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# eDNA analysis reveals high invasion risks in nature reserves in Guangdong Province, China

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**Introduction:** Invasive species can cause ecological and economic damage in various areas, including nature reserves. The invasion risks of aquatic invasive vertebrates in nature reserves, however, remain unclear since this group often hides under the water and is frequently neglected in ecological surveys based on traditional methods.

**Methods:** Environmental DNA (eDNA) provides a promising alternative way to conduct biodiversity surveys in aquatic ecosystems. Here, we collected aquatic eDNA samples from eight nature reserves in Guangdong Province, China to mainly investigate the diversity of aquatic invasive vertebrates and inform their invasion risks in these nature reserves.

**Results and discussion:** We detected a total of 104 aquatic vertebrate species belonging to three classes (Actinopteri, Amphibia and Reptilia), 12 orders, 32 families, and 71 genera, among which nine were invasive species (8.65% of all aquatic vertebrates detected), i.e., *Coptodon zillii*, *Sarotherodon galilaeus*, *Oreochromis niloticus*, *Oreochromis tanganicae*, *Gambusia affinis*, *Clarias gariepinus*, *Chelydra serpentina*, *Trachemys scripta elegans*, and *Rana catesbeiana*. Surprisingly, 55.56% of these aquatic invasive vertebrates (i.e., five species) were found in at least 75.00% samples, and both *C. zillii* and *S. galilaeus* were detected in all samples (100%), suggesting that most invasive species were widely distributed in these nature reserves. In addition, all aquatic invasive vertebrate species ranked very high (top 66 of aquatic vertebrates detected) regarding their relative abundance of sequences, and three of the top 10 species with the highest number of sequences were invasive species (i.e., *C. zillii*, *S. galilaeus*, and *O. niloticus*), suggesting high population size of these invasive vertebrates. Moreover, we also detected 16 endangered/threatened species (15.38% of all vertebrates detected), which demonstrated notable overlaps of geographic distribution with invasive species. The reality of high abundance, wide

geographical distribution and overlaps with the endangered/threatened species indicated considerable risks of aquatic invasive vertebrates in nature reserves in Guangdong Province, which calls for urgent needs for effective management. Our study would provide fundamental insights for the formulation of effective management measures to reduce losses caused by invasive species and promote the protection of endangered/threatened species in nature reserves.

# 1 Introduction

Biological invasion is one of the biggest concerns worldwide, as invasive species can cause huge ecological and economic damage to local ecosystems and economic industries by competing for space and food, preying on native species and transmitting disease (Simberloff et al., 2013; Bellard et al., 2016). Currently, various areas are threatened by invasive species, of which invasive plants and invertebrates can be easily found in farmlands, parks and even protected areas. Whereas invasive vertebrates are hardly found in these areas, since most of them are aquatic species with high mobility and often hide under the water (global invasive species database, GISD) (http://www.iucngisd.org/gisd/). Aquatic invasive vertebrates can invade streams, pools, reservoirs and wetlands, threatening rare or endangered species, because the majority of the most endangered/threatened amphibians and reptiles also live in these habitats (Ficetola et al., 2015; Xin et al., 2024) (http://www.amphibiachina.org/). In addition, many aquatic invasive vertebrates are often carnivorous or omnivorous, even a few individuals can prey on a mass of native and even endangered species, leading to high invasion risks, such as the notorious *Atractosteus spatula* (Li and Zhang, 2023). Hence, to formulate effective management measures, it is critical to understand the composition and distribution of aquatic invasive vertebrates in a given area, especially for areas with endangered/threatened species.

Many endangered/threatened species are protected in nature reserves, which can provide relatively undisturbed environments (Watson et al., 2014; Mi et al., 2023). Whereas many nature reserves have been disturbed by invasive species and an increasing numbers of invasive species have been reported in nature reserves, especially in countries that are highly populated, such as China (Guo et al., 2017). For example, previous investigations using traditional transects and plot surveys revealed a total of 14 and 23 invasive plant species in the Dinghushan National Nature Reserve in Guangdong Province and Shiwandashan National Natural Reserve in Guangxi Zhuang Autonomous Region, respectively (Wei et al., 2006; Song et al., 2009). Similar findings were also reported in many other nature reserves, such as the Bawangling Nature Reserves in Hainan Province, Jinfoshan Nature Reserve in Chongqing City and Lishan Nature Reserve in Shanxi Province (Guo et al., 2017). In addition, a few aquatic invasive vertebrate species (e.g. *Neovison vison*, *Oncorhynchus mykiss* and *Rana catesbeiana*) were also reported in nature reserves in northern China, following traditional observation-based surveys (Gong et al., 2017). However, the traditional observation-based survey methods may underestimate the diversity of aquatic invasive species due to their dwelling under the water (Zhan et al., 2017; Bailey et al., 2020). In addition, only very few studies have focused on aquatic invasive vertebrate species in nature reserves in southern China, which may suffer from severe biological invasions due to intensive anthropogenic disturbances and favorable climates (Yan et al., 2017). The above limitations or facts suggest that the composition of this group and their invasion risks in nature reserves in southern China remain poorly understood.

