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Phylogeographic patterns and population connectivity of *Cephalopholis aurantia* in the spratly islands (Vietnam) inferred from COI gene sequences

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Abstract

The golden hind grouper (*Cephalopholis aurantia*) is a visually striking reef fish of high economic value in the Coral Triangle region, serving both as a food source and as an ornamental species in the aquarium trade. In addition to its economic significance, *C. aurantia* plays an important ecological role in maintaining the balance of coral reef ecosystems. However, the species is facing serious threats due to increasing fishing pressure and exploitation, often carried out without strict regulatory oversight. Assessing genetic variation is thus essential for understanding population diversity and structure, enabling the development of effective conservation strategies, particularly under intensifying environmental and anthropogenic stress. In this study, we examined the genetic diversity and population structure of *C. aurantia* in the Spratly Islands using the mitochondrial Cytochrome c Oxidase subunit I (COI) gene. This gene is a widely recognized marker in population genetics of marine fish. A total of 30 individuals were successfully sequenced, and COI variability was assessed. Results revealed high haplotype diversity but low nucleotide diversity, indicating a genetic disequilibrium within the population. Neutrality tests and population dynamic analyses supported a scenario of post-bottleneck demographic expansion. Phylogenetic and haplotype network analyses further corroborated this hypothesis, showing a dominant haplotype with closely related satellite haplotypes, along with evidence of new haplotypes entering the population. Such patterns may be driven by larval dispersal capabilities, strong reproductive potential, and the influence of oceanic currents facilitating gene flow. Documenting signs of population expansion and immigration is crucial for spatially explicit management and conservation of *C. aurantia*, ensuring the long-term sustainability of this ecologically and economically significant species in the Spratly Islands.

Keywords: *Cephalopholis aurantia;* COI gene; Genetic diversity; Spratly Islands; Marine conservation

1. Introduction

Groupers are among the primary targets of traditional fisheries, the ornamental fish trade, and commercial export-import industries in Southeast Asia [1]. They are highly valued due to their palatable flesh and ease of capture. Groupers exhibit limited mobility and tend to aggregate in large numbers during spawning seasons [2], traits that make them particularly vulnerable to overfishing. The depletion of grouper populations could have severe ecological consequences, as they play critical roles in reef ecosystems by shaping community structure and maintaining ecological balance [2]. As carnivorous predators with proficient hunting skills, groupers influence species abundance, distribution, and diversity. They also serve as an essential food source for scavengers and function as ecological engineers through their burrowing behavior [3]. As of 2018, grouper exploitation showed no significant signs of decline, with an estimated 200,000 tons harvested globally that year [4]. In Asia, Hong Kong remains a major hub for the grouper trade, with over a dozen species reportedly circulated in the market [3]. Groupers are now under increasing fishing pressure [5], with even smaller-

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bodied species being intensively harvested for the aquarium trade. A recent global assessment of groupers indicated that 30% of species are data deficient, 12% are at risk of extinction under current exploitation levels, and 13% are considered near-threatened [6].

The golden hind grouper (*Cephalopholis aurantia*), a member of the subfamily *Epinephelinae*, is a coral reef-associated species commonly found across the Indo-Pacific. *C. aurantia* is harvested in various local regions where it occurs, either for food consumption or the ornamental fish trade, owing to its striking coloration shared with other vividly colored groupers [7]. According to the Food and Agriculture Organization (FAO) [8], *C. aurantia* is directly exploited in several marine regions including the Western Indian Ocean (eastern African coasts), Eastern Indian Ocean, Northwestern Pacific, and Western Central Pacific—encompassing areas such as the Philippines, Papua New Guinea, and Micronesia. Notably, Weh Island in Aceh Province, Indonesia, has been surveyed in detail regarding the exploitation of this species [9]. In this area, *C. aurantia* and other coral reef groupers constitute the primary targets of local fisheries, accounting for up to 80% of the total bony fish biomass [9]. A recent report by Fadli et al. (2021) highlighted that *C. aurantia* commands particularly high market value in Aceh, further intensifying fishing pressure on the species [10]. In Vietnam, *C. aurantia* has been primarily recorded on coral reefs and mesophotic reef systems around the Spratly Islands [11], [12]. Despite its long-standing exploitation, the harvesting of *C. aurantia* in Vietnam remains largely unregulated and insufficiently monitored. This situation reflects the broader global trend for the species, where inadequate attention to lesser-known or data-deficient groupers like *C. aurantia* poses significant conservation risks. Prolonged unregulated fishing of such species may lead to drastic population declines that go unnoticed [13], [14]. Moreover, there is a substantial lack of genetic data on *C. aurantia*, with few targeted studies addressing its population structure or ongoing genetic variation. Enhancing our understanding of the species' genetic diversity and phylogeography is vital for developing informed conservation strategies and sustainable management plans for this ecologically and economically valuable grouper.

