

Phylogeography and population structure of *Lagocephalus spadiceus* (Richardson, 1845) (Tetraodontiformes, Tetraodontidae) in the South China Sea

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Abstract

The Late Pleistocene climate fluctuations have had a major impact on phylogeographic structure and historical dynamics of marine fishes in the marginal seas of the western Pacific Ocean. The puffer fish *Lagocephalus spadiceus*, has high nutritional and economic value in the South China Sea. To allow the examination of the demographic history and population structure of the *L. spadiceus*, the mitochondrial DNA COI and Cyt b gene datasets of 300 individuals from eight populations in the South China Sea was sequenced. High haplotype diversity (0.874 ± 0.013) and low nucleotide diversity (0.00075 ± 0.00058) were observed. The phylogenetic tree and haplotypes network revealed no significant genetic differentiation along the coast of the northern South China Sea. Neutrality tests, mismatch distribution analysis, and Bayesian skyline plots suggested that *L. spadiceus* experienced population expansion during the Late Pleistocene. Ocean currents and climate change play important roles in shaping the geographical distribution and genetic population structure of *L. spadiceus*.

Title

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ABSTRACT

The Late Pleistocene climate fluctuations have had a major impact on phylogeographic structure and historical dynamics of marine fishes in the marginal seas of the western Pacific Ocean. The puffer fish *Lagocephalus spadiceus*, has high nutritional and economic value in the South China Sea. To allow the examination of the demographic history and population structure of the *L. spadiceus*, the mitochondrial DNA *COI* and *Cyt b* gene datasets of 300 individuals from eight populations in the South China Sea was sequenced. High haplotype diversity (0.874 ± 0.013) and low nucleotide diversity (0.00075 ± 0.00058) were observed. The phylogenetic tree and haplotypes network revealed no significant genetic differentiation along the coast of the northern South China Sea. Neutrality tests, mismatch distribution analysis, and Bayesian skyline plots suggested that *L. spadiceus* experienced population expansion during the Late Pleistocene. Ocean currents and climate change play important roles in shaping the geographical distribution and genetic population structure of *L. spadiceus*.

KEYWORDS

Lagocephalus spadiceus; Genetic diversity; Phylogeography; *COI* and *Cyt b* gene datasets; South China Sea

1 INTRODUCTION

In marine species, the phylogeography and genetic differentiation were influenced by historical events, including ocean current systems, vicariance, Pleistocene climatic cycles, and life-history characteristics of organisms (Liu et al., 2007; Ding et al., 2018). It is worth noting that some marine fishes have fragile genetic structure due to their extensive larval and adult dispersal (Ashrafzadeh et al., 2021; Caccavo et al., 2018). Climate oscillations during the Pleistocene greatly altered the environment of marginal seas of the western Pacific, including the South China Sea (SCS). During glacial periods, the SCS formed a semi-enclosed sac-shaped gulf and exposed approximately 0.7 million km² of continental shelf (Wang & Sun, 1994). Previous molecular studies have shown that many marine fishes with high mobility exhibit low genetic structure in the SCS, such as *Cirrhimuraena chinensis* (Li et al., 2014) and *Nuchequula mannusella* (Gao et al., 2019). The phylogeographic study of marine fishes alive in the SCS has particular significance for interpreting the consequences of past events, geological configurations, and modern oceanographic aspects in this environment (He et al., 2010).

Lagocephalus spadiceus is a non-toxic *Lagocephalus* species (Tuney, 2016), belonging to Tetraodontiformes, Tetraodontidae, and *Lagocephalus*. It is a nearshore warm-water demersal fish that inhabits depth between 3 to 200 meters (Tuncer et al., 2008), distributed along the southern coast of Africa in the Indian Ocean, eastward to the Indonesia and the Philippines of the Pacific Ocean, and northward to the coast of China (Liu et al., 2016). In China, it occurs along the coastal areas of the SCS. Almost *L. spadiceus* is imported from China in Japan (Yamaguchi et al., 2013), but this also makes it an easy target for widespread exploitations. Recently, since the continuous increase in fishing intensity and deteriorating environmental conditions, the wild resources of *L. spadiceus* have been drastically reduced (Hardy et al., 2014). There are fewer reports on germplasm resource and genetic diversity evaluation of *L. spadiceus*. To better protect and rationally develop the wild germplasm resources of *L. spadiceus*, it is imperative to conduct a genetic diversity assessment to establish a theoretical basis for the scientific conservation and sustainable utilization of its genetic resources.

