

## Research Article

## Emerging non-native amphibians require immediate prevention management in a megacity of South China

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### Abstract

Biological invasion is one serious threat to global biodiversity, economics and sustainability. Under the era of globalization, emerging non-native species are still accelerating at an unprecedented rate. Identifying new field records of non-native species at early stages is critically important to develop effective prevention and management schemes. Here, we conducted field surveys and applied genetic analysis to identify new recordings of non-native amphibians in Shenzhen (a megacity of South China with enormous trade volume). We recorded a total of three non-native amphibians (*Ceratophrys ornata*, *Hoplobatrachus rugulosus* and *Eleutherodactylus planirostris*) in the field with two having establishment evidence (*H. rugulosus* and *E. planirostris*). Further ecological niche modeling based on climatic and habitat variables also detected a high habitat suitability of the two species with field establishment evidence and a low habitat suitability for the other three species (*C. ornata*, *Rana catesbeiana* and *Xenopus laevis*) lacking establishment evidence or field observation with only records in the market and database. We recommend more systematic surveys covering wider areas to investigate the establishment of non-native amphibians to stop their further invasions in China.

**Key words:** invasive species, amphibian, invasion risk, trade, establishment

### Introduction

Biological invasion is one of the most significant ecological and environmental problems posing significant threats to global biodiversity, economics, and human health (Sakai et al. 2001; Pimentel et al. 2005; Simberloff et al. 2013; Zhang et al. 2022). For example, nearly 33% of animal extinctions listed on

the 2017 International Union for Conservation of Nature (IUCN) Red List of Threatened Species are considered to be related to non-native species (Blackburn et al. 2019). A recent study showed that in the past few decades (1970–2017), biological invasions have caused global economic costs up to \$1,288 trillion, with average annual costs of \$26.8 billion, and this trend is still underestimated with no signs of slowing (Diagne et al. 2021). As it is very difficult to completely eradicate established feral populations (Simberloff 2003), detecting new non-native species at early invasion stages is critically important for implementing effective management strategies.

Amphibians are among the most threatened terrestrial vertebrates, and increasing evidence has shown that invasive non-native species is an important factor causing amphibian declines through competition, predation, and the spread of diseases, such as the notorious chytrid fungus (Kraus 2015). Although there are still data gaps on invasive amphibian impacts compared with other taxa such as birds and mammals, the available data show surprisingly high environmental and socioeconomic impacts of invasive amphibians (Measey et al. 2016). Under the current era of globalization, non-native species are still accelerating with the increased accessibility of new non-native species pools (Seebens et al. 2017). This is especially true for non-native amphibians that are not only intentionally introduced by trade and aquaculture but also unintentionally introduced by human activities, such as transportation and cargo stowaway, due to their close associations with humans (Kraus 2009). Consequently, identifying new field recordings of non-native traded amphibians in time is critically important to develop early prevention and mitigation schemes.

China is undergoing rapid economic development and is under great pressure of non-native species invasions (Ding et al. 2008; Liu et al. 2019). This is especially true for South China, which has been undergoing rapid social and economic development with profound urbanization and habitat disturbances, which are both facilitators of non-native species invasions by providing more introduction opportunities and vacant niches for establishment (McKinney 2008; Wang et al. 2022). For instance, South China has very large pet trade industries generating high propagule pressure by introducing a large number of non-native animals (Lee et al. 2004; Carrete and Tella 2008; Su et al. 2016). In addition, the religious wildlife release is very popular in South China (Chan 2006; Shiu and Stokes 2008; Du et al. 2023), which may unintentionally cause the invasion of released non-native animals (Liu et al. 2012; Su et al. 2016). In recent years, emerging non-native amphibians and reptiles, such as the greenhouse frog (*Eleutherodactylus planirostris* Cope, 1862), the Brown Anole (*Anolis sagrei* Duméril & Bibron, 1837), and the Green or Common Iguana (*Iguana iguana* Linnaeus, 1758), have been recorded in Hong Kong (China), Taiwan (China), and Guangdong Province (Norval et al. 2002; Lin et al. 2017; Wei