DNA barcoding has become the gold standard in supporting taxonomic classification using molecular markers [15]. This technique enables taxonomists to accurately identify species, enhance biodiversity assessments, and improve biological monitoring and regulatory frameworks [16]. In the field of fisheries research, particularly within global ichthyological studies, the mitochondrial COI gene has been widely employed as a genetic marker for both species identification and population-level analyses [17]. In groupers, particularly those in the genus *Cephalopholis*, numerous studies have demonstrated the utility of COI sequences for evaluating genetic diversity and inferring phylogenetic relationships across different marine regions [9], [18], [19], [20], [21]. These findings support the effectiveness of COI markers for both intraspecific genetic assessments and species-level taxonomic resolution. The COI region possesses sufficient variability to achieve two key objectives: first, to reveal intraspecific genetic clustering associated with geographic localities, thereby highlighting fine-scale population structure; and second, to provide enough genetic divergence to distinguish among closely related species within the genus *Cephalopholis*. Based on this foundation, this study aims to investigate the genetic variation in the COI region among *C. aurantia* individuals collected from the Spratly Islands, Vietnam. The findings contribute to a deeper understanding of the species' population genetic structure and support future efforts in conservation and sustainable management of this ecologically and economically important grouper.

2. Materials and Methods

2.1. Sample Collection

Fish specimens were collected from various sites across the Spratly Islands, Vietnam. Sampling locations were selected to represent distinct coral reef habitats within the archipelago. Individuals were captured using handlines or spearguns. Preliminary species identification was conducted in the field based on external morphological characteristics following taxonomic references for *C. aurantia* [3], [8], [22]. A small muscle tissue sample was excised from each individual and stored at −20°C until DNA extraction.

2.2. DNA Extraction

Genomic DNA was extracted from muscle tissues using the TopPURE® Genomic DNA Extraction Kit (HI-112) following the manufacturer's spin-column protocol. The quality and integrity of the extracted DNA were assessed by electrophoresis on a 1% agarose gel using 1X TAE (Tris-acetate-EDTA) buffer.

2.3. Cytochrome c Oxidase subunit I gene amplification

A fragment of the mitochondrial COI gene was amplified using the universal fish primers FishF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and FishR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) [23]. PCR amplification was performed in a 25 μL reaction mixture containing 12.5 μL of Prime Taq Premix (2X) (GENETBIO), 0.5

μL of each primer, 0.5 μL of genomic DNA template, and sterile distilled water to a final volume of 25 μL. The thermal cycling protocol consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. PCR products were visualized on a 1% agarose gel using 1X TAE buffer and a 1 kb DNA ladder to confirm amplification and estimate product size.

2.4. DNA Sequencing

PCR products were purified and sequenced by DNAsequencing (Vietnam) using the Sanger sequencing method [24]. Nucleotide sequences were manually edited and verified using FinchTV version 1.4.0. Cleaned sequences were trimmed and aligned to a reference *C. aurantia* COI sequence obtained from GenBank (accession number: OQ387248.1) using MEGA version 12 [25].

2.5. Phylogenetic analysis

Aligned COI sequences were subjected to phylogenetic analysis using the Maximum Likelihood (ML) method with 1,000 bootstrap replicates in MEGA version 12 [25]. *Cephalopholis boenak* (GenBank accession number: KU722913.1) was included as an outgroup. The best-fit nucleotide substitution model for tree construction was the Kimura 2-parameter (K2P) model.