Genetic diversity is a vital component of biodiversity and prerequisite for the continuous adaptation of species or populations to environment change and survival evolution. Species with a higher genetic diversity possess a greater ability to environmental changes (Roldan et al., 2000). Mitochondrial DNA (mtDNA) constitutes a tiny fraction of organismal genome size but has been widely used as a marker of molecular diversity in animals for the past four decades (Galtier et al., 2009). This tool has been widely embraced by population geneticists, following the works of Avise et al (1987) and Moritz et al (1987), among others. Experimentally, mtDNA is present in most cells in high copy number and is relatively easy, rapid, and inexpensive to sequence (Zink & Barrowclough, 2008). Due to the relationship between the rate and time of evolution, effective information sites are different, and their resolving power is different. Therefore, concatenating *COI* and *Cyt b* markers can increase the number of effective genetic sites, resulting in more accurate and effective information compared to single gene analysis (Halasan et al., 2021; Barrientos-Villalobos & Schmitter-Soto, 2019).

Our study examines the phylogeography, population genetic diversity, and demographic history of *L. spadiceus* using *COI* and *Cytb* gene datasets. As of current knowledge, there has not been a documented report on the population genetic of *L. spadiceus*. This study aims to address the lack of information on *L. spadiceus* in the region by providing a comprehensive background report. Meanwhile, our study has contributed to a better understanding of the evolutionary process that have influenced the phylogeography of coastal marine fishes in China.

2 MATERIALS AND METHODS

2.1 Sample collection

A total of 300 specimens of *L. spadiceus* were obtained from eight geographic locations, including Beihai (BH), Zhanjiang (ZJ), Leizhou (LZ), Danzhou (DZ), Dongfang (DF), Maoming (MM), Shanwei (SW), and Zhangzhou (ZZ) in the South China Sea (Figure 1, Table 1). All *L. spadiceus* Specimens were collected from bottom trawl surveys within the period of April 2022 to April 2023. A morphological analysis was used to identify species (head, dorsal surface, and ventral surface are covered with small spines, the dorsal side of the body is brownish-yellow or yellow-green, caudal fin is white at the upper and lower tips) (Liu et al., 2016;

Chen & Zhang, 2015). Fishes were given access to muscle tissue, which were then stored in 1.5 ml vials with 95% ethanol at -20°C until genomic extraction. Experimental procedures concerning fish were performed following the Experimental Animal Administration Regulations.

2.2 DNA extraction, PCR amplification and sequencing

The total DNA of *L. spadiceus* was extracted from each muscle following the FastPure Cell/Tissue DNA Isolation Mini Kit (Nanjing, China). The purity and concentration of DNA were checked using ultra microspectrophotometer (NanoDrop 2000, United States of America). The primers of mtDNA were adapted from Li et al (Li et al., 2018). The *COI* was boosted by the primers *COI*- F: 5'-AAACCACCGCCTGACACTC-3' and *COI*- R: 5'-GGGATTTTAACCCCGGCAT-3', the *Cyt b* was boosted by the primers *Cyt b* -F: 5'-GCGCCCAAAGTAAGGAGAA-3' and *Cyt b* -R: 5'- GGGATTTTAACCCCGGCAT -3'. PCR amplification volume of 50 μl , including 25 μl $2\times$ *Taq* PCR Master Mix, 2 μl each of primers (10 $\mu\text{mol/L}$), 1 μl DNA template, and 20 μl ddH₂O. PCR cycling conditions were applied: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min (*COI*) or 30 sec (*Cytb*), annealing at 58°C (*COI*) or 56°C (*Cyt b*) for 1 min, extension at 72°C for 1 min, and final elongation at 72°C for 8 min (*COI*) or 5 min (*Cyt b*). Every PCR product was electrophoresed on 1% agarose gel, and PCR products were sent to Guangzhou Ige Biotechnology Ltd (Guangzhou, China) for purification and DNA sequencing.