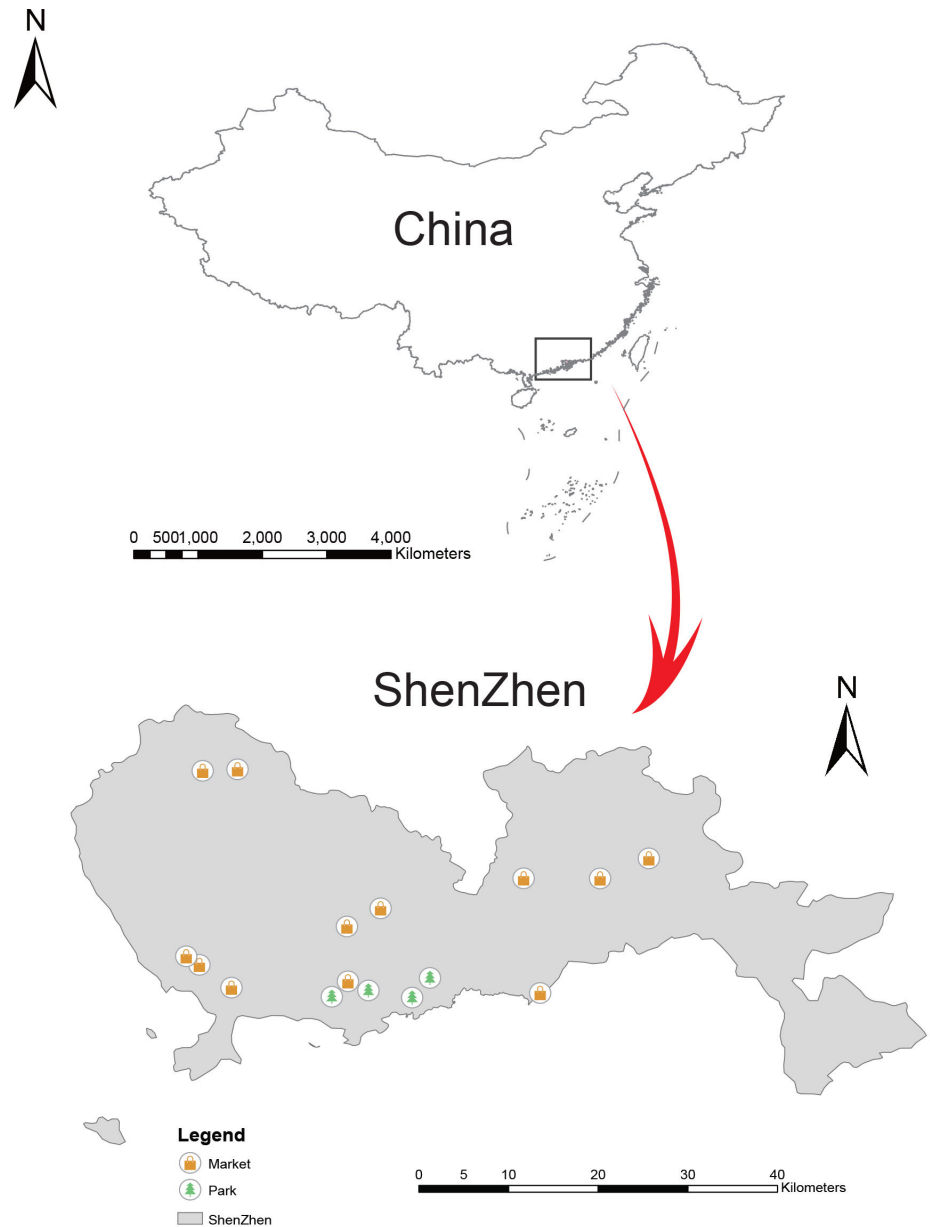
et al. 2017; Lee 2019). However, there are still limited field recordings of non-native amphibian species in representative areas with high biological invasion pressures.

