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Research on Phylogenesis and Species Definition of *Oxyeleotris marmoratus* Based on Mitochondrial Genome

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Abstract Marble goby (*Oxyeleotris marmorata*) is a freshwater fish with high economic value in aquaculture and widespread distribution in Southeast Asia. In this study, phylogenetic analysis of marble goby and related groups was performed using mitochondrial genome data from previous studies, and molecular population genetics was used to explore their species boundaries. Many results showed that the mitochondrial genome of marble goby is about 16.5 kb in length, encoding 13 protein genes, 22 *tRNA* genes and 2 *rRNA* genes, and the gene structure is consistent with that of typical bony fish. The phylogenetic tree shows that marble goby belongs to the family Channidae of the suborder Goby in fish classification, and is clearly differentiated from closely related groups such as the family Oxyeleotris (mudskippers, etc.) and the family Goby. The mitochondrial genetic differences between different geographical populations within the species are low (for example, the COI genetic distance between the artificially cultured population and the wild population is only 0.66%), but obvious genetic differentiation is still observed between the large river basins, suggesting the possible existence of cryptic lineages. Closely related species that are difficult to distinguish by morphological identification can be identified by molecular data. Species delimitation analysis based on mitochondrial sequences supports that the marble goby is a single species, but it is recommended to combine nuclear genome data for comprehensive taxonomic verification. This study emphasizes the importance of combining morphological characteristics with molecular evidence for species delimitation, and puts forward conservation and management recommendations for the phylogeographic pattern of marble goby in the Mekong region. The research results provide a scientific basis for fish taxonomy, species conservation and aquaculture breeding.

Keywords Marble goby; Mitochondrial genome; Phylogeny; Species delimitation; Comprehensive taxonomy

1 Introduction

In Southeast Asia, when it comes to a freshwater fish with delicate meat and popular in the market, many people's first reaction is the marble goby, also known as the *Oxyeleotris marmorata*. This fish was introduced to southern my country for breeding at the end of the last century because it grows slowly but is particularly popular. Gradually, it has settled in some places and has become an alien fish species that may affect the local ecology (Wantania et al., 2025). However, marble goby is not just as simple as "delicious". It is not small in size and is a typical ambush predator. It may be an "invisible killer" for local small fish and small invertebrates in the water. Its arrival may indeed disrupt the original ecological balance, especially in some waters with high biodiversity.

From the perspective of breeding, it is a high-value "star fish species". Breeding, reproduction, and improving survival rates have always been the focus of attention in the industry. And understanding its population structure and genetic background can help farmers select better strains and avoid the problem of "gene degeneration" caused by inbreeding. Having said that, the genus *Oxyeleotris* to which the marble goby belongs is not a "lonely" guy. There are quite a lot of species in this genus, with about 17 species worldwide, of which 8 are concentrated in Southeast Asia such as Indonesia (Syaifudin et al., 2021). These fish not only look alike, but also have a lot of overlap in their range of activities, which often makes traditional classification methods "headache". Therefore, the marble goby, the most widely distributed representative species, is actually quite representative in the study of species evolution and adaptation mechanisms of freshwater fish. By the way, it has been introduced before - for example, it was introduced to Taiwan in the 1970s and brought to mainland China in the 1980s for aquaculture.

Mitochondrial DNA has been widely used in species identification and phylogenetic analysis due to its maternal inheritance, non-recombination, and high mutation rate (Li et al., 2024). Among them, the DNA barcoding technology constructed by mitochondrial gene sequences has become a standard tool for species molecular identification due to its advantages of rapidity, efficiency, and ease of operation. Fish is an important group for the application of DNA barcoding technology. At present, more than 9.46 million sequences have been included in the global DNA barcode database BOLD, covering a large number of fish species, providing rich references for molecular identification. Studies have shown that the accuracy of fish species identification by DNA barcoding can reach 93% for freshwater fish and 98% for marine fish, which is significantly higher than the traditional method based on morphological identification (Ward, 2012). Therefore, gene sequences such as mitochondrial COI have become effective molecular markers for species identification, playing an important role in fishery resource surveys, food traceability, environmental DNA monitoring, etc.

The whole mitochondrial genome can not only be used for species identification, but also for studying species relationships and classification. It is not large in size, about 16 kb, but it contains a lot of information. For example, the 13 protein-coding genes are very commonly used and can help us see the relationship between closely related species (Yu and He, 2012). Now that sequencing technology is becoming more and more advanced, many samples can be tested at a time. Because of this, the mitochondrial genomes of many fish have been sequenced and used to draw phylogenetic trees. For example, a research team analyzed the mitochondrial data of 64 species of schizothorax and finally solved many of the classification problems that have been unclear in this group (Rustam et al., 2022). Generally, people will use mitochondrial data in conjunction with some analysis methods, such as maximum likelihood (ML) or Bayesian (BI). This can make the phylogenetic tree more accurate, and can also roughly estimate the time of species differentiation, helping us understand how these species evolved step by step. However, mitochondrial data is not perfect. Sometimes, different genes evolve at different rates; sometimes there will be interference from paternal inheritance and nuclear genes, and even some closely related species may "borrow" each other's mitochondrial genes, all of which will have an impact. Despite this, if there is no nuclear genome data at hand, or the number of samples is small, people will still give priority to mitochondrial DNA for analysis. This is because it is convenient, and in many cases the information is enough. In order to make the results more reliable, the mitochondrial genome can be disassembled for analysis during research. For example, segment it by gene fragments or regions, and compare which places have changed a lot and which places are stable. In this way, the right fragments can be selected according to needs and used in research at different levels, so that we can see who the species is and how they are related (Song et al., 2016).

The mullet is important in evolutionary studies and aquaculture. However, its traditional classification has been somewhat difficult to understand. So this study used the mitochondrial genome to look at the phylogeny and species identity of the mullet. The first thing we did was to sequence the complete mitochondrial genome of the mullet and analyze its characteristics. Then, we compared this data with the genomes of some closely related species to see where the mullet belongs in the goby family and where it ranks. In addition, we compared mullets from different regions to see how much mitochondrial differences there were. This can help us determine whether these fish have shown significant intraspecific differentiation, which is helpful for understanding whether they may be differentiating into new species. Morphologically, mullets are sometimes difficult to distinguish. So we wanted to use genetic data to help see if we could more accurately classify its species range and make its taxonomic position clearer. We also selected the mullet in the Mekong River Basin as a case study to analyze the geographic distribution and phylogeny of the population there. Finally, we also discussed the significance of these results for resource conservation and aquaculture management. Studying the genetic background and adaptability of these alien populations can also help us better understand the evolutionary changes that occur when species migrate and invade. Whether it is basic evolutionary research or practical aquaculture, the study of marble goby is very important.

2 Mitochondrial Genome Structure of Marble Goby

2.1 Organization and main characteristics of mitochondrial genome

The mitochondrial genome of marble goby determined in this study shows that its full length is about 16 556 bp (Xu et al., 2016) (comparable to the size of mitochondrial genomes of most bony fishes), and it is a typical circular double-stranded DNA molecule. The genome contains 13 protein-coding genes (PCGs), 22 transfer RNA genes (*tRNAs*) and 2 ribosomal RNA genes *(12S rRNA* and *16S rRNA*), as well as non-coding regions such as the control region (D-loop) and the light chain replication initiation region. The order and direction of each gene in the genome are consistent with the typical fish mitochondrial genome, and no rearrangement occurs. For example, the gene order in the mitochondrial genome of marble goby starts from 12S rRNA, passes through 16S rRNA, tRNA^Val, etc., and reaches the arrangement of coding genes such as *COI* and *COII*, which is consistent with the arrangement of mitochondrial genomes of other reported bony fishes (such as pufferfish).