2.3 Data analysis

2.3.1 Genetic diversity

Forward and reverse splicing of all sequences using SeqMan in Larsergene v7.1.0 (Swindell & Plasterer, 1997), and then compare and edit the sequences using the Clustal W method in MEGA v7.0 (Kumar et al., 2016). The *COI* and *Cyt b* sequences were matched one by one for Multi-locus Sequence Analysis (MLSA) using PhyloSuite v1.2.2 (Zhang et al., 2020). Count base composition and content, polymorphic sites (S), and parsimony informative sites (P_i) using MEGA v7.0 (Kumar et al., 2016). The haplotype numbers (H), haplotype diversity (H_d), nucleotide diversity (π), and mean pairwise difference (K) were counted using DnaSP v6.0 (Rozas et al., 2017).

2.3.2 Genetic structure

The Bayesian information criterion (BIC) in jModelTest v2.1.10 (Darriba et al., 2012) was used to establish a substitution model for the haplotype datasets prior to phylogenetic analysis. Subsequently, the mitochondrial *COI* and *Cyt b* gene datasets haplotypes were used to reconstruct the phylogenetic tree using the Bayesian inference (BI). The congeneric species *Lagocephalus laevigatus* was chosen as an out group, from NCBI access number 10400364 (*COI*) and 10400369 (*Cyt b*). Bayesian inference study was carried out using MrBayes v3.2.7 (Ronquist et al., 2012), and one set of four chains was permitted to run concurrently for 20 million generations. Every 1000 generations, a sample of the tree was taken, with the first 25% being eliminated as burn-in. As the sampled generations increased, the log-likelihood maintained a constant level, and stationarity was attained when the split average frequencies' average standard deviation was less than 0.01 (Hall, 2016). Phylogenetic tree editing was done with FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>), and median-joining haplotype network was produced using PopART v1.7 (Leigh & Bryant, 2015).

Genetic distance within and between populations were calculated using MEGA v7.0 (Kumar et al., 2016). Subsequently, AMOVA was used to quantify genetic variation using F -statistics at two geographically distinct levels of subdivision: among and within populations. To test for statistical significance, 10,000 permutations of the fixation index F_{ST} were performed between pairs of populations using Arlequin v3.5 (Excoffier & Lischer, 2010).

2.3.3 Demographic history

The Tajima's D (Tajima, 1989) and Fu's F_s (Fu & Li, 1993) tests were utilized to check for neutral evolution. To examine population growth, the mismatch distribution (Rogers & Harpending, 1992) between the sum of squared deviations (SSD) and Harpending's raggedness index (Rg) was analyzed with Arlequin

v3.5 (Excoffier & Lischer, 2010). Changes in effective population size (N_e) over time were deduced using Bayesian skyline plot analysis in BEAST v2.6.3 (Bouckaert et al., 2019). To account for possible site-specific variations, the rate of clock mutation was fixed at 1×10^{-8} per year, as recommended for reef fishes (Delrieu-Trottin et al., 2017). With a sample every 1000 iterations, 100 million generations of separate independent Markov chain Monte Carlo (MCMC) studies were carried out. The molecular clock was calibrated using an average divergence rate of 2% per million years for mtDNA (Schubart et al., 1998). ESS values were detected until they reached 200, and these parameter values were displayed in Tracer v1.7.1 (Rambaut et al., 2018).

3 RESULTS

3.1 Genetic diversity

A total of 300 *COI* and *Cyt b* gene datasets (2598 bp) were obtained of *L. spadiceus* from eight geographic locations (Figure 1). 94 polymorphic sites were detected among all individuals, including 28 parsimony information sites and 66 singleton variable sites (Table 1). The average nucleotide composition was 23.2% adenine (A), 27.3% thymine (T), 31.6% cytosine (C), and 17.9% guanine (G), with a slightly higher content of A+T (50.5%) than G+C (49.5%), showing a clear anti-G bias. The mutation of DNA was unsaturated for the transition/transversion (Ts/Tv) of bases in the *COI* and *Cyt b* gene datasets, which was 3.73. 95 haplotypes were identified (GenBank accession numbers: OQ970201-OQ970253 and OR428269-OR428309), the majority of which were unique haplotypes (89.47%). Only ten haplotypes were shared between populations (Hap_1, Hap_2, Hap_4, Hap_5, Hap_17, Hap_22, Hap_23, Hap_25, Hap_30, and Hap_65), and the most common Hap_1 (24.0%), Hap_2 (19.3%), and Hap_22 (11.0%) were found at each population (Figure 2).

