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***Research Article** (Open Access)*

MOLECULAR IDENTIFICATION AND ESTIMATION OF GENETIC DIVERSITY USING mtCO1 GENE IN TWO MARINE FOOD FISHES, *CHRYSOCHIR AUREUS* AND *OTOLITHES RUBER*, FROM KAKINADA HARBOUR, ANDHRA PRADESH

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ABSTRACT
Accurate identification of marine fish species is essential for biodiversity conservation and sustainable fisheries management. This study employed mitochondrial cytochrome c oxidase subunit I (mtCO1) gene sequencing to molecularly identify two commercially important marine croaker species, *Chrysochir aureus* and *Otolithes ruber*, collected from Kakinada Harbour, Andhra Pradesh, India. Genomic DNA extracted from muscle tissues was amplified using PCR and sequenced by the Sanger method. The mtCO1 sequences were 706 bp for *C. aureus* and 708 bp for *O. ruber*, with GC contents of 49.29% and 47.74%, respectively. BLASTn analysis confirmed 100% similarity of *C. aureus* with *Chrysochir aurea* voucher ML20 and 99.52% similarity of *O. ruber* with *Otolithes ruber* isolate FGB48. Multiple sequence alignment and Maximum Parsimony phylogenetic analysis demonstrated clear clustering with reference sequences, confirming species identity and genetic relationships. These findings validate mtCO1 DNA barcoding as a reliable tool for molecular identification, genetic diversity assessment, and fisheries conservation.

Keywords: *DNA Barcoding, Cytochrome c Oxidase Subunit I (COI), Otolithes ruber, Chrysochir aureus, Molecular Identification; Marine Biodiversity, Fisheries Management, Phylogenetic Analysis*

INTRODUCTION
Marine ecosystems support diverse plant and animal life that plays vital roles in nutrient cycling and ecosystem health (Alongi, 2002). Fish constitute the most diverse group of vertebrates, exhibiting remarkable variation in morphology, habitat, and ecological function (Kim *et al.,* 2021; Minich *et al.,* 2022). Accurate species identification is therefore essential for biodiversity assessment, ecological studies, conservation, and sustainable fisheries management (Nagelkerken *et al.,* 2008; FAO, 2013).
Conventional morphological identification is often hindered by phenotypic similarity, ontogenetic variation, and the presence of cryptic species, leading to possible misidentification (Hebert *et al.,* 2003). To overcome these limitations, molecular techniques such as environmental DNA analysis, next-generation sequencing, nanopore sequencing, and particularly DNA barcoding have become widely adopted for accurate fish identification (Santanumurti *et al.,* 2024). DNA barcoding is recognized as a rapid, reliable, and cost-effective approach that supports fisheries management, biodiversity conservation, species discrimination, and the detection of seafood mislabelling and illegal trade (Rasmussen and Morrissey, 2008; Bucklin *et al.,* 2011; Elsaied *et al.,* 2021; Ali *et al.,* 2020; Hamza and Mohammed, 2023).
Among molecular markers, the mitochondrial cytochrome c oxidase subunit I (COI) gene is the standard DNA barcode for animals because of its high interspecific and low intraspecific sequence variation, enabling accurate discrimination of closely related species (Hebert *et al.,* 2003; Ward *et al.,* 2005; Ali *et al.,* 2020).
Marine ecosystems support rich biodiversity, with fish being the most diverse vertebrate group. Accurate species identification is essential for biodiversity assessment, conservation, and fisheries management.

However, traditional morphological methods are often limited by phenotypic similarity and cryptic species. Molecular approaches, particularly DNA barcoding using the mitochondrial COI gene, offer a rapid and reliable alternative for species identification due to high interspecific variation. This method is widely applied in fisheries management, biodiversity studies, and detection of mislabelled seafood. Therefore, the present study employed mitochondrial COI gene-based DNA barcoding to molecularly identify the tiger tooth croaker (*Otolithes ruber*) and *Chrysochir aureus* collected from the coastal region of Andhra Pradesh, India.