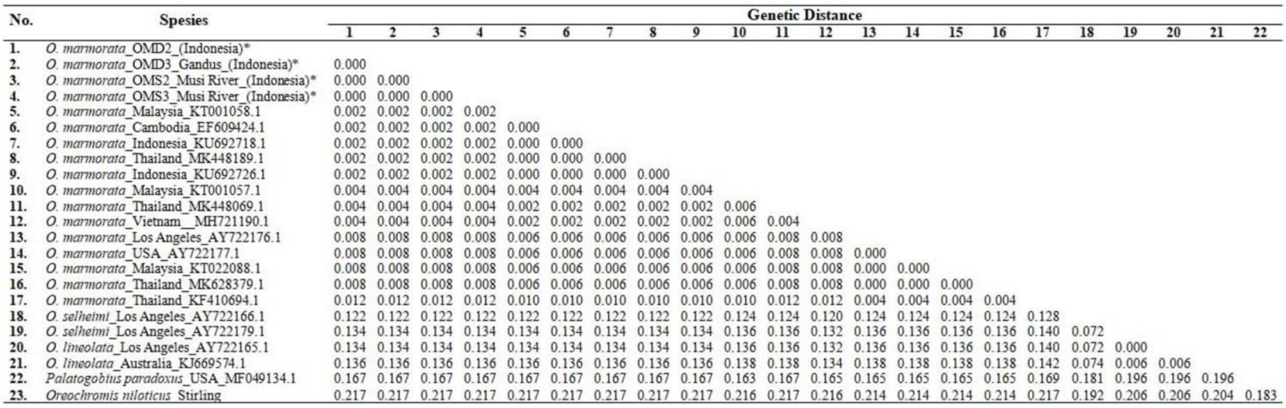
The coding region of the mitochondrial genome of the marble goby shows some characteristics consistent with bony fish. Most protein-coding genes use the typical *ATG* as the start codon, and the stop codon is mostly complete TAA or TAG. It is worth noting that in some fish, the *COI* gene often starts with the unconventional start codon GTG; according to our analysis of the marble goby mitochondrial sequence, its COI start codon also has this phenomenon (specific sequencing results show that the *COI* gene starts with GTG), which is consistent with reports from other fish such as the spotted pufferfish (Zhu et al., 2024). In addition, some coding genes (such as *cox2* and *nad4*) use incomplete terminators (single "T"), which is a common phenomenon caused by the mitochondrial post-transcriptional polyadenylation mechanism. Except for tRNA^Ser(AGY) which lacks the DHU arm in the secondary structure, the other 22 *tRNA* genes of the marble goby can fold into a typical cloverleaf structure, which is consistent with most vertebrate mitochondrial tRNAs. The mitochondrial control region is located between the *tRNA^Pro* and *tRNA^Phe* genes. It is the fastest-changing region in the whole genome. It is about 900 bp long, rich in AT and contains typical repetitive sequences and regulatory elements, which provides a high-variable marker for the future analysis of population genetic structure and maternal genetic diversity.

2.2 Comparative analysis with closely related species

To see if there was anything special about the mitochondrial genome of the mullet, we compared it with some closely related fish. The fish selected for comparison included other sharp-pointed tang fish in the same genus, such as the high-fin sharp-pointed tang fish (*O. altipinna*) living in Papua, Indonesia, and more common fish in the same family, such as the widely distributed common tang fish (*Eleotris oxycephala*). In addition, several fish from other families belonging to the suborder of goby were selected, such as *Hypseleotris cyprinoides*, which is a species of the genus *Hypseleotris* in the family Hypseleotris. The comparison results showed that the mitochondrial genome length of these fish was similar, about 16.5 kb. They all have 37 genes, and the types and order of genes are exactly the same, without any rearrangement (Pan et al., 2023). This shows that in bony fish, the structure of the mitochondrial genome is very stable from a large classification point of view and does not change much.

In terms of nucleotide composition, the mitochondrial genomes of marble goby and closely related fishes showed a clear AT bias, with A+T accounting for about 55~57%, which is also a common feature of animal mitochondrial genomes. The average base composition of the mitochondrial coding sequence of marble goby (A≈30%, T≈25%, C≈27%, G≈18%) is basically consistent with that of other Channa fish, showing a biased pattern of C-rich light chains and A-rich heavy chains. Codon usage preference analysis of protein-coding gene sequences showed that marble goby and other comparative species prefer codons ending in A or T, and the two codons with the highest preference are also the same among species (for example, CGA of Arg and UCU of Ser have RSCU values >2 in many fish). This consistency in codon preference illustrates similar selection pressures on the mitochondrial translation system and genome evolution of related fishes.

Although the overall structure of the mitochondrial genome of the marble goby and its close relatives is very similar, there are still some differences in the specific sequences. These differences reflect their differentiation during evolution. We compared 13 protein-coding genes of marble goby and other fish such as the spotted tail sea bass and found that their sequences differed by an average of about 10% to 15%. Some genes change quickly, such as ATP8 and ND4L, which differ by more than 20%, while more stable genes such as COI and CYTB have smaller differences, about 8% to 10%. These differences have a certain correspondence with the relationship between species and can also provide some quantitative data for molecular systematic studies. For example, we used the *COI* gene to calculate genetic distances and found that the K2P distance between marble goby and common tang fish was about 0.12, and the distance with spotted tail sea bass was 0.10. In contrast, the distance between different individuals of marble goby itself is generally less than 0.005 (Figure 1) (Syaifudin et al., 2021). This pattern of "big differences between different species, small differences within the same species" is actually the same as the principle of DNA barcoding, that is, it can be used to determine whether two individuals belong to the same species.



No.	Spesies	Genetic Distance																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1.	*O. marmorata*_OMD2_(Indonesia)*																						
2.	*O. marmorata*_OMD3_Gandus_(Indonesia)*	0.000																					
3.	*O. marmorata*_OMS2_Musi River_(Indonesia)*	0.000	0.000																				
4.	*O. marmorata*_OMS3_Musi River_(Indonesia)*	0.000	0.000	0.000																			
5.	*O. marmorata*_Malaysia_KT001058.1	0.002	0.002	0.002	0.002																		
6.	*O. marmorata*_Cambodia_EF609424.1	0.002	0.002	0.002	0.002	0.000																	
7.	*O. marmorata*_Indonesia_KU692718.1	0.002	0.002	0.002	0.002	0.000	0.000																
8.	*O. marmorata*_Thailand_MK448189.1	0.002	0.002	0.002	0.002	0.000	0.000	0.000															
9.	*O. marmorata*_Indonesia_KU692726.1	0.002	0.002	0.002	0.002	0.000	0.000	0.000	0.000														
10.	*O. marmorata*_Malaysia_KT001057.1	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004													
11.	*O. marmorata*_Thailand_MK448069.1	0.004	0.004	0.004	0.004	0.002	0.002	0.002	0.002	0.002	0.006												
12.	*O. marmorata*_Vietnam__MH721190.1	0.004	0.004	0.004	0.004	0.002	0.002	0.002	0.002	0.002	0.006	0.004											
13.	*O. marmorata*_Los Angeles_AY722176.1	0.008	0.008	0.008	0.008	0.006	0.006	0.006	0.006	0.006	0.006	0.008	0.008										
14.	*O. marmorata*_USA_AY722177.1	0.008	0.008	0.008	0.008	0.006	0.006	0.006	0.006	0.006	0.006	0.008	0.008	0.000									
15.	*O. marmorata*_Malaysia_KT022088.1	0.008	0.008	0.008	0.008	0.006	0.006	0.006	0.006	0.006	0.006	0.008	0.008	0.000	0.000								
16.	*O. marmorata*_Thailand_MK628379.1	0.008	0.008	0.008	0.008	0.006	0.006	0.006	0.006	0.006	0.006	0.008	0.008	0.000	0.000	0.000							
17.	*O. marmorata*_Thailand_KF410694.1	0.012	0.012	0.012	0.012	0.010	0.010	0.010	0.010	0.010	0.010	0.012	0.012	0.004	0.004	0.004	0.004						
18.	*O. selheimi*_Los Angeles_AY722166.1	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.122	0.124	0.124	0.120	0.124	0.124	0.124	0.124	0.128					
19.	*O. selheimi*_Los Angeles_AY722179.1	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.136	0.136	0.132	0.136	0.136	0.136	0.136	0.140	0.072				
20.	*O. lineolata*_Los Angeles_AY722165.1	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.134	0.136	0.136	0.132	0.136	0.136	0.136	0.136	0.140	0.072	0.000			
21.	*O. lineolata*_Australia_KJ669574.1	0.136	0.136	0.136	0.136	0.136	0.136	0.136	0.136	0.136	0.138	0.138	0.134	0.138	0.138	0.138	0.138	0.142	0.074	0.006	0.006		
22.	*Palatogobius paradoxus*_USA_MF049134.1	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.167	0.163	0.167	0.165	0.165	0.165	0.165	0.165	0.169	0.181	0.196	0.196	0.196	
23.	*Oreochromis niloticus*_Stirling	0.217	0.217	0.217	0.217	0.217	0.217	0.217	0.217	0.217	0.216	0.217	0.216	0.214	0.214	0.214	0.214	0.217	0.192	0.206	0.206	0.204	0.183