Environmental DNA (eDNA) technique can provide a cost-efficient way to investigate species' richness and abundance based on high-throughput sequencing. This technique has proven effective in detecting species diversity of various aquatic groups using group-universal primers (Thomsen and Willerslev, 2015; Valentini et al., 2016; Wang et al., 2021). For example, based on 109 aquatic sampling sites from river systems in Beijing, a total of 75 fish taxa including 23 alien taxa were detected using the fish-universal primer set Ac12S, and the relative sequence abundance of these alien taxa was high, especially in areas that were highly populated (Zhang et al., 2022). Similar studies based on eDNA technique were also performed in plankton (Xiong et al., 2017; Wang et al., 2024) and other groups (Li et al., 2022). Recently, multiple vertebrate-universal primer sets have been developed, such as V12S-U (Wang et al., 2023b), MarVer1 (Valsecchi et al., 2020) and Vert-16S (Vences et al., 2016). These universal primers showed high PCR amplification success and taxonomic discrimination ability, among which V12S-U seems more suitable than other primers and has been used in diversity investigations of aquatic vertebrate species (Wang et al., 2023a, b). In addition, there are abundant 12S sequences of vertebrate species in public database, such as NCBI (National Center for Biotechnology Information) and BOLD (Barcode of Life Data System). Thus, available vertebrate-universal primers combined with sufficient reference sequences of

12S in public databases provide foundations for the reliable investigation of aquatic vertebrate species using eDNA technique.

In the present study, we collected aquatic eDNA samples from eight nature reserves in Guangdong Province with high-density populations to mainly investigate the diversity of aquatic invasive vertebrates using vertebrate-universal primers and inform their invasion risks in these nature reserves. Our study is of critical importance in making effective management measures targeting invasive species and protecting species diversity in nature reserves.

# 2 Material and methods

## 2.1 Sample collection and filtration

We collected aquatic eDNA samples from streams, pools or reservoirs of eight typical provincial nature reserves (Figure 1A) distributed in Guangdong Province in April 2023, i.e., Lianshan Bijiashan Nature Reserve (LBJS), Liannan Bandong Nature Reserve (LNBD), Lianping Huangniushi Nature Reserve (LHNS), Longchuan Fengshuba Nature Reserve (LFSB), Pingyuan Longwen-Huangtian Nature Reserve (PLHT), Chaoan Fenghuangshan Nature Reserve (CFHS), Longmen Nankunshan Nature Reserve (LNKS) and Luhe Nanwan Hongzhuilin Nature Reserve (LHZL). A total of 1 L mixed water was collected at three representative locations of water body from each nature reserve using brand new plastic bottles, and all water samples were placed on ice in coolers and transported to laboratory. We preserved samples at 4°C until filtration, which was completed within 24h of collection.

Each water sample was filtered through 0.45 μm microporous filter membranes (Millipore, USA) with a circulating water vacuum pump in a clean laboratory in Institute of Zoology, Guangdong Academy of Sciences. In addition, 1 L double-distilled $H_2O$ (ddH$_2$O) was also filtered and used as filtration blank. Filtration units were rinsed with 10% commercial bleach before ddH$_2$O to remove potential cross-contamination between samples. The obtained filters were placed in 1.5 mL centrifugal tubes and preserved at -20°C until DNA extraction.