Here, we focused on the city of Shenzhen, which is adjacent to Hong Kong with high trade volume and rapid economic development as a coastal window city of China (113°43′–114°38′E; 22°24′–22°52′N). In addition, as a major member of the Guangdong-Hong Kong-Macao Greater Bay Area, Shenzhen is a typical representative of urban development; thus, there is an urgent need to identify potential new invasions and develop early prevention strategies. We conducted field surveys in several major urban wetlands with frequent human activities, which can facilitate non-native amphibian anthropogenic introductions and invasions (Kraus 2009). Furthermore, we also applied ecological niche modeling (ENM) to evaluate the habitat suitability for establishment of the recorded non-native amphibians in the study area. Specifically, as our field survey also recorded the tiger frog (*Hoplobatrachus rugulosus* Wiegmann, 1834) that is under debate regarding their taxonomic identification on potential introduction from Thailand (Pansook et al. 2012; Yu et al. 2015; Lee et al. 2022), we therefore performed genetic analysis of *H. rugulosus* samples collected in the market and in wild populations to explore the species taxonomic identification and determine the potential introduced source of the species. Our study may provide timely suggestions for the scientific management planning of non-native amphibian prevention to help with the sustainable development.

## Materials and methods

### *Field surveys*

Field surveys were conducted in Shenzhen on September 2–13, 2021 during the frog breeding season (Tang et al. 2015; Song et al. 2022). A standard transect line survey (Scott et al. 1994) was conducted to collect the occurrence data of non-native amphibians in four wetland parks (Xiangmi Park, Lianhuashan Park, Litchi Park, and Shenzhen Honghu Park) (Figure 1). These wetlands are located in highly urbanized areas close to core commercial centers and dense residential areas, which can reflect the potential effect of human activities on non-native amphibian introductions (Lin et al. 2017, 2018). We divided the accessible shorelines of each wetland into 2–4 segments depending on the water area and accessibility. We randomly placed two to five 2 m × 500 m within each segment with half of the transect (i.e., 1 m) in the water and the other half on the shore (Liu et al. 2012). Two investigators simultaneously searched the frogs at night (19:00–22:00) using an electric torch while walking slowly and carefully. We recorded the species identity and the number of individuals at different life history stages, and habitat factors including the cover of submerged vegetation, water depth, area, and shading, that may influence amphibian occurrence along each transect.



**Figure 1.** Market (12 sites, yellow) and field survey (4 wetland parks, green) sites in this study.

### *Genetic analysis to confirm ambiguous species identity*

During our field survey, we collected two potentially non-native species that one is the possibly introduced *H. rugulosus*, and the other is a species suspected of the genus *Ceratophrys*. Among these, the species suspected of the genus *Ceratophrys* has never been recorded in the field of the city. *Hoplobatrachus rugulosus* has been suggested that populations from western, central, and eastern Thailand belong to different evolutionary branches from populations from northern and northeastern Thailand, Laos, Vietnam, and China (Chan-ard 2003; Pansook et al. 2012; Yu et al. 2015). We recorded these two species during field surveys, and collected samples to conduct genetic analyses to confirm their identity. We also collected 22 individuals from 12 food markets to better understand the association between *H. rugulosus* samples collected from the field and those sold as food (Figure 1).

We only collected one individual of *Ceratophrys* spp. in Xiangmi Park (Figure 1). For all individuals of the two species, the third toe of the right hind foot was clipped, and the tissue samples were preserved separately in 95% ethanol and stored at  $-20^{\circ}\text{C}$  in the laboratory (Supplementary material Appendix 1, Table S1).