2.6. Genetic diversity and population structure analysis

Haplotypes were identified from aligned COI sequences using DnaSP version 6 [26]. Analyses of genetic diversity, neutrality tests, and signals of population expansion or contraction were conducted using DnaSP [26] and Arlequin version 3.5.2.2 [27]. A haplotype network was constructed using PopART version 1.7 [28], based on the Minimum Spanning Network algorithm.

3. Results

A total of 30 *C. aurantia* individuals were successfully collected and sequenced for the mitochondrial COI gene region from the Spratly Islands, Vietnam. The average sequence length obtained was 622 bp, with mean nucleotide composition of 29.73% thymine (T), 28.96% cytosine (C), 23.44% adenine (A), and 17.88% guanine (G) (Table 1). This base composition is consistent with previous studies of COI sequences in coral reef fishes, such as those reported by Fadli et al. (2020) [9], supporting the accuracy of target region selection in this study.

Table 1 Nucleotide composition and sequence length of *C. aurantia* COI gene

Sample IDs	T	C	A	G	Sequence length
C.aurantia-ID48	29.74	28.94	23.47	17.85	622
C.aurantia-ID49	29.74	28.94	23.47	17.85	622
C.aurantia-ID50	29.74	28.94	23.47	17.85	622
C.aurantia-ID51	30.02	28.73	23.43	17.82	623
C.aurantia-ID63	29.74	28.94	23.31	18.01	622
C.aurantia-ID64	29.74	28.94	23.47	17.85	622
C.aurantia-ID65	29.74	28.94	23.47	17.85	622
C.aurantia-ID66	29.74	28.94	23.47	17.85	622
C.aurantia-ID67	29.74	28.94	23.31	18.01	622
C.aurantia-ID68	28.99	29.63	23.35	18.04	621
C.aurantia-ID69	29.74	28.94	23.31	18.01	622
C.aurantia-ID70	29.90	28.78	23.47	17.85	622
C.aurantia-ID72	29.74	28.94	23.47	17.85	622
C.aurantia-ID73	29.58	29.10	23.47	17.85	622

C.aurantia-ID74	29.74	28.94	23.47	17.85	622
C.aurantia-ID75	29.74	28.94	23.47	17.85	622
C.aurantia-ID76	29.74	28.94	23.31	18.01	622
C.aurantia-ID77	29.90	28.78	23.31	18.01	622
C.aurantia-ID78	29.74	28.94	23.63	17.68	622
C.aurantia-ID79	29.86	28.89	23.43	17.82	623
C.aurantia-ID80	29.74	28.94	23.47	17.85	622
C.aurantia-ID81	29.74	28.94	23.47	17.85	622
C.aurantia-ID82	29.58	29.10	23.47	17.85	622
C.aurantia-ID88	29.74	28.94	23.47	17.85	622
C.aurantia-ID89	29.74	28.94	23.47	17.85	622
C.aurantia-ID90	29.74	28.94	23.47	17.85	622
C.aurantia-ID91	29.58	29.10	23.31	18.01	622
C.aurantia-ID92	29.74	28.94	23.47	17.85	622
C.aurantia-ID93	29.74	28.94	23.47	17.85	622
C.aurantia-ID94	29.74	28.94	23.47	17.85	622
Average	29.73	28.96	23.44	17.88	622

Haplotype analysis identified 15 distinct haplotypes based on COI sequences, showing an uneven distribution concentrated around a dominant haplotype (Table 2). Genetic diversity analyses revealed a high level of haplotype diversity at 0.7885 ± 0.0795 and low nucleotide diversity at 0.004738 ± 0.002838, suggesting considerable variation within the studied *C. aurantia* population (Table 3). Polymorphism analysis identified a total of 32 variable sites across the COI sequences, of which 22 were singleton mutations (Table 3). Among these were 21 transitions, 8 transversions, and 3 insertions/deletions (indels) (Table 3). This contrasts with the findings of Fadli et al. (2020) [9] , who reported no indels in COI regions among 72 coral reef fish species (including *C. aurantia*), indicating that the Spratly population may have experienced distinct mutation pressures and selection events that contributed to increased genetic divergence. Genetic analysis based on pairwise sequence comparisons revealed a moderate degree of haplotype divergence (Figure 1), with an average inter-haplotype difference of 2.956322 ± 1.591434 nucleotides (Table 3). Notably, haplotype 7 exhibited an exceptional degree of divergence, differing by up to 15 nucleotides from its closest counterpart (Figure 1). Mutation mapping pinpointed this divergence to a cluster of substitutions located within the 550–600 bp region of the COI gene (Figure S1), suggesting a history of accumulated mutations in this lineage.