Total haplotype diversity (H_d) was high ($H_d = 0.874 \pm 0.013$), while nucleotide diversity (π) was low ($\pi = 0.00075 \pm 0.00058$) (Table 1), showing the high haplotype diversity and low nucleotide diversity. The correlation between genetic diversity and longitude and latitude of sampling locations showed that the haplotype diversity ($r = 0.29$) and nucleotide diversity ($r = 0.31$) of *L. spadiceus* populations tend to rise with latitude but did not change significantly with longitude ($r = 0.08$ and 0.04 , respectively) (Figure 3).

3.2 Genetic structure and differentiation

The molecular evolution model with the gamma shape parameter (HKY+I+G) was found to be the best substitution model for the *COI* and *Cytb* gene datasets by the jModelTest. From this result, a BI tree was built to determine phylogenetic relationship across populations (Figure S1). The BI tree was dispersed with haplotypes from each population and lacked well-supported groups. Neither significant genealogy branches nor haplotype clusters could be identified in relation to the sampling locations.

Network analysis and the phylogenetic tree showed similar results. The connection between several haplotypes resembled a star, with certain prominent haplotypes like Hap_1, Hap_2, Hap_4, and Hap_22 (Figure 2). Unnoticeable clades in the network diagram of reticulations do not correlate with sampling locations, suggesting a substantial gene flow among populations and recent population expansion. It was found that relationships between populations were not linked to geological networks, but instead to the haplotypes that were present in each population. According to these results, there was no obvious phylogeographical pattern of *L. spadiceus* in the South China Sea.

The degree of genetic variation between populations was evaluated using F_{st} pairwise comparisons. The F_{st} values were typically low and even negative, as shown in Table 2, only the F_{st} values between LZ population and other populations were higher and significant ($P < 0.05$). The genetic distance between and within populations was at the same level, with little differentiation (Table 2). The range under investigation had no significant genetic structure. Hierarchical AMOVA analysis revealed that genetic variation in all populations existed within populations, while only a small proportion of genetic variation could be attributed to differences between populations (Table 3).

3.3 Demographic history

A unimodal distribution was observed in the mismatch distribution analysis (Figure 4a), which was consistent

with the expected distribution under a sudden expansion model ($R_g = 0.0536$, $P > 0.05$, Table 4). It is also possible that populations had undergone expansion in the past (as indicated by the star-like networks). The sum of the squared deviation (SSD) showed that there was no significant deviation from the growth and expansion model ($P > 0.05$). The Tajima's D and Fu's F_s tests of *L. spadiceus* were significantly negative (Tajima's $D = -1.905$, $P < 0.05$; Fu's $F_s = -10.543$, $P < 0.01$, Table 4). Typically, such values indicated that *L. spadiceus* may have experienced population expansion.

Bayesian skyline plots supported demographic scenarios explaining the recent population expansion of *L. spadiceus* (Figure 4b). The population experienced a significant increase, followed by a period of demographic stability. The calculated population expansion time is approximately from 0.025 Mya to 0.010 Mya during the Late Pleistocene (Figure 4b).

4 DISCUSSION

4.1 Genetic diversity

Haplotype diversity (H_d) and nucleotide diversity (π) are two important indicators to measure genetic diversity, and π represents the proportion of each haplotype in the populations, which can reveal the polymorphism of mtDNA in the populations more accurately (Chen et al., 2022). Grant & Bowen (1998) concluded that H_d was higher than 0.5 and π was greater than 0.005, which indicated higher species diversity. This study revealed high levels of haplotype and low levels of nucleotide diversity, which is common among some marine fish species (Avise et al., 1987; Zhang et al., 2006).

High genetic diversity plays a crucial role in the exploitation and restoration of fishery resources (DeWoody et al., 2021). Assessing genetic diversity is an effective approach the adaptability and survival ability of species in response to environmental changes (Schmitt and Hewitt, 2004), which is essential for species management and conservation. The fish of *L. spadiceus* is an economic species and has been caught for a long time. Compared with other marine fishes in the same sea area, *L. spadiceus* had a lower nucleotide diversity (Yi et al., 2021; Niu et al., 2019; Xu et al., 2021), reflecting that the *L. spadiceus* population in the South China Sea has a fragile genetic diversity and requires conservation as well as a sustainable development planning from fishery management.