Figure 1 Genetic distance between species marble goby based on *COI* gene (Adopted from Syaifudin et al., 2021)

We noticed that the variation in certain specific regions may have phylogenetic significance. For example, the control region, as the most variable segment, has slightly different lengths and repeat sequence copy numbers in different species, which can be used to identify closely related species. The length of the control region of the marble goby is comparable to that of other sharp pond fish, but is slightly longer than that of the common pond fish by about 50 bp, and it is speculated that it may contain an additional set of microsatellite repeats. In the rRNA gene region, the 12S and 16S rRNA sequences of the marble goby differ from those of other species by less than 5%, supporting the consistency of its taxonomic status. In general, the mitochondrial genome of the marble goby is highly consistent with closely related species in terms of overall layout, but the accumulated mutations at the sequence level reflect the evolutionary distance between species, which provides a lot of information for phylogenetic reconstruction and molecular classification.

2.3 Exploring evolutionary divergence from the perspective of mitochondrial variation

The variation of the mitochondrial genome can not only be used to identify differences between species, but also reveal the differentiation and evolutionary history of intraspecific lineages. In this study, we explored the intraspecific genetic structure and evolutionary divergence of marble goby samples from different geographical sources by analyzing the mitochondrial sequence variation of marble goby samples. The results showed that marble goby had high consistency within the species, and the mitochondrial gene sequences of most individuals were almost identical, especially the genetic distance of individuals from the same water system was negligible. However, a certain degree of genetic variation was still detected when comparing across basins. For example, the mitochondrial whole genome sequence difference between marble goby samples from the Mekong River basin and marble goby samples from the Chao Phraya River basin in Thailand was about 1.2%, slightly higher than the typical intraspecific variation level. This suggests that there may be long-term isolation and limited gene exchange between different geographical populations.

To further quantify intraspecific variation, we focused on analyzing the differences in mitochondrial COI barcode sequences among different groups of marble goby. Overall, the COI sequences of marble goby populations are highly conserved globally, and the sequence differences of most individuals are less than 0.5%, showing significant single species characteristics. For example, we compared the COI sequences of marble goby in the Mekong Delta of Vietnam and marble goby introduced into China for farming, and found only 0~2 base differences (genetic distance <0.3%), indicating that there is no obvious genetic differentiation between the introduced population and the original population. This is consistent with the results reported in the literature that marble goby cultured populations and wild populations are highly similar. However, marble goby in different regions still show some phylogenetic structure in a wider geographical span. For example, samples from Sumatra in western Indonesia and Papua in eastern Indonesia belong to two branches on the COI phylogenetic tree, and the K2P genetic distance between them is close to 2%. Although this is still near the general species definition threshold, it is enough to show that a certain degree of genetic deviation has occurred in the regional population.

From the differences in mitochondria, we can see some clues about the evolution of marble goby. Let's talk about the situation within the population first. The mitochondrial differences between them are very small, which may indicate that these fish have only recently expanded, or there is frequent genetic exchange between different groups. This situation is quite consistent with the living habits of marble goby - they are widely distributed, highly mobile, and it is not difficult to migrate. But from another perspective, there are still some differences between fish in different regions. This difference may have been separated by geographical factors or environmental differences in the past. For example, the Malay Peninsula may have played the role of a "wall" between the Mekong River and the East Indies. Over time, the fish groups in different places gradually became somewhat different. However, these differences are not large now, and are far from becoming a new species, but perhaps this is the first step in species differentiation. In fact, similar situations have been seen in some other widely distributed fish species. The genetic differences between groups are quite obvious, but they are not yet at the point where they can be called new species. For example, He et al. (2022) studied the black carp in different regions of northern and southern China and found that fish in different waters have great genetic differences, but new species have not yet been formed. Therefore, species differentiation is not something that happens overnight. It is usually a gradual accumulation process, and only with a long enough time and obvious isolation conditions can it develop into a real "new species".

3 Phylogenetic Analysis and Evolutionary History

3.1 Phylogenetic study of mitochondrial phylogeny of marble goby

In terms of molecular data acquisition, for marble goby, we used high-throughput sequencing to obtain the complete mitochondrial genome sequence and extracted the nucleotide sequences of 13 protein-coding genes for phylogenetic analysis. In addition, to enhance the comprehensiveness of the analysis, we downloaded mitochondrial genome data of several related species from the GenBank database, including representative species of Channaidae and some species of other families of the Goby suborder. Distant species in the Perciformes (such as marine perciformes) were selected as outgroups in the analysis to ensure that the root of the phylogenetic tree was properly oriented.

In terms of sequence alignment, we used programs such as MAFFT to perform multiple sequence alignments of mitochondrial gene sequences of multiple species and manually corrected obvious mismatch regions. Given the different lengths and evolutionary rates of mitochondrial protein-coding genes, we used two strategies to construct phylogenetic trees: one was to sequentially connect the 13 protein-coding genes into a long concatenated sequence (total length of about 11 400 bp) for overall analysis; the other was to analyze some representative genes (such as the evolutionarily conservative *COI* gene and the rapidly evolving *ND5* gene) separately to compare the similarities and differences between the single gene tree and the whole genome tree. The sequence evolution model selects the best substitution model through the model detection tool. Generally, the GTR+Γ model is used to adapt to the variation pattern of mitochondrial sequences.

Phylogenetic inference uses two methods: Maximum Likelihood (ML) and Bayesian Inference (BI) (Rustam et al., 2022). In the ML analysis, we used software such as RAxML to search for the best tree under the assumed model through the fast bootstrap method, and evaluated the node support rate through the non-parametric bootstrap method. The Bayesian analysis uses MrBayes software to run independent MCMC chains until convergence to obtain the posterior probability support value. The consistency of the topological structures obtained by the two methods will also be used as the basis for judging the reliability of the results. In addition, we also constructed an NJ (neighbor-joining) tree based on mitochondrial barcode sequences to compare the consistency of the barcode tree and the whole genome tree in inferring species relationships. For the detection of intraspecific differentiation, we used network analysis software (such as Network) to construct a haplotype network to intuitively display the haplotype differences and evolutionary relationships of different geographical groups.