Table 2 Haplotype analysis in COI gene of *C. aurantia*

Haplotype numbering	Count (Frequency)	Sample IDs
Haplotype 1	1 (3.33%)	*C.aurantia*-ID48
Haplotype 2	1 (3.33%)	*C.aurantia*-ID49
Haplotype 3	1 (3.33%)	*C.aurantia*-ID50
Haplotype 4	1 (3.33%)	*C.aurantia*-ID51
Haplotype 5	2 (6.67%)	*C.aurantia*-ID63, *C.aurantia*-ID67
Haplotype 6	14 (46.67%)	*C.aurantia*-ID64, *C.aurantia*-ID65, *C.aurantia*-ID66, *C.aurantia*-ID72, *C.aurantia*-ID74, *C.aurantia*-ID75, *C.aurantia*-ID80, *C.aurantia*-ID81, *C.aurantia*-ID88, *C.aurantia*-ID89, *C.aurantia*-ID90, *C.aurantia*-ID92, *C.aurantia*-ID93, *C.aurantia*-ID94

Haplotype 7	1 (3.33%)	*C.aurantia*-ID68
Haplotype 8	1 (3.33%)	*C.aurantia*-ID69
Haplotype 9	1 (3.33%)	*C.aurantia*-ID70
Haplotype 10	1 (3.33%)	*C.aurantia*-ID73
Haplotype 11	1 (3.33%)	*C.aurantia*-ID76
Haplotype 12	1 (3.33%)	*C.aurantia*-ID77
Haplotype 13	1 (3.33%)	*C.aurantia*-ID78
Haplotype 14	1 (3.33%)	*C.aurantia*-ID79
Haplotype 15	1 (3.33%)	*C.aurantia*-ID82
Haplotype 16	1 (3.33%)	*C.aurantia*-ID91