4.2 Genetic structure and differentiation

Genetic distance is a crucial factor in determining the genetic relationship between species (Mather et al., 2017). Shaklee et al (1982) proposed a classification of fish genetic distance at the population level (0.05), species level (0.30), and genus level (0.90). In this study, it was found that the genetic distance between populations were small, indicating a close genetic relationship between these populations. According to the coalescent theory (Crandall & Templeton, 1993), more diverse populations have longer coalescence times and larger coalescent effective population sizes than less diverse populations (assuming the same mutation rate). Therefore, the ancestral haplotype was the most widely distributed. In the *COI* and *Cyt b* gene datasets, Hap_1 and Hap_2 were found to be the dominant haplotypes (Figure 2), and may be the origin of *L. spadiceus*.

In the phylogenetic analysis, the haplotypes of eight populations were randomly distributed. The haplotype network and phylogenetic tree also showed no clear pedigree structure corresponding to geographical location. This pattern suggests that the species went through a bottleneck event followed by a population expansion (Grant & Bowen, 1998). The dispersal of larvae with ocean currents is an important cause of the limited genetic differentiation of marine fishes that have a geographically large distribution range (Strathmann et al., 2002). In this study, *L. spadiceus* were caught in the spring and autumn, during this time, the China Coastal Current and the South China Sea Warm Current flowed northward into the East China Sea (Figure 1) (Yang et al., 2008; Wang et al., 2015). Previous studies have reported that extensive gene exchange occurs over a wide geographical range in marine fishes (Grant & Bowen, 1998; Niu et al., 2019; Yi et al., 2021). F_{st} is a significant measure in evaluating genetic diversity among populations, as emphasized by Allendorf (1983). A higher F_{st} value suggests a greater level of genetic differentiation. According to Wright (1951)

classification, F_{st} value of $0 \sim 0.05$ suggests no differentiation, $0.05 \sim 0.15$ suggests little differentiation, $0.15 \sim 0.25$ suggests moderate differentiation, and F_{st} value greater than 0.25 suggests significant genetic differentiation. The F_{st} between the BH and LZ population was the highest value among all populations (Table 2), indicating the greatest genetic differentiation is between these populations, other populations were lower.

4.3 Demographic history

This study utilized the Tajima's D , Fu's F_S tests, and mismatch analysis to suggest that a population expansion event of the *L. spadiceus* population may have occurred from 0.025 Mya to 0.010 Mya in the Late Pleistocene. *L. spadiceus* is mainly distributed less 50 m depth, and spawns in coastal habitats and shallow shorelines. Therefore, the *L. spadiceus* distribution is closely related to historical sea level fluctuations. When sea level was 120 m lower than the present level during the last glacial maximum of the Pleistocene, the northern South China Sea included Beibu Gulf, which was part of the South China continent, Hainan Island, and Taiwan Island were connected to mainland China. The entire South China Sea was separated from the Indian Ocean to form a semi-closed basin (Wang, 1990). The survival range of marine fish decreased sharply; therefore, the *L. spadiceus* population may have moved and survived in one or more glacial refuges during this period, such as the semi-closed South China Sea. In the Late Pleistocene, the glaciation began to disappear and *L. spadiceus* might have experienced rapid population expansion when favorable conditions occurred.

Many studies have demonstrated a weak genetic differentiation between the geographical populations of surface marine fish that can migrate long distances or swim. This can be attributed to the free dispersal of floating eggs, fish larvae, juveniles, and adults, as well as the absence of significant geographical obstacles in the open ocean environment. Consequently, gene exchange occurs extensively and widely among these marine fish populations (Canfield et al., 2022; Hewitt, 2000; Palumbi, 1994). However, it should be noted that *L. spadiceus*, being a benthic fish, does not exhibit a long-distance migration behavior according to its life history. Therefore, the observed panmixia among populations may be attributed to their early life habits. The active diffusion of fish larvae and juveniles as well as marine environmental factors, such as ocean circulation and climate change in the Late Pleistocene, have played crucial roles in shaping the systematic geographical pattern and population genetic structure of *L. spadiceus*.