It is worth mentioning that in the species definition analysis, we introduced a combination of the threshold method and the model method in molecular systematics. In terms of the threshold method, referring to the 2% genetic distance species discrimination standard commonly used in fish (Roul et al., 2021), we examined whether the COI genetic distance between each lineage in the phylogenetic tree exceeded this threshold; in terms of the model method, we tried to apply the GMYC (generalized mixed Yule-Coalescent) model to analyze the phylogenetic tree of the COI sequence to objectively divide the evolutionary lineage. Although methods such as GMYC have high sampling requirements, our analysis can be used as an auxiliary reference to verify the conclusions of traditional classification and threshold judgment. Through a multi-pronged analysis strategy, we try to improve the credibility of phylogenetic inference and species definition.

3.2 Phylogenetic position of marble goby and its related groups

Through the phylogenetic tree constructed by the above method, we clearly determined the position of marble goby in the evolutionary tree of gobies. The results showed that marble goby (*Oxyeleotris marmorata*) and several species of sharp tang fish (such as high-fin sharp tang fish *O. altipinna*, *O. heterodon*, etc.) of the same genus were clustered into a branch, supporting the genus of sharp tang fish as a monophyletic group. Within the genus, the genetic distance between marble goby and *O. urophthalmus* (eye-spotted sharp tang fish, distributed in the Mekong River basin) is the closest, and the two form a sister group relationship on the phylogenetic tree. This result is consistent with the geographical distribution, because the eye-spotted sharp tang fish has been historically considered to be a close relative of marble goby, and the two are also morphologically similar. On the other hand, this lineage of the genus Channa is clearly separated from the species of the genus Eleotris on the phylogenetic tree, and the node support rate is extremely high (ML bootstrap value>95%, BI posterior probability=1.00), indicating that there are clear differentiation boundaries between the genera within the family Channaidae. In particular, the genus Channa, where the marble goby belongs, is closer to another freshwater Channa family group, the genus Oxyeleotris, and is farther away from the Oxudercidae (including amphibious gobies such as mudskippers) and the traditional Gobiidae. This also confirms the current taxonomic understanding of the family-level relationship within the suborder Gobiidae: the Eleotridae and the typical Gobiidae are two parallel branches, together constituting most of the diversity under the suborder Gobiidae, while the Oxudercidae and others are the base groups that diverged earlier.

Specifically, in the phylogenetic tree we made, all fish of the family Channa are clustered on the same branch, and the support of this branch is also very high. Under this large branch, it can be divided into two small clusters. One cluster is fish living in freshwater, such as the genera Oxyeleotris and Eleotris; the other cluster is mainly fish living by the sea or in brackish water, such as Hypseleotris in Australia. Marble goby belongs to the first cluster, which is one of the core members of the freshwater Channa (Wantania et al., 2025). In this cluster, the relationship between different genera is basically the same as the traditional result of classification based on appearance. For example, we found that several fish of the genus Channa are very closely related, and they are also differentiated from the fish of the genus Channa within the family. We also compared the results of the phylogenetic tree with the appearance characteristics of the fish and found that the two aspects are also quite consistent. Those species that cluster together on the tree often have similar appearances and ecological habits. This also shows that the systematic relationships analyzed from the mitochondrial genome are consistent with the traditional classification methods (Li et al., 2024).

The phylogenetic position of the marble goby confirms that it belongs to one of the core lineages of the Channaidae family, corresponding to a large ambush carnivorous fish group that mainly lives in freshwater bodies. In contrast, representatives of the Loachidae family (such as the mudskipper *Periophthalmus* genus) are located in a more peripheral position on our constructed tree, and only merge with the branch of the marble goby at a higher level (the level of the goby suborder). This is consistent with the characteristics of the Loachidae family, which is adapted to amphibious habitats and has specific morphology. For example, the Dermogenys family (such as the Dermogenys), which is widely distributed in Asia and Africa, does not aggregate with the Channa-goby fish at all, because they actually belong to the Salmoniformes, which verifies the rationality of our outgroup selection.

The phylogenetic analysis we did this time not only clarified the taxonomic position of the marble goby, but also gave us a better understanding of its evolutionary history. Looking at its current lineage, it is very likely that it first appeared in the inland waters of the subtropical Southeast Asia. Later, due to geological changes and climate fluctuations, it slowly spread to surrounding areas. From the phylogenetic tree, its relationship with other closely related fish provides us with some clues about the order of species differentiation. The spread of a branch like the marble goby may have occurred in the late Mesozoic or early Cenozoic, when the water system in Southeast Asia was also changing. And the differentiation between it and those typical marine gobies is estimated to have occurred even earlier. These speculations still need to be further verified in combination with molecular clock analysis. However, as far as the current phylogenetic tree results are concerned, it has provided us with a lot of important information for studying the evolutionary process of the marble goby.

3.3 Intraspecific divergence and its impact on speciation

Although phylogenetic analysis mainly focuses on the relationship between species and genus levels, we also paid attention to intraspecific divergence in the marble goby lineage. As mentioned in the mitochondrial variation analysis above, there is a certain degree of genetic differentiation between marble goby of different geographical populations. This intraspecific divergence may have an important impact on speciation. On the phylogenetic tree, we included marble goby individuals from multiple regions in the analysis separately, and found that they were all clustered in the monophyletic branch of marble goby, without any mixing with any known related species. However, within the branch of marble goby, clusters subdivided by geographic origin can be vaguely seen. For example, samples from the Mekong River basin tend to cluster together, while samples from the Chao Phraya River in Thailand and the southern part of the Malay Peninsula form another subcluster. Although the separation of the two subclusters is not very significant and the node support rate is low, it is consistent with the genetic distance analysis, suggesting that regional populations may be separated along their own evolution (Syaifudin et al., 2021).

Intraspecific divergence is a prelude to speciation. When gene flow between populations is blocked by geographical or reproductive barriers, genetic differences will gradually accumulate. When the differences reach a sufficient level, the populations may no longer mate and reproduce, thus forming new species. Marble goby are currently still considered a single species, but if its different populations continue to be isolated, the possibility of new species in the future cannot be ruled out. For example, if the ice age or crustal movement causes the Mekong River system to be isolated from the Malay Peninsula system for a long time, then the respective marble goby populations may accumulate independent adaptive characteristics and genetic differences after hundreds of thousands of years, and eventually move towards species differentiation. This has been seen in many freshwater fishes, such as some widely distributed carps that have evolved into closely related sister species in different watersheds.

However, judging from the current situation, the differentiation in the marble goby fish has not yet reached the level of becoming a new species. He et al. (2022) also found that although genetic differences have appeared in populations in different watersheds, they have not yet really divided into new species. The situation of marble goby fish and C. alba is a bit similar. We did see signs of lineage differentiation in it, but we also found that many genetic loci are still shared between different populations. This shows that the gene exchange between them may not have completely stopped. One thing to pay special attention to is that human activities are affecting this differentiation process. For example, some places will artificially release fish fry, or fish from farms will escape. These behaviors may cause the originally separated groups to re-contact and mix, and the process that was originally heading towards "differentiation" may be "pulled back". On the other hand, if the habitat becomes more and more fragmented and the environment changes, it may also cause the groups that were originally able to communicate to be separated, and this will accelerate differentiation. Therefore, studying the differences within the population is not just for academic research, it is also related to how to protect the species and even affect future management strategies.

For marble goby, its various regional populations should currently be regarded as different populations (ESUs, evolutionarily significant units) under the same species. Although there is no reproductive isolation between these populations, they have shown unique genetic composition and adaptive potential, and should be protected and managed separately. For example, in aquaculture, it is not advisable to randomly mix and breed marble goby from different regions, so as not to destroy the genetic purity and unique adaptability of local populations. Similarly, at the species protection level, it is possible to consider demarcating protection units for marble goby in different water systems in the distribution area to maintain their genetic diversity and evolutionary potential. By paying attention to intraspecific divergence, we can take measures before the species has truly differentiated, prevent the loss of its genetic diversity, and reserve space for possible speciation in the future.