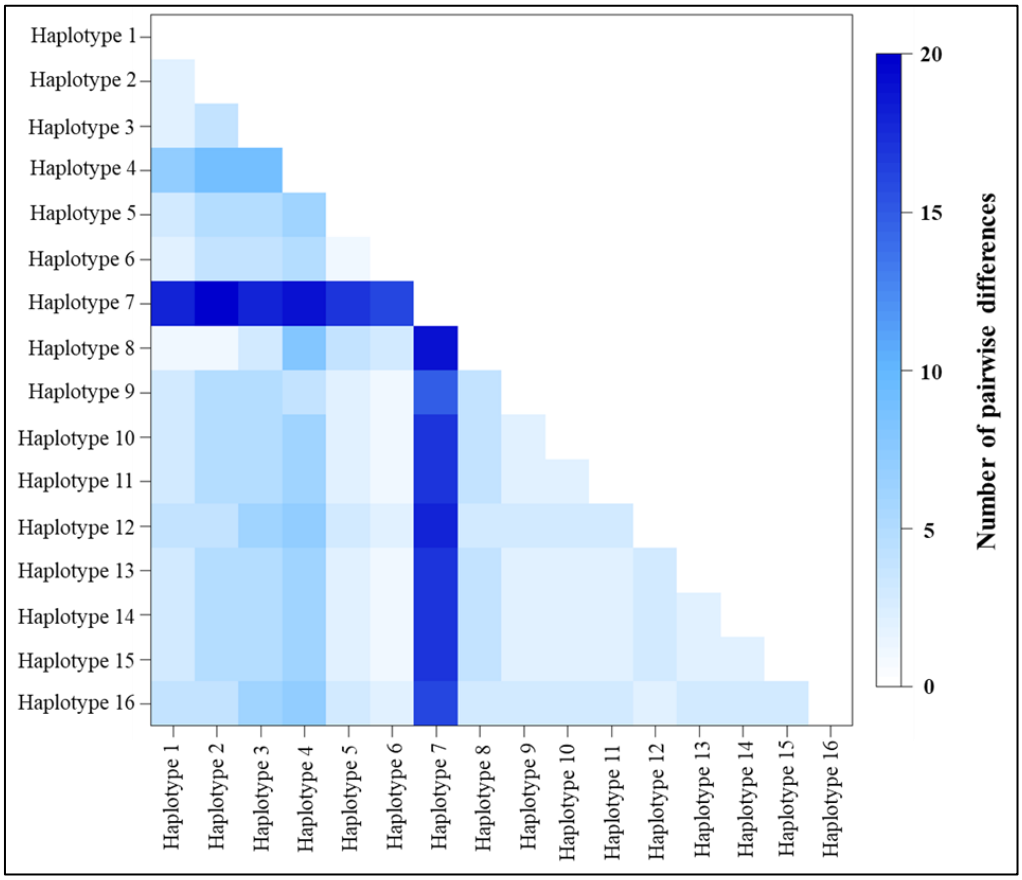

Figure 1 Nucleotide pairwise differences in COI gene of *C. aurantia* haplotypes

Table 3 Genetic diversity analysis of the COI gene

Measurements	**Values**
Haplotype diversity	0.7885 ± 0.0795
Nucleotide diversity	0.004738 ± 0.002838
Number of pairwise differences	2.956322 ± 1.591434

Polymorphic sites	32
Number of singleton mutations	22
Number of observed transitions	21
Number of observed transversions	8
Number of observed indels	3

We further investigated population dynamics and mutational patterns in the COI gene to assess their potential implications for the genetic structure of *C. aurantia*. Statistical analysis results of consistently negative values across multiple statistical tests: Tajima's D was significantly negative (–2.24695, $p < 0.01$); Fu's Fs was strongly negative (–7.379); Fu & Li's D* and F* were both negative with significance at $p < 0.02$; and low values were observed for the raggedness index ($\boldsymbol{r}$ = 0.024) and the Ramos-Onsins and Rozas $\boldsymbol{R2}$ (0.0787) (Table 4). These findings, along with the high number of singletons detected (Table 3), align with scenarios of population growth or directional selection acting on the population. Despite the strong indications of a demographic expansion, certain haplotypes display unusually high divergence levels, deviating from the expected smooth, unimodal distribution characteristic of a recent population expansion model (Figure S2). This suggests the possibility of secondary contact or introgression from genetic distinct source populations, potentially leading to asynchronous demographic expansions among different subgroups that have yet to fully exchange genetic material.

Table 4 Summary of DnaSP neutrality and population dynamics test results

Neutrality tests	**Values**
Tajima's D	-2.24695, ($p < 0.01$)
Fu's Fs	-7.379
Fu and Li's D*	-3.28228 ($p < 0.02$)
Fu and Li's F*	-3.47211 ($p < 0.02$)
Raggedness, $\boldsymbol{r}$	0.0240
Ramos-Onsins and Rozas, $\boldsymbol{R2}$	0.0787

To gain deeper insights into the genetic relationships and potential origins of the observed haplotypes, we constructed a phylogenetic tree and a haplotype network. Maximum Likelihood (ML) analysis based on COI sequences of *C. aurantia* revealed clear genetic differentiation, forming two major clades (Figure 2A). Clade I contained the majority of the samples, displaying a compact and closely related structure, whereas Clade II exhibited more pronounced branching and greater genetic distances among certain sample pairs. The haplotype network further clarified the relationships among haplotypes. Clade I exhibited a typical star-like pattern, characterized by multiple satellite haplotypes surrounding a central, dominant haplotype, each differing by only a single nucleotide. This suggests that the surrounding haplotypes likely arose through recent mutational events. In contrast, Clade II comprised fewer individuals and presented a more dispersed network topology, with singletons connected by multiple mutations (mostly two nucleotide differences), indicative of an older lineage that has accumulated mutations over time. Additionally, several peripheral haplotypes showed marked divergence from the main cluster, suggesting that they may have originated from external sources. These haplotypes likely represent migrant or introgressed lineages that have entered the population, potentially triggering complex, non-uniform demographic expansions.

