–21.9)	17.7±0.6 (17–18.4)	19.1±1.9 (17.3–21.7)	0.873	0.127
FL	14.1±1.2 (12.8–17)	12.7±0.7 (12–13.6)	14.1±1.5 (13–16.3)	0.516	0.127
HLL	54.7±5.8 (44.5–62.7)	44.9±13.6 (21–54)	51.5±6.6 (46.2–60.5)	0.253	0.127
TD/ED	0.7±0.1 (0.6–0.9)	0.6±0 (0.6–0.7)	0.7±0.1 (0.7–0.8)	0.446	0.248
TIB/SVL	0.4±0 (0.4–0.4)	0.4±0 (0.4–0.5)	0.4±0 (0.4–0.4)	0.450	0.346

TABLE 4. Morphological characteristics of *Boulenophrys brachykolos* from Lantau Island, Hong Kong Island, and Kowloon.

Morphological characteristics	Lantau Isl.	Hong Kong Isl.	Kowloon
Tubercles on upper eyelids	Present	Present	Present
Vomerine teeth	Absent	Absent	Absent
Vomerine ridge	Present	Present	Present
Tongue	Not notched	Not notched	Not notched
Overlapping of heels when limbs flexed	No overlap	No overlap	No overlap
Lateral fringe on toes	Absent	Absent	Absent
Degree of webbing on toes	Rudimentary	Rudimentary	Rudimentary
Canthus rostralis	Well developed	Well developed	Well developed
Conical spine on temporal region	Absent	Absent	Absent
Subarticular tubercles at each finger and toe base	Distinct	Distinct	Distinct

pared to the other two populations. Male frogs from Lantau produce calls with statistically higher pulse and note rate (while accounting for temperature as a covariate). These call differences provide a foundation for the potential evolution of reproductive isolation. It is important to acknowledge that temporal parameters of advertisement calls are often more variable among conspecific whereas dominant frequency tends to be more consistent within a species and varies between species, making it a more reliable indicator for species differentiation

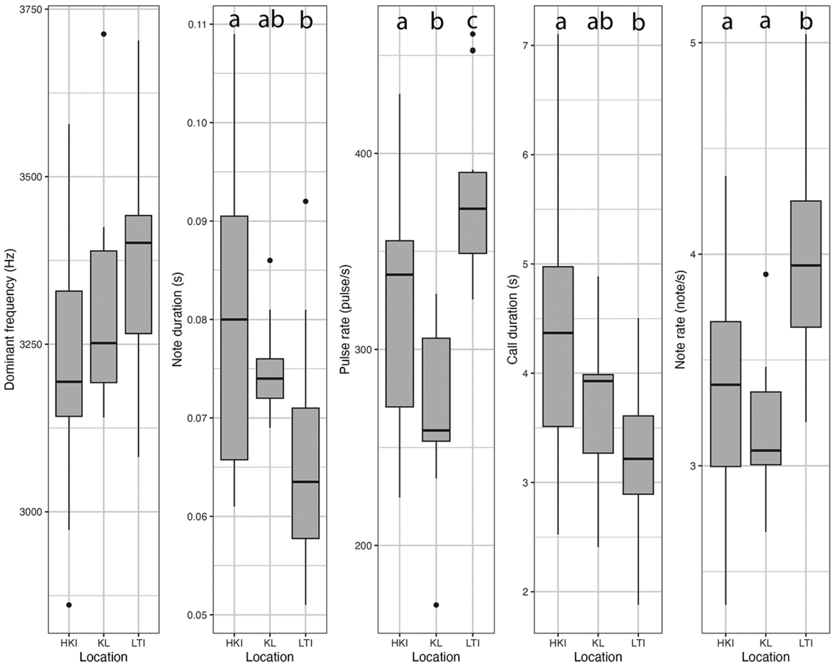

FIG. 3. Median dominant frequency, note duration, pulse rate, call duration, and note rate of male advertisement calls of *Boulenophrys brachykolos* recorded in Hong Kong Island (HKI), Kowloon (KL), and Lantau Island (LTI). Different letters (a, b, and c) indicate statistically significant differences between groups tested using ANCOVA (P<0.05).

(Gerhardt, 1994). Although our data show the Lantau toads exhibited a tendency to produce calls with slightly higher frequencies, the range of dominant frequency overlap among the three geographic regions (Fig. 3).

Despite the observed genetic and acoustic differences, we did not discover any distinct morphological features that differentiate the Lantau toads. Vieites et al. (2009) suggests that candidate species should have >3% (sometimes 1–2%) genetic divergences and either a qualitative difference in mating calls, or a diagnostic morphological characteristic. Following this guideline, we believe that the *B. brachykolos* individuals from Lantau Island represent a case of speciation in progress, rather than a candidate species. Nonetheless, we do not rule out the possibility that the Lantau lineage can be described as a new species if new data from more detailed external morphological or skeletal analyses indicate its distinctness.

Overall, our results support that the Lantau Island lineage is likely undergoing speciation, but has not yet reached the threshold for recognition as a distinct species. Our findings underscore the importance of treating the Lantau population as a separate conservation unit in future management efforts (Dufresnes et al., 2023).

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