Genomic DNA was extracted using the Universal Genomic DNA Kit (catalog no. CW2298M; Beijing, CoWinBiotech Co., Ltd, Beijing, China) following the manufacturer's standard instructions. We speculated that the sampled *H. rugulosus* might be introduced into China for aquaculture in recent years and that *Ceratophrys* spp. might be introduced through the pet trade (Deutsch et al. 2021). To further explore the potential geographic origin of the sampled individuals of *H. rugulosus* and infer the exact species of the *Ceratophrys* spp. sample, we amplified a 750 bp fragment of the mitochondrial cytochrome b gene (CYTB) for *H. rugulosus* (Kosuch et al. 2001) and amplified a 550 bp fragment of the 16S rRNA for *Ceratophrys* spp. (Appendix 1, Table S2). The PCR products were then separated by electrophoresis on 2% agarose gels. The resulting PCR products were directly sequenced by Beijing Liuhe Bgi Co., Ltd, Beijing, China with the same primers used for amplification. All genes obtained for each specimen sequence were compared with the available homologous sequences from GenBank (<https://www.ncbi.nlm.nih.gov/>) using the Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with the default parameters. All the obtained consensus sequences were aligned using the default parameters in Clustal X 1.81 (Thompson et al. 1997), and then MEGA 7.0 software was used to compare the sequences of each gene (Tamura et al. 2011). For *H. rugulosus*, the 11 CYTB published sequences (representing individuals from mainland China, Vietnam, Laos, and Thailand) from NCBI were downloaded, and there were a total of 24 sequences obtained in this study. We used three sequences of *Hoplobatrachus litoralis* (Hasan et al. 2012) and *Hoplobatrachus tigerinus* (Alam et al. 2008) as outgroups. A total of 38 sequences were tested for quality and were calibrated manually using SeqMan in the LASERGENE 7.0 software package (Ahmed et al. 2016) to generate consistent sequences. The phylogenetic relationships of *H. rugulosus* were reconstructed based on CYTB sequence data using maximum likelihood (ML) and Bayesian (BI) analyses. The "best" model of sequence evolution for the sequences was inferred using the Akaike Information Criterion (AIC) as implemented in PhyloSuite (Darriba et al. 2012; Zhang 2020). For the *Ceratophrys* spp. samples, 16S universal primers were used for amplification and sequencing. The sequence results were compared with the sequences reported in the GenBank database (BLAST: Basic Local Alignment Search Tool), and the species was identified by combining molecular evidence and morphological characteristics (Sung et al. 2014). All newly obtained sequences are deposited in GenBank.





*Hoplobatrachus rugulosus*



*Ceratophrys ornata*



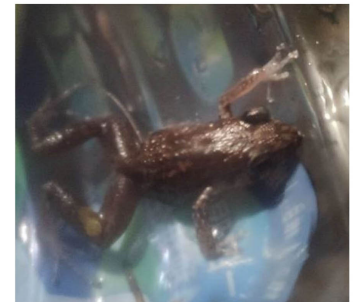
*Eleutherodactylus planirostris*



*Hoplobatrachus rugulosus*



*Ceratophrys ornata*



*Eleutherodactylus planirostris*

**Figure 2.** Non-native amphibian species recorded through field surveys in the city of Shenzhen, Guangdong Province, China.