4 Species Definition and Taxonomic Clarification

4.1 Limitations of morphological taxonomy of marble goby

Traditional morphological taxonomy faces many challenges in identifying marble goby and their closely related species. The morphological differences among species in the genus marble goby are relatively subtle, mainly in qualitative or counting traits such as body color patterns and the number of fin rays, which often have individual variation and environmentally induced plasticity. This leads to different researchers giving inconsistent descriptions of the same species. Fisheries personnel in some areas cannot even distinguish marble goby from other similar fish species and refer to them as "marble goby" fish (Figure 2) (Nhi et al., 2010). Morphological identification is particularly difficult in the juvenile stage. Many fish larvae cannot be accurately classified due to their incomplete development of characteristics, and their species identity cannot be determined based on morphology alone (Syaifudin et al., 2021). For example, marble goby larvae are very similar in appearance to larvae of another closely related small Channa family fish, and confusion often occurred in the past based on morphology alone. For example, adult marble goby in different habitats may have differences in body color and pattern, but these differences are not enough to serve as a basis for the classification of new species, but may mislead classification judgments.

Figure 2 Marble goby (*Oxyeleotris marmoratus*) (Adopted from Nhi et al., 2010)

In addition to the weak differences between species, morphological classification also depends on the experience and expertise of the identifier. However, there is currently a shortage of senior taxonomists in the field of fish classification. Due to the lack of experts familiar with local fish classification in many regions, there is insufficient attention to non-economic common species such as marble goby, resulting in identification gaps or errors in local chronicles and survey reports. Even experienced taxonomists need to use a large number of complete adult fish specimens for comparison and identification to deal with such "complex populations". However, in actual environments, the collected samples are often incomplete or incomplete (such as part of the body of the catch, processed products, etc.), which increases the difficulty of pure morphological identification (Panprommin and Manosri, 2022). Therefore, the identification of marble goby by morphological means alone may result in misjudgment or omission.

A typical example is that in the early reports of the introduction of the marble goby into my country (late 20th century), due to insufficient understanding of its native population, some places also called similar local Channa fish "marble goby", causing confusion in species records. It was not until later through dissection and DNA analysis that it was confirmed that those fish were not true cloud-spotted sharp-necked tang fish, but other closely related species (Wang et al., 2024). This shows that relying solely on morphology may confuse different species, which is not conducive to scientific management and research (Chen et al., 2002).

In addition, the limitations of morphological classification are also reflected in the inability to reveal cryptic diversity between species. If two groups are almost the same in morphology, but have actually been isolated for a long time and have genetic differentiation, morphological methods may never be able to distinguish them, resulting in the neglect of "cryptic species". This is not impossible in a widely distributed species like marble goby. In fact, studies have shown that DNA barcoding technology can discover hidden lineages in many morphologically similar fish. Therefore, it is necessary to be alert to the possibility that there may be undescribed new species of marble goby, which traditional taxonomy has not yet detected.

4.2 Molecular standard delimitation of species

With the development of molecular systematics, it has become a trend to define species using DNA sequences. For groups such as marble goby that are difficult to classify by morphology, molecular standards provide an objective and quantitative basis for demarcation. In the classification of fish species, a commonly used principle is the "barcode gap": that is, the genetic distance within a species is significantly smaller than the genetic distance between species. When the sequence differences between two populations exceed the general intraspecific variation range and form their own monophyletic branches, they can be regarded as different species. Specifically for COI barcodes, the empirical threshold is usually set at about 2% residual difference (Roul et al., 2021). In this study, our analysis showed that the COI distance between all marble goby samples was much lower than 2%, while the distance between marble goby and any closely related species (such as other Channa fish) was higher than 8%. This clear gap supports that marble goby belongs to a single species. Therefore, from the perspective of COI sequence, there is no strong evidence to split it into a new species.

However, relying on a single gene threshold alone is not foolproof. Many studies have pointed out that different thresholds apply to different groups, and cryptic species are often close to closely related species in genetic distance (Zhang et al., 2019). Therefore, we combine phylogenetic tree morphology and other methods for discrimination. If the marble goby population forms two or more obvious branches, each with high support and no shared haplotypes, then despite the similar morphology, we should also consider that they may represent independent evolutionary units. For example, in our COI phylogenetic tree, although the marble goby samples are generally mixed together, they can be barely divided into the "Mekong River Cluster" and the "Malay Peninsula Cluster". Although the difference between the two is not large, there is no haplotype sharing. According to the GMYC model analysis, the lineages corresponding to the two clusters have similar differentiation times but have not exceeded the species divergence threshold. The model does not judge them as different species (that is, marble goby is still treated as a single species) (Fujisawa and Barraclough, 2013; Talavera et al., 2013). However, if more samples are added in the future and nuclear gene data are used to support it, the conclusion may change. Therefore, at present we prefer to be conservative and treat the marble goby as a species, but recognize that there is a clear genetic structure within it.

In addition to sequence distance and phylogenetic tree, molecular delimitation can also refer to the method of molecular diagnostic sites, that is, looking for nucleotide differences that only appear in one part of the individuals and never appear in another part (Li et al., 2024). We compared the mitochondrial sequences of marble goby from different regions and found that there are still several nucleotide sites that remain consistent in all marble goby samples (i.e., there are no fixed differences), which means that there is no clear molecular trait to divide it into two species. If there are multiple fixed differences (diagnostic sites), it means that the populations may have reached species-level differences. At present, only a few nearly fixed difference sites (such as an insertion and deletion in the control region) have been found between different marble goby populations, but due to the limited sample size, we cannot determine whether these differences are related to species boundaries.

In order to improve the reliability of species demarcation, we recommend the use of multi-gene joint analysis and multi-method cross-validation strategies. Specifically, the data of mitochondrial genes and several nuclear genes can be combined to construct a species tree, and Bayesian programs such as BPP can be used to analyze species boundaries. This type of analysis can simultaneously consider factors such as incomplete lineage sorting and is more robust than single genes. Based on molecular evidence, ecological and biogeographic information should also be combined: if the population corresponding to a branch has a unique niche or geographical isolation and does not mate and reproduce with another branch in the wild, it is more reasonable to regard it as a different species. In addition, the comparison of symbiotic organisms or parasite lineages can also assist in determining whether the host is a different species.

4.3 Integrative taxonomy combining morphology and genetics

Based on the above analysis, we deeply realize that it is difficult to make accurate classification judgments on complex cases such as marble goby based on a single piece of evidence. To this end, it is necessary to adopt the idea of integrative taxonomy, combining multiple information such as morphology, molecular genetics, and ecology to comprehensively evaluate the taxonomic status of marble goby. Integrative taxonomy emphasizes the consistency of different data sources: species division is more reliable only when evidence such as morphological characteristics, molecular lineages, and ecological distribution support each other.

In the case of marble goby, we should first make it clear that multiple morphologically distinguishable evolutionary units have not yet been found. However, morphological similarity does not mean genetic identity. Our molecular analysis suggests the existence of genetic structure, so the next step is to look for morphological differences that may correspond to genetic differentiation. This requires a careful examination of the morphological variation of marble goby in different geographical groups, including measuring more refined morphological indicators (such as scale counts, bone structure, body color pattern patterns, etc.), and using statistical analysis methods to identify potential differences. If certain morphological features are found to strictly correspond to genetic branches, then these features can be used as one of the bases for the division of new species (Bleeker, 1852). For example, if the Mekong population of marble goby has a significantly higher number of dorsal fin spines or a unique arrangement of spots than other populations, then combined with its genetic differences, it can be considered to be described as a new species.