## 2.2 DNA extraction and high throughput sequencing

The filter-based eDNA was extracted using DNeasy Blood & Tissue Kits (QIAGEN Ltd., West Sussex, United Kingdom) according to the manufacturer's protocol with slight modifications. Briefly, the filters were cut into small pieces using clean surgical scissors in a 2 ml centrifuge tube with 360 μm ATL buffer and 40 μm proteinase K. The obtained mixtures were incubated at 56°C for 2 h, and then 400 μm AL buffer and 400 μm ethanol were added. The new mixture was centrifuged at 6000 rpm for 1 min, the obtained supernate was transferred into a DNeasy Mini spin column and centrifuged at 8000 rpm for 1 min. The eDNA reserved in the DNeasy Mini spin column was successively washed using 500 μm AW1 buffer and 500 μm AW2 buffer by centrifuging at 8000 rpm for 1 min and 13000 rpm for 1 min, respectively. The eDNA was eluted using 80 μm AE buffer by centrifuging at 13000 rpm for 3 min, and the concentration and quality were determined using a Nanodrop One spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Metabarcoding of aquatic invasive vertebrate species in nature reserves was performed using the vertebrate-universal primer pair V12S-U (Forward primer: GTGCCAGCNRCCGCGGTYANAC, Reverse primer: ATAGTRGGGTATCTAATCCYAGT), which targets an ~208 bp 12S ribosomal RNA gene fragment and has shown efficient detection of vertebrate diversity in aquatic realms (Wang et al., 2023a, b). We performed three PCR replicates for each extract of eDNA samples and filtration blanks to avoid biased amplification. Each PCR in a 25 μl total volume included 12.5 μl of 2 × Taq PCR Master Mix (Cat: B639295, Sangon Biotech, Shanghai, China), 10 pmol each of forward and reverse primers, 1 μl DNA that was diluted 5-fold to reduce the PCR inhibition. The PCR program consisted of an initial denaturation step at 95°C for 5 min; then 35
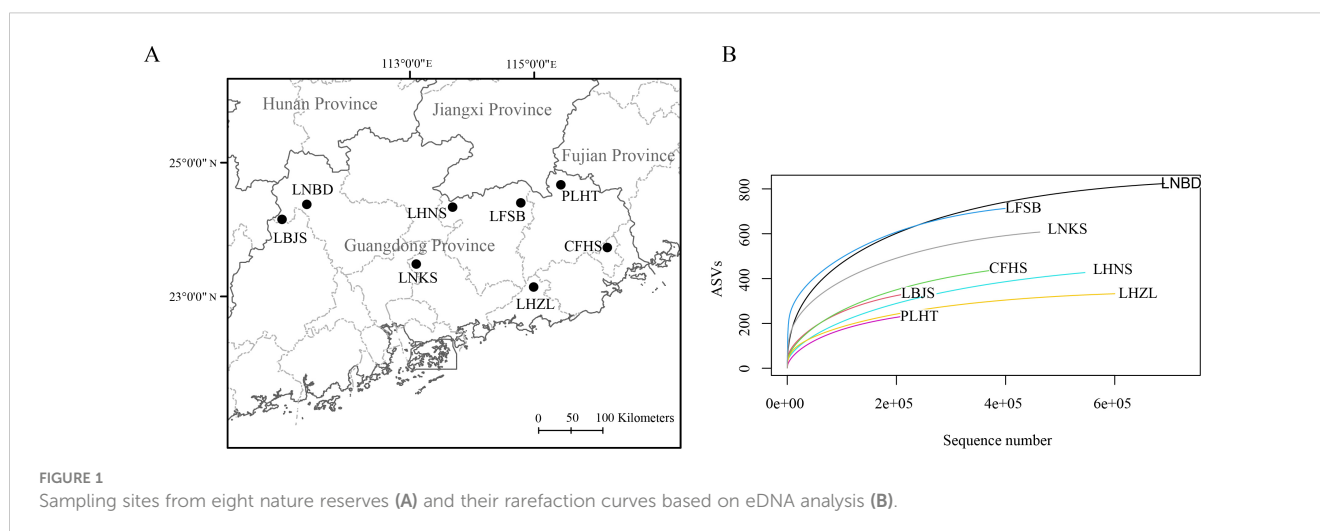


FIGURE 1
Sampling sites from eight nature reserves **(A)** and their rarefaction curves based on eDNA analysis **(B)**.

cycles of 95°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec; and a final elongation step at 72°C for 5 min. PCR products were checked using 2.0% agarose gel and no bands were detected for the filtration blanks. PCR products of the three replicates for each sample were pooled and purified using the SanPrep Column PCR Product purification kit (Cat: B518141, Sangon Biotech, Shanghai, China). The purified PCR products-based sequencing libraries were sequenced using 2 × 150 bp paired end sequencing on an Illumina NovaSeq 6000 Sequencing System (Illumina, San Diego, CA, USA).

## 2.3 Bioinformatics processing

Raw sequence reads were filtered to remove adapters and low-quality reads (average Phred quality score per read < 20) using Trimmomatic (Bolger et al., 2014). Reads shorter than 100 bp were also discarded. The obtained clean pair-end reads were then analyzed using the program VSEARCH v 2.27.1 (Rognes et al., 2016). The pair-end reads were merged into sequences using "–fastq_mergepairs" command with default settings. The primers and sequences with possible sequencing errors were removed from merged sequences using "–fastx_filter" command with the expected error threshold of 0.5. The "–derep_fulllength" command was used to remove redundant sequences. Unique amplicon sequence variants (ASVs) were generated using the "–cluster-unoise" command with minimum abundance of 2 (–minsize = 2). Chimeras were removed using the "–uchime_ref" command and ASV table was generated by running the "–usearch_global" command. To determine whether the sequencing data for each sample was sufficient to cover the biodiversity, the ASVs-based rarefaction curve was plotted using R package Vegan (Dixon, 2003) and ggplot2 (Ginestet, 2011).

The relative proportion of each ASV was calculated in each sample and used as the proxy of relative ASV abundance for subsequent analyses (Hirai et al., 2015). The ASVs were annotated according to the best-hit by searching against the NR database from NCBI using SEED v1.46 (Větrovský and Baldrian, 2013) with the parameters of e value <10$^{-80}$, minimum query coverage >99% and similarity >98% (Cilleros et al., 2019). ASVs assigned to non-freshwater vertebrate species were discarded. Here, aquatic invasive vertebrate species and endangered/threatened species were determined according to "List of Invasive Alien Species in China" and "China's Red List of Biodiversity: Vertebrates (2020)", respectively, published by the Chinese government.