### Ecological niche modeling

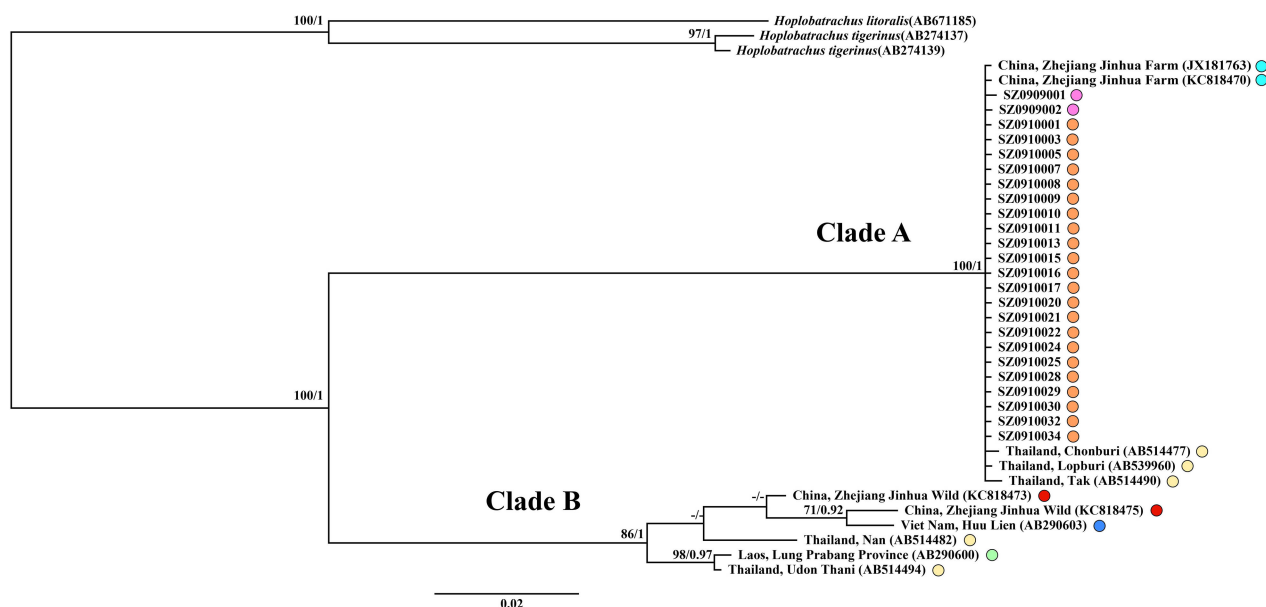
As some species (e.g., *Ceratophrys ornata* Bell, 1843) we recorded in the field lacked establishment evidence and some species (e.g., *Rana catesbeiana* Shaw, 1802; *Xenopus laevis* Daudin, 1802) with literature recording were not recorded during this survey, we therefore conducted further ecological niche modeling (ENM, Appendix 2, Text S1) analyses at a spatial resolution of 0.5 arc-minutes to evaluate the habitat suitability for the establishment of all recorded non-native amphibians in our field survey or only reported in the market or database.

### Results

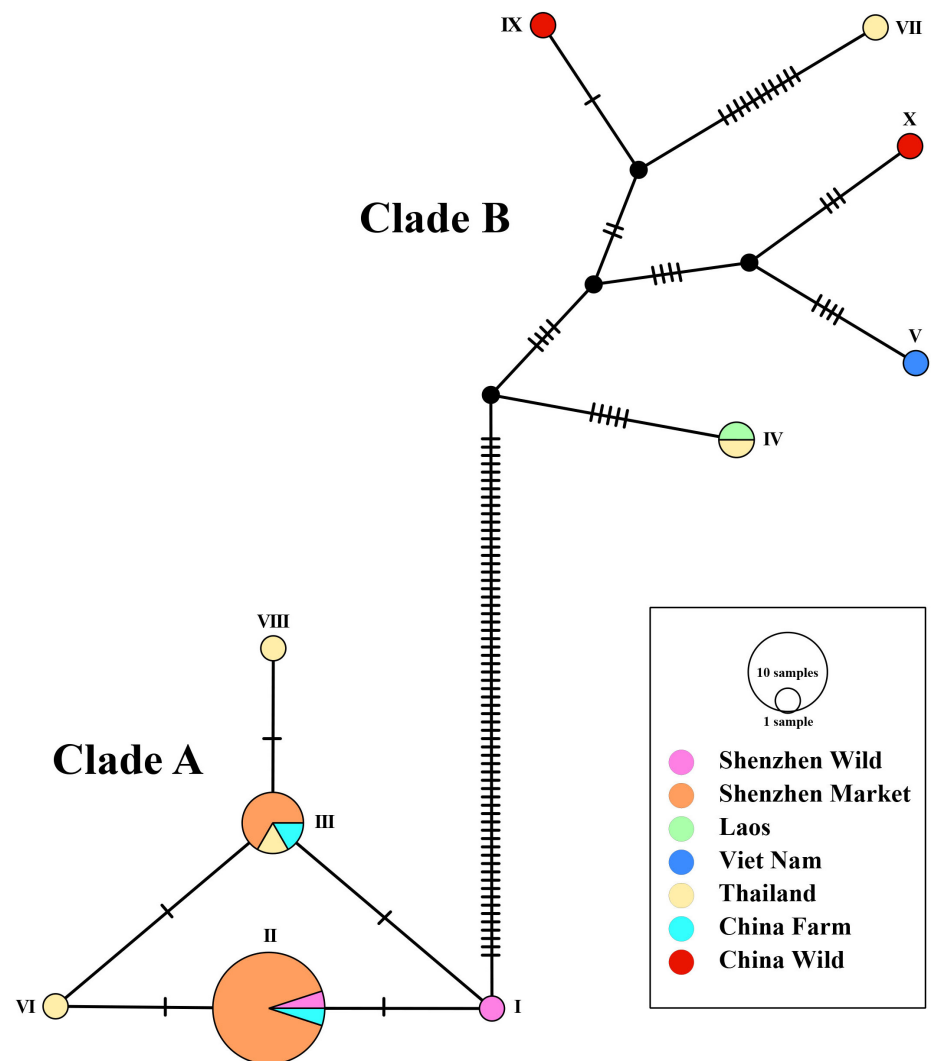
A total of three non-native amphibian species were recorded in the field (Figure 2), including one newly recorded species *Ceratophrys* spp., and two previously reported species (*E. planirostris* and *H. rugulosus*). Specifically, in Lianhuashan Park, we encountered three *H. rugulosus*, two of which we captured are amplexus individuals. This indicated that the *H. rugulosus* may have established here. In Xiangmi Park, we caught a *Ceratophrys* spp., and did not observe any tadpoles or hear calls. Therefore, we did not have evidence on the establishment of this species in the park. At Xiangmi Park, we caught four *E. planirostris* individuals including two adults and two subadults, indicating that this species had established in Shenzhen. Unfortunately, despite earlier field records of *R. catesbeiana* and *X. laevis* in literature and database, neither of these two species were observed during our field surveys (Table 1).