4.4 The choice of mtDNA.

Surprisingly, despite the popularity of mtDNA as a marker in evolutionary studies, this assumption only relies on a handful of comparisons involving mostly vertebrate's species (Nabholz et al., 2009). Depending on species, mtDNA mutation rate was much higher or lower than nuclear DNA (nuDNA) rate. For example, it is not always clonal, far from neutrally evolving and certainly not clock-like, and the ratio of mitochondrial to nuclear mutation rate varies widely among animals (Allio et al., 2017; Galtier et al., 2009). Despite these long-acknowledged concerns, similar results were obtained in several studies that have employed mtDNA and nuDNA to investigate genetic structuring and demographic history in populations of marine fishes (Machado-Schiaffino et al., 2009; Vinas et al., 2010; Adams et al., 2006; Mccusker & Bentzen, 2010; Yang et al., 2022).

5 CONCLUSIONS

In this study, we present the first exploration of the genetic structure of *L. spadiceus* in the SCS. The mtDNA sequence analysis of specimens from the SCS revealed no significant genetic differentiation among sampling sites, with low F_{st} values indicating genetic homogeneity, which probably reflected widespread and recent historical interconnections during the post-glaciation. Hainan Island and Leizhou Peninsula had no effect on gene flow of *L. spadiceus* in the SCS. In its demographic history, it experienced a low effective population size during the Quaternary period that increased sharply after the last glacial maximum (LGM). The phylogeographic pattern of *L. spadiceus* may be attributed to past population expansion and long-distance larval dispersal facilitated by present-day ocean currents. Given the maternally inherited characteristics of mtDNA, which cannot accurately provide the overall population structure. To gain a

better understanding of the population structure of *L. spadiceus* , further investigation using more precise nuclear genetic markers like microsatellites and single nucleotide polymorphisms is needed. These markers would help determine more accurate and refined management units, which can then inform the development of an effective management policy.

AUTHOR CONTRIBUTIONS

Hao Xu : Data curation (lead); Formal analysis (lead); Investigation (equal); Methodology (equal); Software (lead); Writing-original draft (lead); Writing -review & editing (lead). **Laingliang Huang and Tao Chen** : Methodology (equal); Writing -review & editing (lead). **Zhiqiang Wu, Bin Kang, Yunrong Yan, and Xiuguo Zhang**: Writing -review & editing (lead). **Caiguang Wang, Yanan Cheng, and Qiongyuan Su**: Formal analysis (lead); Investigation (equal).

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The haplotype DNA sequences were deposited in GenBank under accession numbers OQ970201-OQ970253 (*COI*) and OR428269-OR428309 (*Cytb*). The data have been uploaded into Dryad under the following <https://doi.org/10.5061/dryad.cjsxksnc3>.