# 3 Results

## 3.1 Composition of freshwater vertebrates

High throughput sequencing produced a total of 4,258,886 raw paired end reads (NCBI SRA sample accession: SAMN42018080-SAMN42018087). After quality filtering, de-replication, and ASV detection, a total of 2130 ASVs were retained, and rarefaction curves were saturated for all samples (Figure 1B). Among the 2130 ASVs, 1216 ASVs were successfully annotated as vertebrates. After removing ASVs assigned to non-freshwater species and ASVs with similarity < 98%, a total of 211 ASVs were kept for downstream analyses (Supplementary Table S1).

Based on the 211 ASVs, we identified a total of 104 freshwater vertebrate species (Supplementary Table S2), belonging to three classes: Actinopteri, Amphibia and Reptilia, of which Actinopteri was the most abundant class (87 species), followed by Amphibia (11 species) and Reptilia (6 species). From the three classes, we identified 12 orders, of which Cypriniformes (56 species), Siluriformes (11 species), Anura (10 species), Gobiiformes (7 species) and Testudines (6 species) were identified as the first five dominant orders. A total of 32 families were retrieved, and the top six were Xenocyprididae (13 species), Cyprinidae (9 species), Gobionidae (8 species), Acheilognathidae (7 species), Gobiidae (6 species) and Bagridae (6 species). A total of 71 genera were detected, and the top six were *Rhinogobius* (6 species), *Acheilognathus* (5 species), *Tachysurus* (5 species), *Channa* (4 species), *Mauremys* (3 species) and *Hemiculter* (3 species).

Among the 104 freshwater vertebrate species, we detected a total of nine (8.65%) invasive species: *Coptodon zillii, Sarotherodon galilaeus, Oreochromis niloticus, Oreochromis tanganicae, Gambusia affinis, Clarias gariepinus, Chelydra serpentina, Trachemys scripta elegans,* and *Rana catesbeiana* (Table 1). In

TABLE 1 Invasive species and their relative abundance at eight nature reserves.

| Invasive species | LNBD | LBJS | LFSB | LHNS | PLHT | LNKS | LHZL | CFHS |
|---|---|---|---|---|---|---|---|---|
| *Coptodon zillii* | 227 | 6788 | 7276 | 110 | 112 | 2102 | 122 | 69593 |
| *Sarotherodon galilaeus* | 15 | 37 | 4561 | 33 | 8 | 2800 | 5 | 28904 |
| *Oreochromis niloticus* | 40 | 7768 | 15327 | 27 | 42 | 3579 | 0 | 113 |
| *Gambusia affinis* | 0 | 12 | 60 | 16477 | 29 | 0 | 0 | 34 |
| *Rana catesbeiana* | 6897 | 1693 | 7 | 244 | 0 | 22 | 0 | 10 |
| *Chelydra serpentina* | 0 | 3104 | 0 | 5 | 0 | 0 | 0 | 0 |
| *Trachemys scripta elegans* | 0 | 91 | 53 | 10 | 58 | 2 | 1750 | 56 |
| *Clarias gariepinus* | 16 | 0 | 32 | 0 | 0 | 307 | 0 | 5 |
| *Oreochromis tanganicae* | 0 | 0 | 62 | 0 | 0 | 0 | 0 | 0 |

addition, we also detected 16 (15.38%) endangered/threatened species, including four critically endangered species (*Acipenser schrenckii*, *Mauremys mutica*, *Mauremys nigricans*, and *Mauremys sinensis*), two endangered species (*Pelodiscus sinensis*, *Hoplobatrachus chinensis*), five vulnerable species (*Xenocypris fangi*, *Silurus soldatovi*, *Odontobutis haifengensis*, *Onychostoma macrolepis*, and *Sinocrossocheilus labiatus*) and five near threatened species (*Channa striata*, *Pterocryptis cochinchinensis*, *Sylvirana guentheri*, *Sylvirana maosonensis*, and *Paramesotriton hongkongensis*) (Table 2).