**Table 1.** Non-native amphibian species recorded in the field and trade surveys and the habitat suitability for their establishment based on ecological niche modeling.

Non-native Species	Field Surveys			Literature and Database Surveys (Whether have recorded)	Trade Surveys (Number of market stores and pet shops)	ENM	
	Sampling Locality (Latitude, Longitude)	Time	Evidence of Establishment or Field Observation			Mean	SE
<i>C. ornata</i>	Xiangmi Park (22.55, 114.03)	September 2–13, 2021	One adult	NA	15 online pet shops	0.5552	0.0010
<i>E. planirostris</i>	Xiangmi Park (22.55, 114.03)	September 2–13, 2021	two adults and two subadults	Present	NA	0.8883	0.0017
<i>H. rugulosus</i>	Lianhuashan Park (22.56, 114.07)	September 2–13, 2021	Three adults; and two of them are in amplexus	Present	22 market stores	0.9884	0.0008
<i>R. catesbeiana</i>	NA	September 2–13, 2021	NA	Present	17 market stores	0.1127	0.0005
<i>X. laevis</i>	NA	September 2–13, 2021	NA	Present	2 market stores and 3 online pet shops	0.0328	0.0002


**Figure 3.** Bayesian/maximum likelihood phylogenetic tree of *H. rugulosus* inferred from a fragment of the CYTB gene. “-” denotes low support by Bayesian posterior probabilities (BPP < 95%) and bootstrap analysis (BS < 70%). *H. litoralis* and *H. tigerinus* were used as outgroups. Clade names follow Pansook et al. (2012). Samples collected from Shenzhen in this study are represented by purple (Wild) and orange (Market).

For the *H. rugulosus* and *Ceratophrys* spp. recorded in the field, we further conducted genetic analysis to confirm their identity with the aid of morphological characteristics. After comparing the sequence with the reported sequence in the GenBank database, our sampled *Ceratophrys* spp. matched with *C. ornata* 100% (query cover = 100%; E-value = 0; Appendix 1, Table S3). Combined with the morphological characteristics, we confirm that there is a high probability of the collected sample being *C. ornata*. For *H. rugulosus*, we analyzed the CYTB sequence, which was composed of 750 bp of all 24 individuals (22 market individuals and 2 wild individuals). The results showed that it is clear that all our sampled individuals belong to a strong monophyletic group (Bootstrap = 100/1; Figure 3). According to Pansook et al. (2012), there are two major evolutionary branches (Bootstrap =



**Figure 4.** Median-joining network of *H. rugulosus* haplotypes based on sequence data of the mitochondrial cytochrome b gene (CYTB) constructed in PopART to show relationships among the network of haplotypes of *H. rugulosus*. Haplotypes are represented by circles, and the size of the circles is proportional to the number of individuals. Different colors represent the potential geographic origins of the samples.