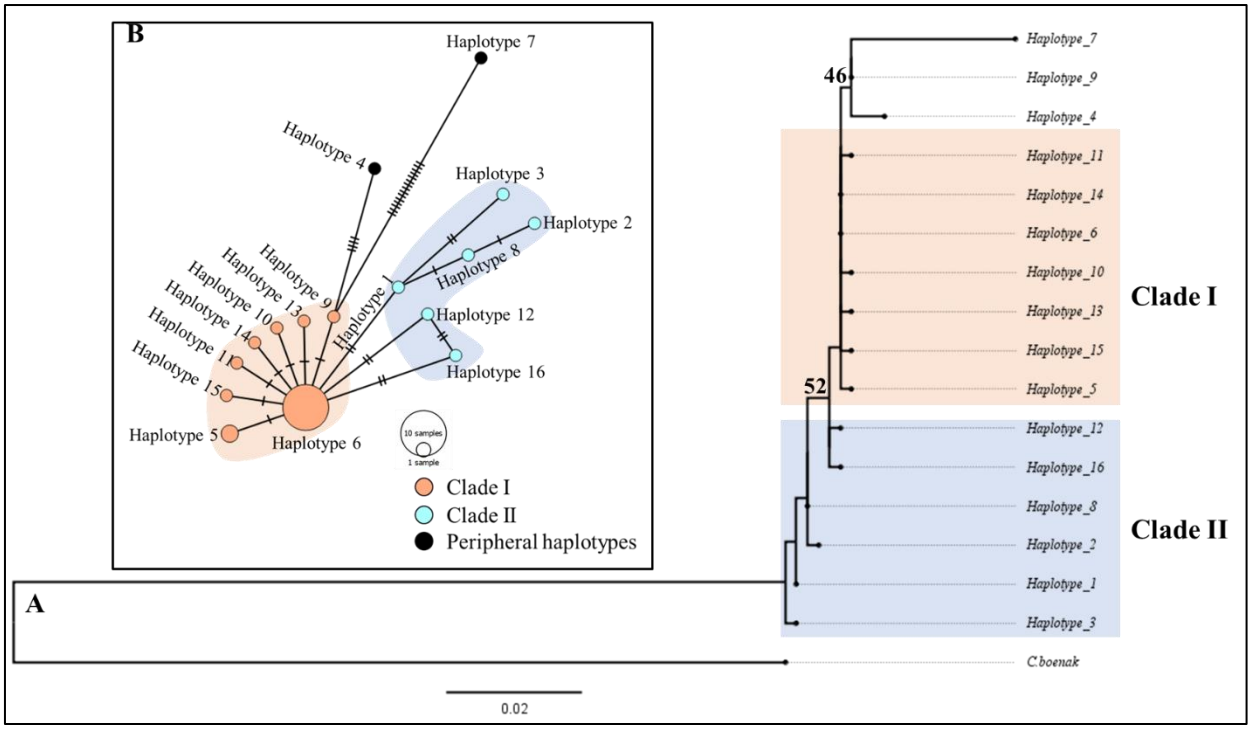

Figure 2 The phylogenetic tree and haplotype network of *C. aurantia* based on COI gene