## 3.2 Distribution and abundance of invasive species

These invasive species distributed widely with five (55.56%) (*C. zillii*, *S. galilaeus*, *O. niloticus*, *T. s. elegans*, and *R. catesbeiana*) detected in at least six (75.00%) nature reserves (Figure 2A). Both *C. zillii* and *S. galilaeus* were detected in all eight nature reserves (LBJS, LNBD, LHNS, LFSB, PLHT, CFHS, LNKS, and LHZL). *O. niloticus* and *T. s. elegans* were detected in all nature reserves except for LHZL and LNBD, respectively. *R. catesbeiana*, *G. affinis*, and *C. gariepinus* were detected in six (LBJS, LNBD, LHNS, LFSB, CFHS, and LNKS), five (LBJS, LHNS, LFSB, PLHT, and CFHS) and four (LNBD, LFSB, CFHS, and LNKS), respectively. *C. serpentine* and *O. tanganicae* were only detected in two (LBJS and LHNS) and one (LFSB), respectively (Table 1). The highest invasive species richness was found in LFSB (eight species), followed by LBJS (seven), LHNS
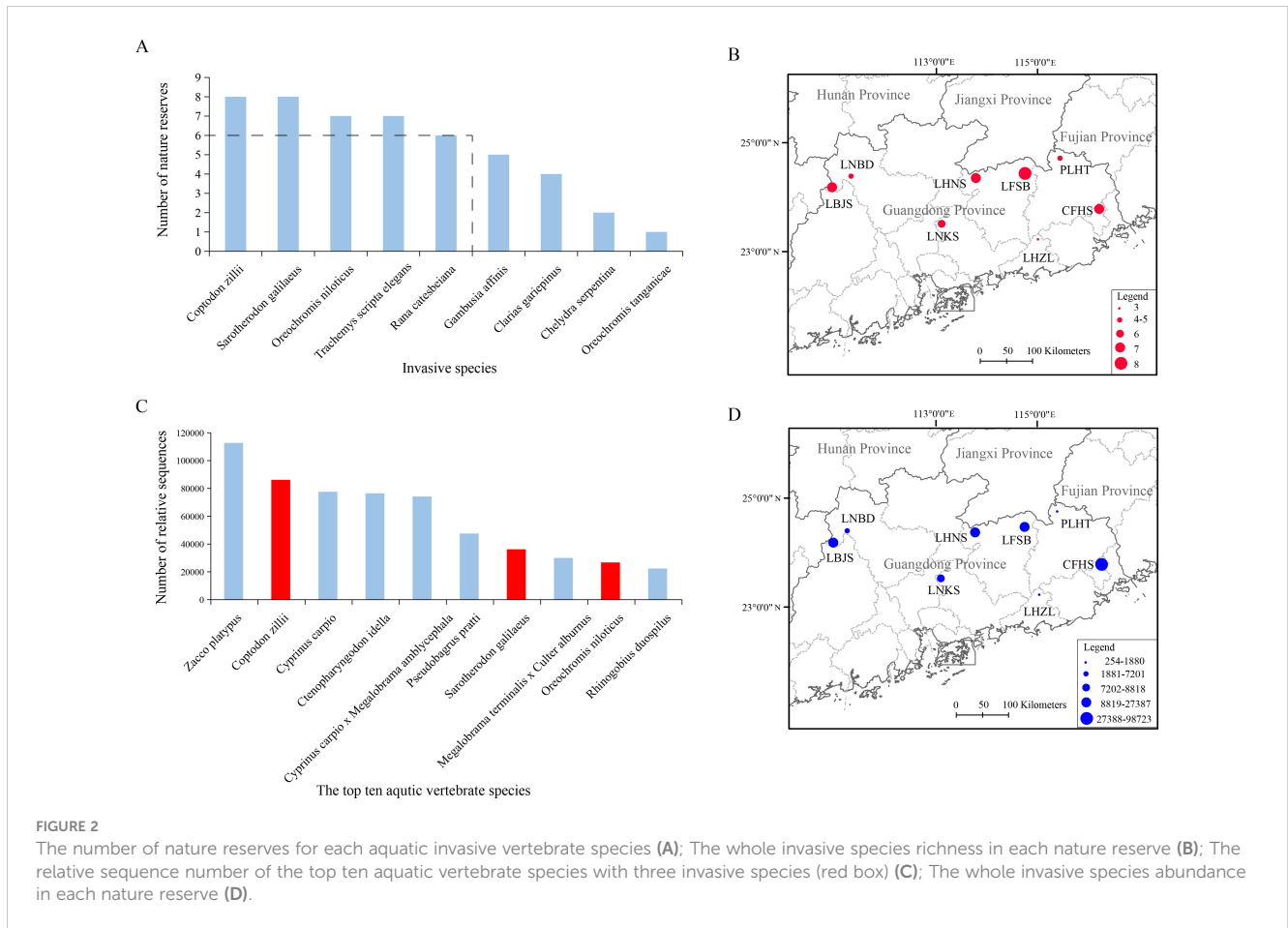
(seven), CFHS (seven), LNKS (six), PLHT (five) and LNBD (five), while LHZL (three) had the lowest number of aquatic invasive vertebrate species (Figure 2B; Table 1).