86/1–100/1) in *H. rugulosus*: evolutionary branch A and evolutionary branch B (Pansook et al. 2012). Yu et al. (2015) suggested that *H. rugulosus* may be a cryptic species complex. The Thailand population and the Chinese wild population are two separate genetic lineages (Clade A: Thailand, and Clade B: China) (Pyron and Wiens 2011; Pansook et al. 2012; Yu et al. 2015). Individuals collected from 13 locations in our present study (Figure 1) were confirmed to be members of the branch of Clade A, indicating that these 24 samples collected from Shenzhen have the same haplotype with those from Thailand (Figure 4). Further analyses based on ML and BI trees constructed from the gene sequence combined with haplotype networks further suggested that *H. rugulosus* in Thailand might be their most likely source population.

ENM analyses showed that the two non-native species with establishment evidence had high habitat suitability (*E. planirostris*:  $0.8883 \pm 0.0017$ , mean  $\pm$



standard error, Figure S1a; *H. rugulosus*:  $0.9884 \pm 0.0008$ , Figure S1b), whereas the three non-native species without establishment or field observation evidence tended to have lower values of habitat suitability (*C. ornata*:  $0.5552 \pm 0.0010$ , Figure S1c, *R. catesbeiana*:  $0.1127 \pm 0.0005$ , Figure S1d, and *X. laevis*:  $0.0328 \pm 0.0002$ , Figure S1e).

## Discussion

In this study, we recorded a total of three non-native amphibian species (*H. rugulosus*, *C. ornata*, and *E. planirostris*) in the field and there are another two non-native amphibians (*R. catesbeiana*, *X. laevis*) recorded only in the market and the literature or database. For the potentially introduced individuals of *C. ornata*, this is the first field report of their presence in mainland China. We speculated that these species recorded in the field may be from local market, pet shops and online stores that have been posing a high invasion risk of non-native species (Carrete and Tella 2008; Su et al. 2016; Reino et al. 2017). For example, our intensive market and database surveys (Appendix 2, Text S2) found that a total of 35 pet shops or live animal markets and 18 online pet shops are selling the five non-native amphibians recorded in our field survey. We recorded *X. laevis* as pets in 2 market stores and 3 online pet shops, *R. catesbeiana* as food in 17 market stores, *H. rugulosus* as food in 22 market stores, and *C. ornata* as pets in 15 online pet shops (Table 1). For the *E. planirostris*, our survey confirmed the presence of established populations in Shenzhen as reported in previous studies (Lin et al. 2017; Wang et al. 2020; Hong et al. 2022). Importantly, ENM analyses supported our field surveys that the two species with establishment evidences (*E. planirostris* and *H. rugulosus*) also tend to have high predicted habitat suitability, whereas the other three species without establishment evidences or field observations (*C. ornata*, *R. catesbeiana*, and *X. laevis*) tend to have low predicted habitat suitability (Table 1).

Non-native *H. rugulosus* is an economically important aquaculture species provided as human food in many parts of China, especially in southern regions, such as Hainan and Guangdong provinces (Weng et al. 2002; Herath et al. 2021). Literature reviews found that the non-native *H. rugulosus* in Chinese markets might be both from local aquacultures in China and through aquaculture transportation from wild individuals in Thailand (Yu et al. 2015). Our genetic analysis provides molecular evidence that *H. rugulosus* individuals collected both in the field and in the market all belong to the genetic members of the nonnative branch A from Thailand (Figure 3). They might be escaped from aquaculture enclosures (Liu and Li 2009) or released into the wild as mercy releases have contributed to the establishment of invasive American bullfrogs and other non-native species (Liu et al. 2012; Su et al. 2016) as Guangdong is one of the Chinese provinces