4. Discussion

We conducted a genetic diversity survey of the mitochondrial COI gene in *C. aurantia* individuals from the Spratly Islands, Vietnam. The results showed that the studied population exhibits a relatively high haplotype diversity (0.7885 ± 0.0795, > 0.5), but a low nucleotide diversity (0.004738 ± 0.002838, < 0.005). This pattern is indicative of a population that is not in genetic equilibrium [29]. Additionally, the presence of substantial nucleotide differences, a high variance in mean pairwise differences, and a predominance of singleton mutations (22 out of 29 polymorphic sites) typically suggests a scenario of complex (multi-phase) population expansion or selective pressures that promote the retention of rare variants. Population dynamics analyses and neutrality tests consistently yielded significantly negative values, reinforcing the hypothesis that *C. aurantia* populations in the Spratly Islands are experiencing a genetic disequilibrium caused by demographic expansion [30], [31], [32], [33], [34]. These findings support a scenario in which the current population has likely undergone a historical bottleneck, followed by a post-bottleneck expansion and potential immigration from genetically distinct sources. This hypothesis is further supported by the haplotype network and phylogenetic analyses. The network shows a characteristic pattern of population expansion, with a dominant central haplotype surrounded by multiple satellite haplotypes differing by single mutations. This star-like configuration is commonly associated with rapid growth from a single ancestral lineage. The ML phylogenetic tree also revealed two major clades: a large group (Clade I) of highly similar individuals, likely representing the dominant expanding lineage; and a smaller, more genetically diverse group (Clade II) along with several independent haplotypes. These more divergent lineages may reflect older genetic lineages or isolated populations shaped by geographic, ecological, or historical evolutionary barriers.

Multiple factors may have contributed to the observed gene flow and complex population structure of *C. aurantia* in the Spratly Islands. This archipelago is situated within the Coral Triangle, a region that is widely recognized for its extraordinary biodiversity. It is characterized by an extensive coral reef system that provides a diverse range of habitats and ecological niches. Such environmental heterogeneity plays a significant role in reducing interspecific competition and promoting population diversification [35]. This environmental mosaic likely fosters local adaptation, resulting in genetic differentiation among populations inhabiting distinct reef environments. In addition to natural environmental factors, the species' biological traits may also play a significant role in shaping its genetic structure. *C. aurantia* exhibits high fecundity [3], with the potential for year-round reproduction, particularly intense during the December to March period [13]. Its larvae are known for their robust dispersal capabilities, long pelagic duration, and broad environmental tolerance, which facilitate effective gene flow and mitigate the effects of genetic drift even among geographically isolated populations [36], [37], [38], [39].

Importantly, signals of deeply diverged genetic variants and evidence of a complex population history have been detected in our study population. These findings indicate that *C. aurantia* may have undergone a period of population decline, potentially driven by environmental stressors or anthropogenic impacts, followed by demographic recovery and subsequent expansion. This highlights the importance of considering the current genetic structure of *C. aurantia* populations in the Spratly Islands in the context of conservation and sustainable fisheries management. Recognizing the existence of genetically distinct and recovering populations is critical for implementing effective conservation and exploitation strategies [40]. Although our study is limited by sample size and the use of a single mitochondrial marker (COI), it nonetheless provides valuable insights into the genetic variability and population dynamics of *C. aurantia* in a biologically and geopolitically significant region. Future studies are urgently needed to assess the extent of population expansion and to obtain a more comprehensive understanding of this species' evolutionary history. Such efforts should include broader spatial sampling, increased sample sizes, and multilocus analyses involving nuclear DNA markers, microsatellites, or even complete mitochondrial genome sequencing.

5. Conclusions

The analysis of COI gene diversity in *C. aurantia* populations from the Spratly Islands reveals signs of demographic imbalance, characterized by high haplotype diversity coupled with low nucleotide diversity and substantial genetic differentiation among haplotypes. Neutrality tests and population size change assessments support a scenario of post-bottleneck population expansion. These findings underscore the urgent need for effective management and regulation of *C. aurantia* fisheries to support conservation efforts and ensure the long-term sustainability and resilience of this ecologically valuable and dynamic population. Future research should be expanded to monitor temporal changes in genetic diversity and to assess potential population shifts associated with environmental stressors or fishing pressure. Such efforts will be essential for developing adaptive management strategies that protect the genetic integrity and ecological function of *C. aurantia* populations in the region.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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Appendix