These invasive species ranked top 66 (63.46%) of aquatic vertebrate species regarding their relative sequences, and three invasive species (*C. zillii*, *S. galilaeus* and *O. niloticus*) were found among the top 10 aquatic vertebrate species, suggesting high abundances of these invasive species (Figure 2C; Table 1). The average of relative sequence reads per invasive species was 20,070, ranging from 62 relative sequence reads for *O. tanganicae* to 86,331 relative sequence reads for *C. zillii*. The top four invasive species were *C. zillii* (86,331), *S. galilaeus* (36,363), *O. niloticus* (26,896) and *G. affinis* (16,613), followed by *R. catesbeiana* (8,873), *C. serpentine* (3,109) and *T. s. elegans* (2,021), and the relative sequence reads of both *C. gariepinus* (361) and *O. tanganicae* (62) were relatively less. Both *C. zillii* (69,593) and *S. galilaeus* (28,904) were detected with the most sequence reads in CFHS, followed by LFSB, where both *O. niloticus* and *O. tanganicae* were detected with the most sequence reads. Other five invasive species were detected with the highest abundance in different nature reserves, *G. affinis*, *R. catesbeiana*, *C. serpentine*, *T. s. elegans*, and *C. gariepinus* were detected with the highest abundance in LHNS (16,477), LNBD (6,897), LBJS (3,104), LHZL (1,750) and LNKS (307), respectively (Table 1). The highest abundance of all invasive species was found in CFHS (98,723), followed by LFSB (27,387), LBJS (19,501), LHNS (16,913), LNKS (8,818), LNBD (7,201) and LHZL (1,880), and the lowest abundance were found in LHNS with only 254 relative sequence reads of all invasive species (Figure 2D).

TABLE 2 Endangered/threatened species and their relative abundance at eight nature reserves.

| Endangered/ threatened species | Catergory | LNBD | LBJS | LFSB | LHNS | PLHT | LNKS | LHZL | CFHS |
|---|---|---|---|---|---|---|---|---|---|
| *Onychostoma macrolepis* | Vulnerable | 0 | 8 | 51 | 34 | 25 | 0 | 0 | 14470 |
| *Sinocrossocheilus labiatus* | Vulnerable | 0 | 8 | 60 | 10106 | 12 | 0 | 0 | 22 |
| *Mauremys mutica* | Critically endangered | 247 | 0 | 1393 | 12 | 4 | 34 | 1172 | 7 |
| *Xenocypris fangi* | Vulnerable | 0 | 4 | 2831 | 2 | 4 | 0 | 0 | 5 |
| *Channa striata* | Near threatened | 0 | 0 | 1938 | 2 | 0 | 430 | 0 | 10 |
| *Hoplobatrachus chinensis* | Endangered | 1941 | 0 | 0 | 0 | 0 | 18 | 0 | 0 |
| *Acipenser schrenckii* | Critically endangered | 0 | 0 | 1500 | 3 | 8 | 0 | 0 | 0 |
| *Sylvirana maosonensis* | Near threatened | 642 | 0 | 0 | 0 | 0 | 104 | 0 | 0 |
| *Pterocryptis cochinchinensis* | Near threatened | 0 | 8 | 7 | 19 | 17 | 0 | 0 | 15 |
| *Silurus soldatovi* | Vulnerable | 0 | 0 | 2 | 19 | 21 | 0 | 0 | 12 |
| *Paramesotriton hongkongensis* | Near threatened | 0 | 12 | 0 | 2 | 25 | 0 | 0 | 12 |
| *Mauremys nigricans* | Critically endangered | 8 | 0 | 7 | 7 | 4 | 2 | 0 | 2 |
| *Odontobutis haifengensis* | Vulnerable | 0 | 8 | 7 | 0 | 0 | 0 | 0 | 5 |
| *Mauremys sinensis* | Critically endangered | 0 | 4 | 12 | 0 | 0 | 0 | 0 | 2 |
| *Pelodiscus sinensis* | Endangered | 0 | 4 | 5 | 3 | 0 | 0 | 0 | 2 |
| *Sylvirana guentheri* | Near threatened | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 |

**FIGURE 2**
The number of nature reserves for each aquatic invasive vertebrate species **(A)**; The whole invasive species richness in each nature reserve **(B)**; The relative sequence number of the top ten aquatic vertebrate species with three invasive species (red box) **(C)**; The whole invasive species abundance in each nature reserve **(D)**.

## 3.3 Distribution and abundance of endangered/threatened species

Unlike invasive species, only two (12.50%) endangered species (*M. mutica* and *M. nigricans*) were detected in at least six (75.00%) nature reserves (Table 2). Most (11, 68.75%) endangered/threatened species (*P. cochinchinensis*, *X. fangi*, *O. macrolepis*, *S. labiatus*, *P. sinensis*, *C. striata*, *P. hongkongensis*, *S. soldatovi*, *A. schrenckii*, *M. sinensis*, and *O. haifengensis*) were distributed in less than five nature reserves, such as *P. cochinchinensis* detected in LBJS, LHNS, PLHT, and CFHS, *O. haifengensis* detected in LBJS, LFSB and CFHS. Both *H. chinensis* and *S. maosonensis* were detected in LNBD and LNKS, while *S. guentheri* was only found in LNKS. For the richness of endangered/threatened species, the top two highest nature reserves were LFSB and CFHS, both had 12 endangered/threatened species, followed by LHNS (11), PLHT (9), LBJS (8), LNKS (6) and LNBD (4), the lowest richness of endangered/threatened species was detected in LHZL, which had only one endangered species (*M. mutica*) (Figure 3A).