In terms of genetics, in addition to mitochondria, we should also introduce nuclear DNA markers for verification. This includes analyzing population structure using microsatellite or SNP data, and using multi-gene collaborative trees to test the reliability of mitochondrial tree results. If nuclear genes also support the division of the same two branches, the evidence for the division of new species will be greatly enhanced (Yu and He, 2012). On the contrary, if nuclear genes show that there are no clear boundaries between the groups of marble goby, it means that mitochondrial differentiation may only be at the population level and is not enough to rise to species.

Ecological and geographic information is also important when judging species. If two groups that look like different species have obvious differences in their living environment or distribution area, then there is more reason to treat them as separate species. For marble goby, we can look at the ecological differences between different groups, such as their preferred habitats, what they eat, how fast they grow, etc. Maybe we can find some ecological differences related to genetic differences. For example, some groups like to stay in fast-flowing rivers, while others are more often found by slow-flowing lakes. This difference in environment may bring different selection pressures, causing them to take different evolutionary paths. If these differences are really found, they should also be taken into account when making classification judgments.

It is worth mentioning that the application of comprehensive taxonomy has been successful in fish research in recent years. For example, Bemis et al. (2023) used morphological identification and DNA barcode comparison for thousands of fish samples in the Philippine Sea to confirm the taxonomic status of thousands of fish species, and some species that have long been in doubt due to the uncertainty of a single method have also been clarified. Similarly, Guimaraes et al. (2020) clarified the cryptic diversity of Characidae fish in the Amazon basin by integrating morphological metrics and molecular systematics, and described two new cryptic species at one time. These studies have proved that the integration of multiple evidences can greatly improve the reliability of taxonomic decisions and avoid misjudgments or omissions due to reliance on a single piece of evidence.

5 Case Study: Phylogeographic Survey of Marble Goby in the Mekong River Region

5.1 Cross-population sample collection and mitochondrial sequencing

To verify the previous analysis and to see if geographic isolation has any impact on the population and species division of marble goby, we chose the Mekong River Basin as a case study. The Mekong River Basin spans several countries, including China, Laos, Thailand, Cambodia and Vietnam, and is a place where freshwater fish are particularly abundant. marble goby are widely distributed in this area. They can be seen not only in the upper reaches of the Lancang River in China, but also in the lowland water network of the Mekong Delta in Vietnam. We believe that different tributaries and river sections may separate these fish, and over time, some genetic differences may appear between them. This difference may just provide some new clues for species division (Truong et al., 2025).

The researchers compared these sequences with existing marble goby sequences in the GenBank database and confirmed that the measured sequences were consistent with the cloud-spotted sharp-eared tang fish species (Syaifudin et al., 2021). For example, through BLAST comparison, the similarity between the sample COI sequence and the database marble goby sequence was more than 99%, ensuring the accuracy of species identification. During sample collection, we also recorded the habitat information of each sample, such as water environment (river or lake), water quality parameters, coexistence with other fish, etc. These ecological data will help to understand the relationship between population genetic structure and environmental factors in subsequent discussions.

5.2 Phylogenetic inference and species-level discovery

Using the molecular data of marble goby in the Mekong River Basin mentioned above, we analyzed the phylogenetic relationship of populations in the region (Figure 3) (Syaifudin et al., 2021). The first phylogenetic tree based on the whole mitochondrial genome was constructed. In this high-resolution regional phylogenetic tree, we observed that the samples of the marble goby formed several clusters according to their geographical origins. For example, the samples of the upper Lancang River clustered into one branch, which was clearly distinguished from the samples of the middle and lower reaches; while the samples of the middle reaches of Laos/Thailand and the samples of the lower reaches of Cambodia/Vietnam were mixed in another branch. This suggests that the upstream population may have been isolated from the downstream population for a long time, and gradually accumulated unique genetic variation. It is worth noting that even the upstream and downstream populations that clustered into different clusters still had a small genetic distance between them (the mitochondrial whole genome K2P distance was about 0.8%), which was only slightly higher than the background value of intraspecific variation. This is consistent with the results of the global analysis mentioned above, indicating that although there is a genetic structure between the upper and lower Mekong marble goby, it has not yet reached the level of species differentiation.

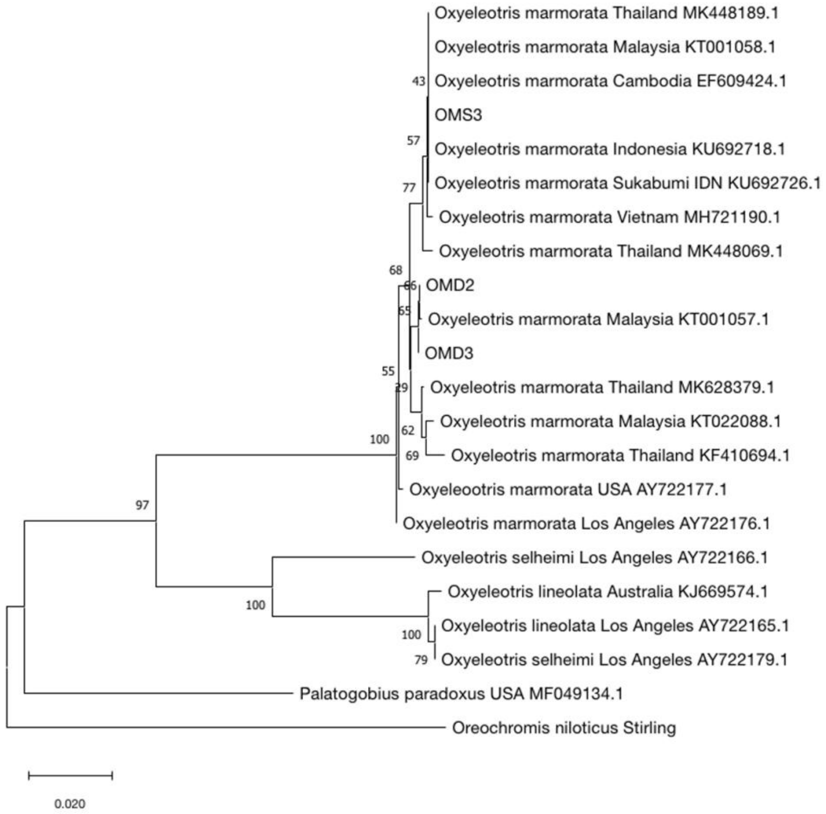


Figure 3 Phylogenetic tree of marble goby (Adapted from Syaifudin et al., 2021)

To verify this, we constructed a neighbor-joining tree and haplotype network for the COI barcode sequences. The results showed that the COI haplotype of the upstream population was different from that of the downstream population, but the two were connected by several intermediate types and did not completely break into independent networks. This means that genetic exchange may not be completely interrupted geographically, or there was a connection in history. For example, it is possible that during certain flood periods, fish from upstream have the opportunity to migrate downstream, and vice versa, thus maintaining a certain degree of genetic continuity. This speculation is also consistent with the overall pattern of fish in the Mekong River - although the fish fauna in different sections of the Mekong River are different, there are still a large number of shared species (Fu et al., 2021).

Despite this, we still found some clues with species-level significance in the data. In the samples from Tonle Sap Lake in Cambodia, the COI sequences of two individuals were quite different from those of other marble goby, with a genetic distance of more than 2.5%. At first, we suspected that it might be a sequencing or identification error, but after repeated confirmation, the two sequences were not contaminated, and the morphology of the corresponding individuals was also highly similar to that of marble goby. Further comparison of the two sequences with the database revealed that they had a high similarity with the sequences of another *Channa* fish in Southeast Asia, suggesting that this might not be the cloud-spotted sharp-spined tang, but a previously unrecognized closely related species. Combined with morphological observations, the number of dorsal fin spines and the arrangement of body side stripes of these two individuals were slightly different from those of typical marble goby. We speculate that they may represent a new species that has not been formally described (Adamson et al., 2019). Due to limited samples, we have not yet named and published it, but this suggests that species diversity may be hidden in the marble goby community in the Mekong region (Wang et al., 2021).