MATERIAL AND METHODS:

Sample Collection

Fresh and healthy two tiger toothed croaker fish species, which were morphologically identified as *Chrysochir aureus* and *Otolithes ruber* were collected from fishing boats working from a fishing harbour (Long: 83º18' 8.85" E; Lat: 17º 41' 44.71" N) located on the North-East coast of Andhra Pradesh, adjoining the Bay of Bengal, Visakhapatnam, India, between August 2025 and September 2025. The collected fish samples were designated as *Chrysochir aureus* isolate DANU-1 and *Otolithes ruber* isolate DANU-2 and the samples were transferred to sterile zip pouches and maintained in iceboxes until they were brought to the laboratory, where they were maintained in a refrigerator at -20ºC until further analysis.

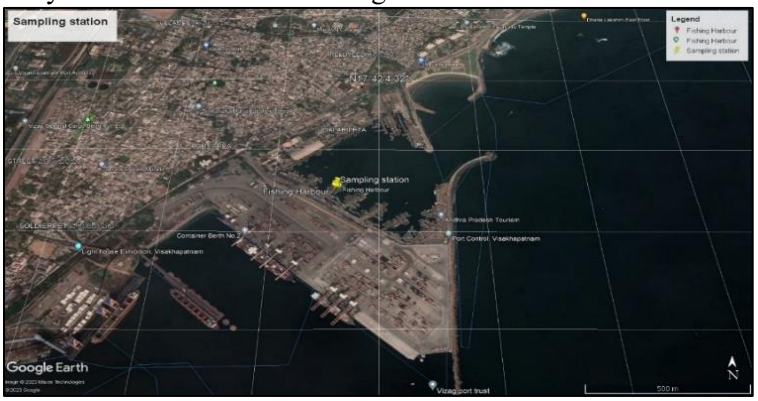

Figure 1. General map of the sample collected area.

Sample Preparation

To prepare the specimen for research studies, the collected fishes were rinsed with running tap water and then with distilled water. Followed by washing, the excess water was removed with blotting paper. The fish were then dissected, and the non-edible organs of the fish such as the offal, bones, and scales were quickly removed to avoid decomposition. The dissected muscle tissue was washed and preserved at -20ºC for future experiments.

Figure 2. Dissected edible muscle tissues of two selected fish species.

Chemicals

All chemicals and reagents used in the study were of analytical grade and procured from Sigma-Aldrich and Merck. Experiments were conducted at room temperature unless otherwise specified.

Molecular Identification

Two marine fish species were identified using mitochondrial cytochrome oxidase subunit I (mtCOX1) gene sequencing. The process involved genomic DNA isolation, PCR amplification of the mtCO1 gene, sequencing, multiple sequence alignment, and phylogenetic analysis.

Genomic DNA Extraction

Genomic DNA was extracted from ~100 mg of muscle tissue using the phenol–chloroform–isoamyl alcohol method (Sambrook and Russell, 2001) with minor modifications. Tissue was homogenized in lysis buffer (Tris-HCl, EDTA, NaCl, SDS), followed by RNase treatment (37°C, 30 min) and proteinase K digestion (55°C, 3 h). After extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1), DNA was precipitated using sodium acetate and cold ethanol. The DNA pellet was air-dried, dissolved in TE buffer, and stored at −20°C. DNA concentration was measured using a UV spectrophotometer.

Qualitative Analysis of DNA

DNA quality was assessed by 0.8% agarose gel electrophoresis in 1× TAE buffer. Samples were loaded with DNA loading dye along with a 1 kb ladder and run at 70 V for 1 hour. DNA bands were visualized using a UV transilluminator and documented**.**

PCR Amplification and Sequencing of mtCO1 Gene

The mtCO1 gene was amplified using specific primers:
Forward: 5′-TCAACCAACCACAAAGACATTGGCAC-3′
Reverse: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′

PCR was carried out in a 50 µL reaction mixture containing template DNA, primers, PCR buffer, Taq DNA polymerase, and dNTPs. Thermal cycling conditions included initial denaturation at 95°C (5 min), followed by 40 cycles of denaturation (95°C, 30 sec), annealing (60°C, 15 sec), and extension (72°C, 30 sec), with a final extension at 72°C (10 min).

Amplified products were verified on 1.5% agarose gel and compared with DNA ladder. PCR products were sequenced using the Sanger dideoxy method on an ABI Prism 3700 DNA analyzer using BigDye Terminator v3.1 kit.