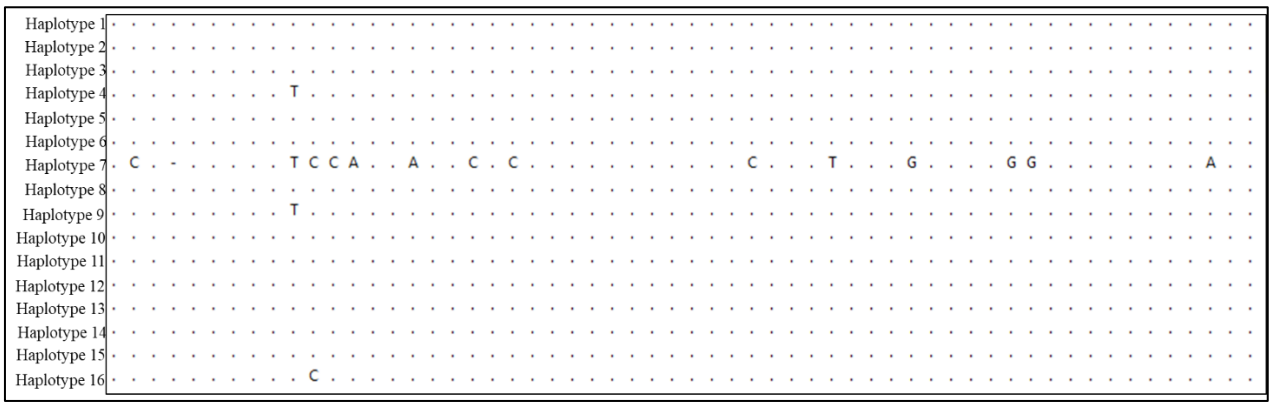

Figure S1 The mutation in the haplotype 7

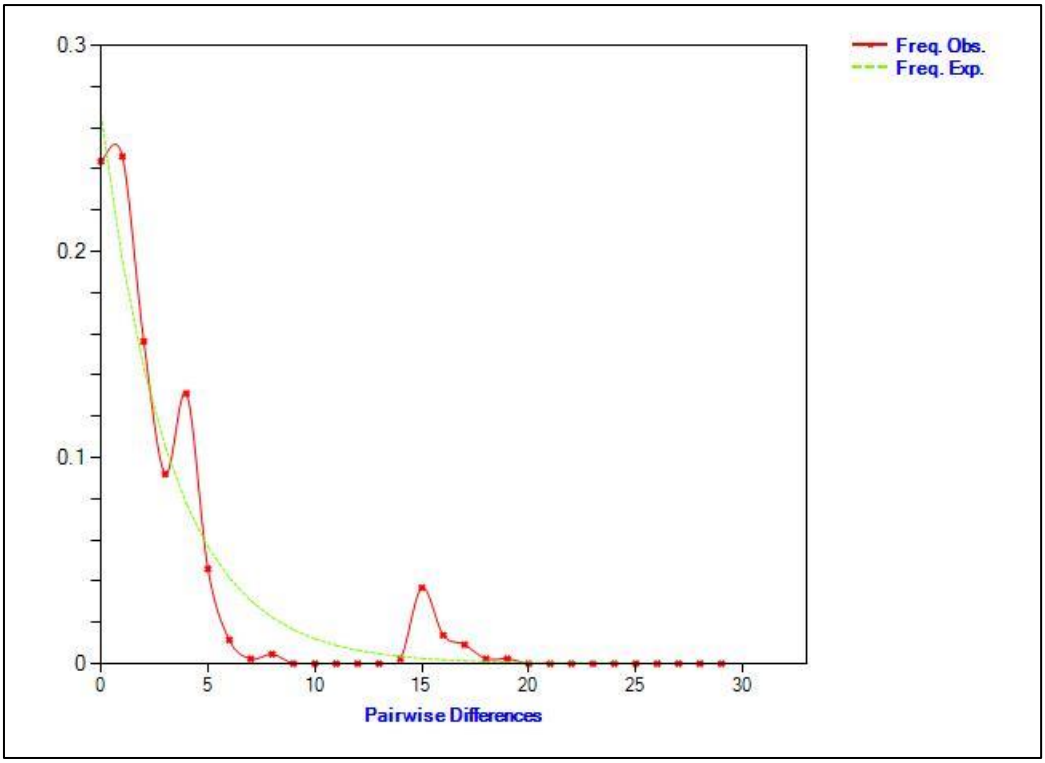

Figure S2 The pairwise differences (mismatch distribution) in COI of *C. aurantia* by DNAsp6