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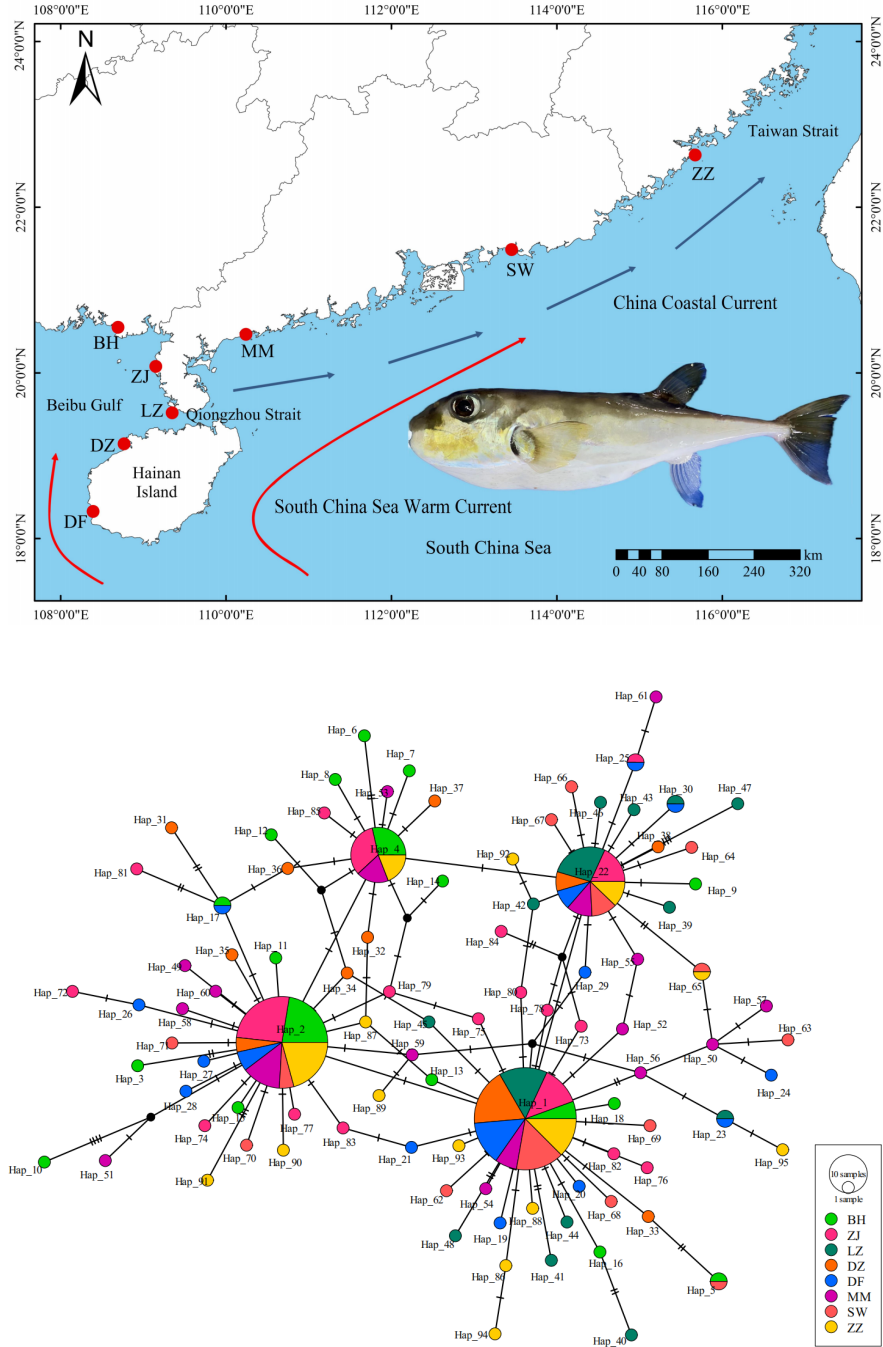
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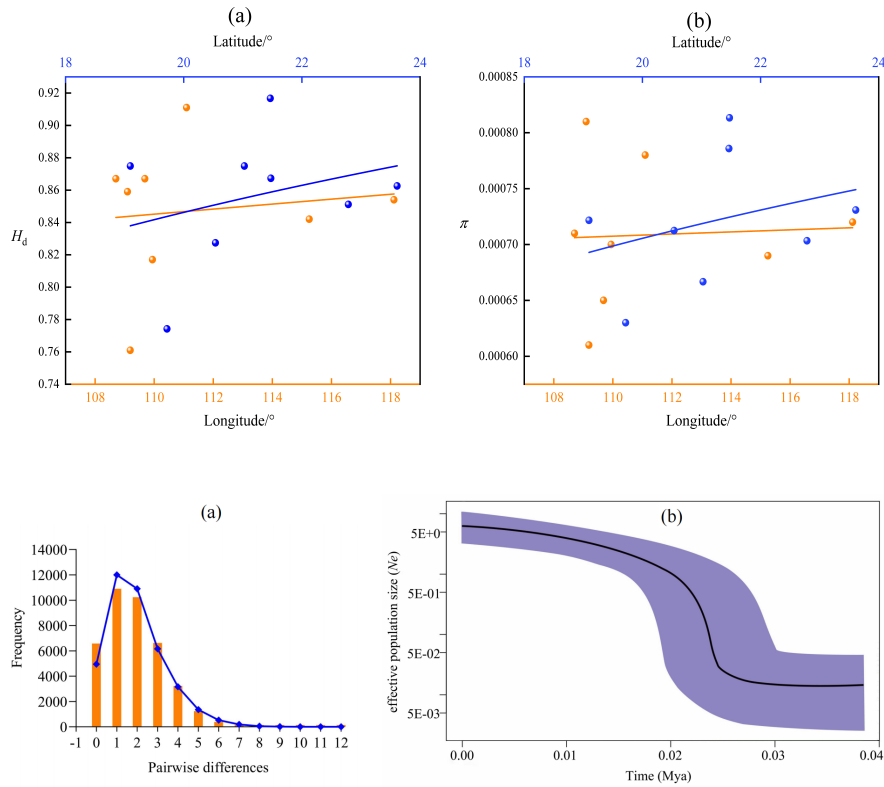
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