*O. macrolepis* and *S. labiatuswas* were identified as the top two highest abundant endangered/threatened species with 14,589 and 10,209 sequence reads in total, respectively, followed by *M. mutica* (2,870), *X. fangi* (2,846), *C. striata* (2,379), *H. chinensis* (1,959), *A. schrenckii* (1,511) and *S. maosonensis* (746). The rest endangered/threatened species had extremely low abundance with less than 100

sequence reads in total for each species, the top two lowest abundant species were *P. sinensis* (15) and *S. guentheri* (10). The most endangered/threatened species with highest abundance were distributed in different nature reserves, while the rest endangered/threatened species with highest abundance were found in the same nature reserves. For example, CFHS was found with the highest abundance of *O. macrolepis* with 14,470 sequence reads, LHNS was detected with the highest abundance of *S. labiatuswas* with 10,106 sequence reads, and LNBD was found with the highest abundance of *H. chinensis* with 1,941 sequence reads. While, six species (*M. mutica*, *X. fangi*, *C. striata*, *A. schrenckii*, *M. sinensis*, and *P. sinensis*) were detected with the highest abundances in LFSB (Figure 3B).

## 3.4 The comparison between invasive species and endangered/threatened species

To investigate the potential threats of invasive species to local endangered/threatened species, we compared the richness and abundances of both groups in the same nature reserves. We found high overlaps of geographic distribution between endangered/threatened species and invasive species (Figures 2, 3). Nature reserves with both high richness and abundances of invasive
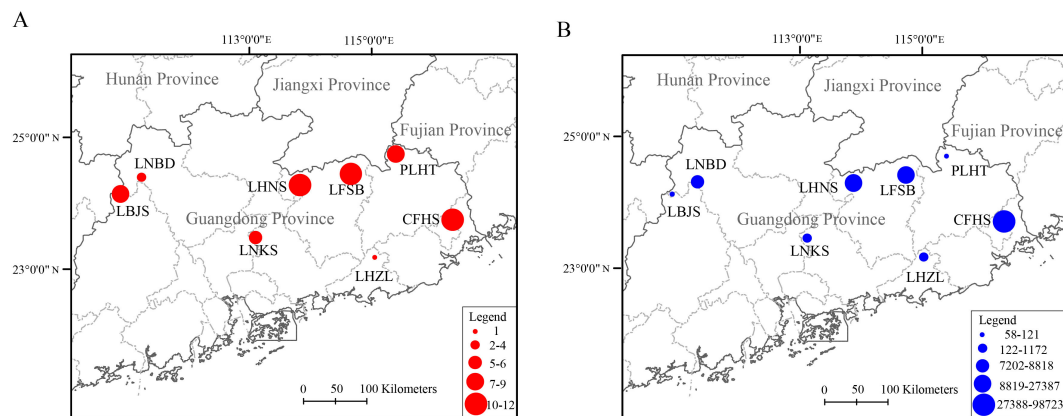
FIGURE 3

The whole endangered/threatened species richness **(A)** and abundance **(B)** in each nature reserve.

species were also detected with both high richness and abundances of endangered/threatened species, and vice versa. For example, the top three highest nature reserves in endangered/threatened species diversities [LFSB (12), CFHS (12) and LHNS (11)] were also detected with the top three largest invasive species richness [LFSB (8), CFHS (7) and LHNS (7)]. While, the top two lowest nature reserves in endangered/threatened species diversities [LNBD (4) and LHZL (1)] were also detected with the top two lowest invasive species richness [LNBD (5) and LHZL (3)].

# 4 Discussion

Aquatic invasive vertebrates often hide under the water and are frequently neglected by traditional ecological surveys in nature reserves, leading to witting invasion risks exerted on nature reserves. Here, based on eDNA technique, we detected nine invasive species and 16 endangered/threatened species in eight nature reserves in Guangdong Province. Surprisingly, these invasive species had a wide distribution and high abundance in nature reserves. In addition, we found high overlaps of geographic distribution between invasive species and endangered/threatened species. These observations indicated high invasion risk in nature reserves in Guangdong Province, highlighting urgent needs to manage these invasive species.