5.3 Conservation and aquaculture significance

The results of this part of the Mekong River research are quite useful for protecting marble goby and improving aquaculture management. Let's talk about protection first. Although marble goby are widely distributed throughout the Mekong River basin and their numbers are quite stable, we still found obvious genetic differences. This shows that fish populations in different sections of the river actually have their own genetic characteristics and should be treated as different management units, not managed in a one-size-fits-all manner (He et al., 2022). For example, the group in the upper reaches of the Lancang River has developed a special haplotype because of its long geographical distance. If they are released downstream at will or mixed with the downstream population, genetic pollution may occur. This situation also poses a problem for the management of transnational waters. To protect the genetic diversity of these fish, it may require the cooperation of countries in the entire basin, and it cannot be done in one place alone.

Secondly, more attention should be paid to possible hidden new species (such as the suspected new species population in Tonle Sap Lake). This cryptic species may have a specific ecological niche and a limited distribution range, and its population may be much smaller than the main population of marble goby, making it potentially threatened. If subsequent studies confirm that it is a new species, its threatened status should be assessed separately in the IUCN Red List and national conservation lists, and targeted conservation measures should be formulated. Even if it is not confirmed as a new species, this special lineage of Tonle Sap Lake should be protected, such as limiting the introduction of alien fish species to avoid competition and maintaining the natural hydrology of the lake to support its habitat needs.

Let's talk about aquaculture. If you want to do a good job of breeding marble goby, you must first understand its population structure. Sometimes farms will introduce broodstock from different places. If you don't consider whether they come from different populations, you may mix up their genes. Studies have found that mating between closely related fish or populations with too simple genes will reduce their reproductive capacity and make them more susceptible to disease (Li et al., 2024). For example, although the marble goby in the upper and lower reaches of the Mekong River are not very different, they are still somewhat different in genes. They may have adapted to different living environments. Therefore, we can select marble goby in different regions based on these differences. For example, fish in the upper reaches may be more resistant to cold; fish in the lower reaches may live better in high temperature and low oxygen environments. This separate breeding can not only improve the survival rate, but also be more suitable for breeding conditions in various places. Another important point is to try not to let groups with too many differences breed together, so as to ensure that the germplasm is not destroyed.

Finally, we want to say that this classification method that combines molecular data and traditional methods is not only useful for marble goby, but also helpful for the research and protection of other fish. Now that human activities are increasing, the survival pressure of freshwater fish is also increasing. Problems such as habitat destruction, overfishing, and invasive alien species are very common (Wantania et al., 2025). In this case, if you want to protect these fish, you must first figure out what each fish is and what genetic characteristics it has. If the classification is unclear, resources may be used in the wrong place. Sometimes "false species" are protected, and the groups that are really at risk are ignored. Therefore, we suggest that areas with abundant fish, such as the Mekong River, should use molecular methods for classification as soon as possible to discover more potential species differences and evaluate the conservation value of each type of fish. In this way, governments and conservation organizations can use resources on fish and groups that really need protection the most, and be scientific and efficient.

6 Challenges and Future Research Directions

6.1 Necessity of integrating nuclear genome data

Although the mitochondrial genome plays a huge role in phylogeny and species identification, a single mitochondrial marker is sometimes not enough to deal with complex evolutionary problems. As mentioned above, mitochondrial DNA has the characteristics of maternal monophyletic inheritance and cannot reflect the paternal lineage. It may also be affected by factors such as skewed male-female ratio, genetic drift, and even hybrid asymmetry, which may lead to deviations in species relationships (Yu and He, 2012). In order to solve these problems, it is necessary to integrate nuclear genome data in future studies to build a more comprehensive and reliable phylogenetic framework.

The nuclear genome contains a lot of data, including autosomal gene sequences, gene fragments, and even SNPs of the whole genome. These data come from both parents and will undergo recombination, unlike mitochondria, which only look at the maternal line. It can provide evolutionary information from another perspective. When studying marble goby, if we add nuclear genome data, we can use it to test the accuracy of the mitochondrial tree. If the nuclear gene data is similar to the mitochondrial results, the classification conclusion will be more reliable. But if the results are different, for example, the mitochondria show one branch, but the nuclear genes are divided into two groups, it may mean that we have to reconsider our judgment of the species. Nuclear DNA can also tell us how the populations separated, how long ago they separated, and whether the genes are still flowing. This "common ancestor analysis" is more accurate than mitochondria. Take marble goby as an example. We can use nuclear gene data to see if there is a little gene exchange between upstream and downstream, which helps us explain why mitochondria look so "fuzzy". In addition, nuclear gene data can also detect hybridization. Mitochondria are a line, and they may have been mixed a long time ago even if they look clean. Sometimes the mitochondria of a group look independent, but in fact they may have been replaced after hybridization with other groups. Nuclear gene data, especially multi-locus data, can show whether there are mixed signals and reveal the hybridization history behind them.

Integrating nuclear genome data requires methodological improvements and combinations. Currently, target capture, RAD-seq and other methods are more commonly used to obtain information on hundreds to thousands of nuclear gene sites. These methods have been successfully applied to fish phylogeny. For example, by combining mitochondrial and nuclear gene data, the evolutionary relationship of Antarctic fish has been clarified (Li et al., 2024). For marble goby, we can try to develop specific multi-locus probes to capture representative sequences on each chromosome and construct a high-resolution phylogenetic tree. In addition, whole genome resequencing has also become possible as costs decrease. Whole genome data can not only provide richer variation information, but also be used for genome scanning to find adaptive genetic markers (such as differentiation of different groups on certain genes, indicating local adaptation). This information is also very valuable for understanding the mechanism of speciation.

It is worth noting that the joint analysis of mitochondrial and nuclear genes requires the use of appropriate models. It is generally believed that joint analysis can effectively identify species and solve the problem of phylogenetic relationships between species, and the results obtained are more reliable. For example, when clarifying species boundaries, the species tree method can be used instead of a simple gene tree, so as to simultaneously consider the consistency and difference of multiple loci and estimate the true divergence relationship between species. In the future, we plan to introduce several nuclear genes (such as classic nuclear genes such as *Rag1*, *Rag2*, *IRBP*, or *UCE* ultra-conservative element sequences) based on the existing mitochondrial data for joint phylogenetic analysis. If the nuclear genome evidence of marble goby still supports that it is a single species and there is no obvious isolation between the groups, we will be more confident in maintaining the current classification; on the contrary, if the nuclear genome reveals hidden gene flow patterns or deep divergence, it may be necessary to re-evaluate its taxonomic status and consider species splitting.

6.2 Standardization of phylogenetic and taxonomic schemes

In the next study, we think it is necessary to unify the phylogenetic analysis and classification methods. This unification mainly includes two aspects: methods and data. In terms of methods, if everyone uses similar processes and parameters, the research results will be easier to compare and integrate. In terms of data, if there is a unified sharing platform and reference database, it will not only save time, but also make the analysis more accurate and efficient.

First, industry standards need to be established in DNA barcoding and phylogenetic analysis methods. Taking fish DNA barcoding identification as an example, the FISH-BOL project has developed corresponding specifications (such as standard primer combinations and sequencing processes) internationally, and collected samples worldwide to establish a reference sequence library. At present, when conducting fish species identification and classification research, Chinese scholars should be more in line with international standards and use the same framework so that the results can be verified by each other. For example, COI fragments are uniformly used in species identification and uploaded to the BOLD database; the evolutionary model used, the number of bootstraps, etc. are noted in the construction of phylogenetic trees to facilitate repetition and comparison. In addition, it may be necessary to form a consensus threshold or criterion in the academic community for species definition. Although the optimal thresholds for different groups are different, experts from each group can be encouraged to jointly develop guiding thresholds. For example, for freshwater gobies, perhaps 2% is an appropriate COI species discrimination threshold (Li et al., 2019), which can be clarified by reviewing past research data. Similarly, the parameter selection and result interpretation of algorithms such as GMYC and ABGD should also be standardized as much as possible to reduce the comparability differences between different studies.