with the highest frequency and money spent on prayer animal releases (Du et al. 2023). Considering Shenzhen is within the natural ranges of *H. rugulosus*, the potential introduced individuals may cause a high risk of hybridization with other local clades with close phylogenetic relationships and thus produce genetic contamination issues (Pagano et al. 2003; Lee et al. 2022). We strongly recommend that it should be strictly managed to prevent aquaculture individuals from escaping into the wild and prohibited from being released to avoid potential ecological and evolutionary impacts on native species (Liu and Li 2009; Liu et al. 2013; Lee et al. 2022).

Our field surveys provided additional evidence of the establishment of the greenhouse frogs (*E. planirostris*) in Shenzhen. This non-native amphibian was first recorded in 2017 and now had established in Shenzhen (Lin et al. 2017, 2018; Wang et al. 2020). The source of this exotic amphibian is regarded as very complex due to its small body size and cryptic introductions with human activities but it most likely was introduced through the transport of garden seedlings (Lin et al. 2017, 2018) with complex introduction sources, possibly from adjacent Hong Kong (China), the Philippines, Panama and Florida (USA) (Lee et al. 2016; Hong et al. 2022). This non-native species has been detected as a natural host of the zoonotic disease agent *Angiostrongylus cantonensis* which can be transmitted to humans and cause public health concerns (Niebuhr et al. 2019). Therefore, we recommended that Shenzhen citizens can participate in the eradication of the established greenhouse frogs with the aid of professional guidance from the scientific institutions (Davis et al. 2018).

For *C. ornata*, previous studies have shown that this species along with the other two widely introduced species (*R. catesbeiana* and *X. laevis*) can carry *Batrachochytrium dendrobatidis* (Une et al. 2008; Daszak et al. 2004; Weldon et al. 2004), which has caused the rapid decline of global amphibians (Scheele et al. 2019). Although we did not have establishment evidence in the field survey, ENM analysis indicated that Shenzhen may have a medium habitat suitability for *C. ornata*, which warrants more intensive field surveys covering more areas to identify its invasion status in the future. In addition, we encourage that environmental DNA tools can be applied to detect the species occurrence especially when there might be high population density in suboptimal habitats (Ficetola et al. 2008).

Overall, our study implies that traded non-native amphibians in markets or pet shops have been posing high invasion risks in the field of China. Shenzhen is a megacity with huge trade import and export volume (Wu 2022). A recent study has identified that there are nearly one hundred non-native amphibians that have been introduced into mainland China (Du et al. 2023). We acknowledge that our present study only focuses on some major habitats due to the limited sampling effort during the COVID-19 period, and strongly recommend taking immediate action to conduct more systematic and regular field surveys to investigate the establishment of non-native amphibians and prevent further invasions.

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## Authors' contribution

Zhiqiang Lin: investigation and data collection; writing – original draft; data analysis and interpretation. Yanhua Hong: investigation and data collection; writing – original draft; data analysis and interpretation. Shengnan Chen: investigation and data collection. Yuanbao Du: investigation and data collection. Lixia Han: investigation and data collection. Qing Zhang: investigation and data collection. Shimin Gu: investigation and data collection. Weishan Tu: investigation and data collection. Zhiyong Yuan: writing – review and editing. Shengwei Hu: project supervision. Xuan Liu: research conceptualization; sample design and methodology; writing – review and editing; ethics approval; funding provision; project supervision.

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### Supplementary material

The following supplementary material is available for this article:

#### Appendix 1:

**Table S1.** Species information for Genetic Analysis in Shenzhen.

**Table S2.** Primers and PCR conditions used in this study.

**Table S3.** Results of comparison of amplified sequences of *Ceratophrys* spp. samples to sequences reported in NCBI.

#### Appendix 2:

**Text S1.** The detail of ecological niche modeling.

**Text S2.** The detail of literature and database surveys and trade surveys.

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