Invasive species can cause huge damages to local biodiversity and ecosystems, and are often characterized by wide distributions and high abundances (Fristoe et al., 2021). Here, eDNA analysis detected a total of nine aquatic invasive vertebrate species, among which tilapia species (*C. zillii*, *S. galilaeus*, and *O. niloticus*) showed both the widest distribution and highest abundance. Tilapias are notorious invaders and their invasions can alternative aquatic communities and ecosystem functions by preying on native aquatic species' eggs, larvae, and even adults, competing for food resources and habitat, and deteriorating water quality, potentially causing population decline and even extinction of native species (Canonico et al., 2005; Martin et al., 2010; Sanches et al., 2012; Yongo et al., 2023). For example, previous field investigations

showed that the invasion of *O. niloticus* could reduce the food resources available for local species and can significantly reduce the abundance and body size of native fishes, such as *Cirrhinus molitorella*, *Megalobrama terminalis* and *Hemiculter leucisculus* (Gu et al., 2015; Shuai et al., 2019; Shuai and Li, 2022). Further manipulative experiments revealed the inhibited growth of native *C. molitorella* in the presence of *O. niloticus* (Gu et al., 2015), thus providing experimental evidence of serious damages caused by tilapia species to native species. Hence, the wide distribution and high abundance of these tilapia species in the present study indicated that their invasions may have caused population decline and even extinction of native species in nature reserves in southern China.

Although both *T. s. elegans* and *R. catesbeiana* had lower abundance (less than 10,000 relative sequence numbers) compared to the above three tilapia species (more than 25,000 relative sequence numbers), they had wide distributions and were detected in at least 75.00% nature reserves. They are also notorious invaders and have been listed as the world's 100 worst invasive species compiled by the International Union for the Conservation of Nature (IUCN) Invasive Species Specialist Group (ISSG) of the Species Survival Commission. Mounting evidence showed that both invasive species can cause huge damages to native species by preying on them (Kraus, 2015), competing for food and basking sites with native species (Cadi and Joly, 2003, 2004), compromising integrity of native species through hybridizing with native genetically closed species (Kraus, 2015), and spreading pathogens (Daszak et al., 2004; Gong et al., 2014; Martínez-Ríos et al., 2022; Hossack et al., 2023). For example, *T. s. elegans* has invaded into most wetlands in Europe where the native specie European pond turtle (*Emys orbicularis*) also lives, when *E. orbicularis* were cultured together with *T. s. elegans* for three years, the weight of *E. orbicularis* lost significantly ($p < 0.05$) and its mortality was significantly higher ($p < 0.001$) than control groups (Cadi and Joly, 2004). Hence, its reasonable to speculate that the introduction of *T. s. elegans* into these nature reserves may have caused decreased weight and increased mortality of native endangered turtles, such as *M. mutica* and *M. nigricans* in the present study. In addition, one

investigation of frog community in 65 permanent lentic waters on islands showed that sites with *R. catesbeiana* invasion had lower native frog abundance and species richness than sites without *R. catesbeiana*, and these indexes of native frogs were negatively related to post-metamorphosis *R. catesbeiana* abundance, suggesting that post-metamorphosis *R. catesbeiana* can negatively influence native frog communities, and that the influence extents are proportional to post-metamorphosis *R. catesbeiana* abundance (Li et al., 2011). Hence, the low abundances of both *T. s. elegans* and *R. catesbeiana* in the present study suggested that their damages to native common species may be not severe in nature reserves, but potential huge damages to endangered amphibians, reptiles and other rare species, given their wide distributions in nature reserves, high overlaps of geographic distribution with endangered/threatened species, and carnivorous diets with the big appetites (Wang et al., 2008; Nishizawa et al., 2014). There were similar concerns for other aquatic invasive vertebrate species at least in a few nature reserves, such as *G. affinis* in LHNS, since *G. affinis* was abundant in LHNS.

In conclusion, based on eDNA technique, we detected a total of nine aquatic invasive vertebrate species, most of which showed high abundances and were widely distributed in nature reserves in southern China. In addition, we also detected 16 endangered/threatened species, which showed high overlaps of geographic distribution with invasive species. These findings indicated high invasion risks of these invasive species in nature reserves in Guangdong Province, highlighting urgent measures to manage these invasive species and prevent their further spread. Our study would provide fundamental insights for the formulation of effective management measures to curb potential damage and losses caused by invasive species and protect endangered/threatened species in nature reserves. Moreover, our study detected a total of four endangered turtle species in eight nature reserves based on eDNA technique, which was also successfully used for the detection of endangered turtle *Platysternon megacephalum* in nature reserves in Hong Kong (Lam et al., 2022), thus providing evidence for that eDNA technique is a promising tool for the conservation of endangered turtle species. However, although some species such as *O. macrolepis* (VU species) were detected in some nature reserves based on eDNA technique in the present study, their presence based on morphological characteristics was absent in these nature reserves (Zhao et al., 2011). Hence, further investigations based on both eDNA technique and morphological methods can provide more comprehensive insights into invasion risks of alien species and protection of endangered/threatened species in nature reserves.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because DNA sequences were collected from water samples.

## Author contributions

YG: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. YW: Data curation, Investigation, Writing – original draft, Writing – review & editing. CW: Data curation, Writing – review & editing. SZ: Formal analysis, Software, Writing – review & editing. ZX: Methodology, Visualization, Writing – review & editing.

## Funding

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1462123/full#supplementary-material

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