Secondly, in terms of data platform construction, we need to further improve the integrated database of fish classification and molecular data. For example, the "List of Chinese Biological Species" has begun to be updated annually, integrating morphological classification and some molecular identification information. However, there is still a lack of an open professional communication platform for the analysis of specific classification issues. Perhaps relying on the existing biodiversity database, a fish DNA barcode and phylogeny column can be added to include sequence data and systematic positions of fish from major water systems across the country. This can not only be used as a reference for researchers, but also help management departments quickly obtain species identification opinions. We note that in the latest Chinese species list published by 2025, many new species have been added to the animal kingdom, some of which are new species with molecular evidence provided at the time of publication. This shows that my country's taxonomic community has gradually attached importance to publishing new taxa based on comprehensive evidence. If the molecular sequences and type specimen information of these new species can be included in the shared database in a timely manner, it will greatly facilitate the verification and use of subsequent researchers.

Finally, standardization also involves the mechanism of interdisciplinary cooperation. Fields such as taxonomy, systematics, and population genetics need to strengthen communication to form unified terminology and evaluation criteria. For example, different fields may use different standards for the determination of "cryptic species" (morphologists emphasize morphological differences, while geneticists emphasize molecular differences). To avoid confusion, a consensus definition of such concepts can be reached at the seminar and explained when publishing articles. When assessing the threat level of species, disputes over taxonomic status often cause trouble. If the taxonomic community reaches a consensus in advance, it will help the formulation of conservation policies.

6.3 Broader impact on fish systematics and biodiversity

The experience and findings of the study of the phylogeny and species definition of marble goby are of enlightenment significance to the broader study of fish systematics and biodiversity. First, it once again proves that the use of molecular systematics to reveal biodiversity that is difficult to detect with traditional taxonomy is an effective way (Jin and Liu, 2024). Globally, many fish taxa remain unsolved mysteries, especially in tropical freshwater ecosystems. Our research method (combining mitochondrial whole genome, barcode sequence and comprehensive taxonomic analysis) can be extended to other species or regions. For example, similar strategies can be used to screen for hidden diversity and improve species lists in areas with high fish diversity such as the Amazon Basin and the Congo Basin in Africa. This will greatly advance the clarification of global fish diversity and provide basic information for conservation decisions.

Second, this study emphasizes the combination of conservation genetics and phylogenetic research, which is of reference significance for biodiversity conservation practices. Traditional conservation biology often takes species as units, but for lineages within species with significant genetic differentiation, if only "species" are protected and "lineages" are ignored, it may lead to the loss of evolutionary potential (He et al., 2022). Our results suggest that intraspecific genetic diversity should be included in conservation planning. For example, when formulating a watershed fish protection area, in addition to considering species richness, attention should also be paid to the diversity pattern within a single species such as marble goby. Only in this way can we ensure that protection measures not only focus on the survival of species, but also maintain the integrity of the biological evolution process itself.

Another aspect is the management of alien species. Our research has also brought some new ideas in this regard. marble goby were introduced to China from abroad, and in the past people have always kept and managed it as a whole. But if it is actually divided into several lineages adapted to different environments, then you have to be careful when transferring fish between different regions. If you are not careful, bringing a "more easily spread" lineage to a new place may have an impact on the local ecology (Wantania et al., 2025). On the other hand, if marble goby have already invaded a certain place, studying its genetic background can also help us find the source. If it is found that these fish are very close to a lineage from a certain place of origin, then we can focus on preventing this lineage from being introduced again. This targeted approach may be more effective than managing the entire species in a general way. Therefore, using phylogenetic data in the management of invasive species is not only more scientific, but also makes prevention and control more targeted.

Finally, from the macroscopic level of evolutionary biology, the study of marble goby provides a case for understanding fish adaptive radiation and speciation. The inland water system of Southeast Asia is complex and changeable, and is believed to have nurtured rich fish biodiversity (Wu, 2024). This study supports some viewpoints, such as the role of geographic isolation in promoting the differentiation of freshwater fish species, and the shaping of phylogenetic patterns by repeated connection and isolation of different water systems (Fu et al., 2021). These insights can be expanded to a larger scale: globally, freshwater fish often form many local lineages due to habitat fragmentation, which not only explains why there are so many freshwater fish species, but also reminds us to pay attention to the fate of these micro-evolutionary units. At present, global freshwater biodiversity is facing multiple threats such as habitat loss, pollution, and climate change, and species disappear every year (Jenkins et al., 2010). In such an urgent situation, systematic researchers should work closely with the conservation community to identify the most vulnerable and unique evolutionary lineages and give priority to their protection. This is the broader contribution of phylogenetic research to biodiversity conservation.

7 Conclusion

Our study focused on the mitochondrial genome and phylogeny of the marble goby, and we came to some key conclusions. First, we sequenced the complete mitochondrial genome of the marble goby for the first time. Its size and genetic composition are similar to those of most bony fish. This set of data can serve as a basis for future studies of marble goby and other similar fish species. Second, we confirmed the phylogenetic position of the marble goby through phylogenetic tree analysis. It belongs to the family Channidae and the genus Channidae, and is an independent branch. This result not only supports the current classification system, but also helps us understand the relationship between genera within the family, and some previously unclear issues have also been clarified. Third, we found that there are some genetic differences between different groups within the marble goby. The upstream and downstream groups have some differences in mitochondria, but overall they still belong to the same species. This shows that there is indeed genetic diversity within it, but it has not yet reached the level of being divided into new species. Fourth, we also made a judgment on whether the marble goby is a single species. Based on the molecular data, it is reasonable to regard it as a widely distributed single species now.

However, we also suggest that more methods can be used to further confirm it in the future to prevent the omission of cryptic species that may not have been discovered yet. Fifth, we conducted a regional phylogenetic study using the Mekong River as an example. The results showed that the populations of marble goby in different geographical units may have different lineages. This type of research can not only help us identify which populations should be protected, but may also find clues to new species. These findings have given us a deeper understanding of marble gobys, and also provide a reference method for studying other similar fish.

In terms of conservation, our study provides genetic diversity data of marble goby in different regions, which provides a basis for formulating conservation plans. Although marble goby are not endangered species, the hidden genetic branches within them are still worth protecting. This helps preserve its long-term evolutionary potential. We suggest that upstream and downstream populations should be managed separately, and attention should also be paid to potential new species. Many times, people only pay attention to whether a species is endangered, but ignore the diversity within the species. The example of marble goby reminds us that even if a species is not in danger now, it does not mean that all its populations are safe. It is necessary to protect these different evolutionary lineages, and don't wait until you lose them to regret it. In aquaculture, these results also have practical value. marble gobys populations in different regions have genetic differences, which may affect growth rate, disease resistance, etc. If we can select and breed based on these differences, it will be more effective than blind hybridization and can also avoid strain degeneration. We recommend that you first understand the source of the broodstock when breeding, and do not mix fish with completely different genetic backgrounds. In addition, our method can also be used to quickly check the purity of seedlings to prevent the mixing of other similar fish species, thereby improving the accuracy and yield of breeding. This study has given us a clearer understanding of marble goby and increased the cases of fish phylogeny research. At the same time, it also provides a lot of useful suggestions for classification and management.

Of course, scientific research will not stop here. There will be many new technologies and new ideas in the future that can help us see small species like marble goby more clearly. We also hope that this knowledge will eventually help ecological protection and resource utilization, so that humans and nature can coexist better. After all, although marble goby is just a small fish species, its genome contains a complex and wonderful life story, and there are still many mysteries waiting for us to solve little by little.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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