Figure 3. PCR program for the amplification of mtCOI gene.

Sequence Analysis and Phylogenetic Tree Construction

The biologics Corp web server (https://www.biologicscorp.com/tools/GCContent/) was used to compute the nucleotide composition of the mtCO1 gene from the two fish species. The genetic identification and phylogenetic study of the collected fish species were conducted by creating a phylogenetic tree using closely associated species and an outgroup species. The mtCOX1 gene sequence from each species was analysed using the NCBI server's BLASTn to detect homologous species. Depending on the degree of homology with the query gene sequence, similar species were selected from the NCBI database. The gene

sequences used for the evaluation and building of the phylogenetic tree were obtained from the nucleotide database of GenBank. The homology and phylogenetic parameters of each fish species mtCO1 gene sequence was analysed by multiple sequence alignment (MSA) using an advanced cluster approach UPGMA. The MSA was done using Muscle software incorporated in MEGA-X. A distance matrix was computed in order to analyse the variations among the sequences. A maximum-parsimony phylogenetic tree was generated using MEGA-X based on the discrepancies shown in the distance matrix. The tree was subjected to 1000 replicates of the bootstrap method to obtain an accurate result.

RESULTS

Molecular and Phylogenetic Characterisation

Marine fish exhibit high diversity, traditionally identified using morphological traits. However, these features are often lost in processed fish products, leading to misidentification and species substitution (Danezis *et al.,* 2016; Barbosa *et al.,* 2021). To address this, molecular approaches have been widely adopted for accurate species authentication and monitoring of fisheries (Helyar *et al.,* 2014).

Among these, mitochondrial cytochrome oxidase subunit I (mtCO1) gene sequencing has proven to be an effective tool for species identification and taxonomic studies. In the present study, mtCO1 gene amplification using PCR was employed to identify the collected fish species. Molecular characterisation also facilitated the analysis of evolutionary relationships among species, and phylogenetic divergence was estimated using the Maximum Composite Likelihood method (Tamura *et al.,* 2004).

DNA Quality and Quantity

The results are presented as mean ± standard deviation (SD) of three independent experiments. Genomic DNA was successfully extracted from both fish species. The A260/A280 ratios for *C. aureus* (DANU-1) and *O. ruber* (DANU-2) were 1.75 ± 0.01 and 1.83 ± 0.01, respectively, indicating high purity within the acceptable range (1.7–1.9).

DNA quantification was performed using UV spectrophotometry with a standard calibration curve. The DNA concentrations were 712 ± 13 µg/g for *C. aureus* and 659 ± 18 µg/g for *O. ruber*. The integrity of extracted DNA was confirmed by the presence of distinct bands on 1% agarose gel (Fig. 4).

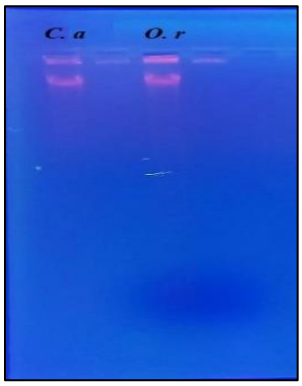

Figure 4. Genomic DNA of two collected fish species on 1% agarose gel [*C. a* - *C. aureus* isolate DANU-1; *O. r* - *O. ruber* isolate DANU-2].

PCR Amplification and Sequencing of 16s rRNA Gene

In the PCR experiment, mtCO1 gene amplification of the genomic DNA of all the three fish species was performed by using two primers such as Forward -5' TCAACCAACCACAAAGACATTGGCAC-3' and Cyt oxidase Reverse 5'- TAGACTTCTGGGTGGCCAAAGAATCA-3'. The mtCO1 gene has been regularly employed for phylogenetic analysis due to its high primer specificity and discriminatory strength. The primers used to amplify the mtCO1 of the two fish species produced distinct single bands with good quality. Figure 5 shows the PCR amplified mtCO1 gene bands of the fish species in a 1.5% agarose gel. In

1.5% agarose gel, the first well contains marker DNA, while the remaining wells had an amplified gene product. All the amplified mtCO1 genes exhibited their size in the range of 600 to 700 bp length. The PCR amplified mtCO1 gene products were for used for further studies, including nucleotide sequencing and phylogenetic analysis.

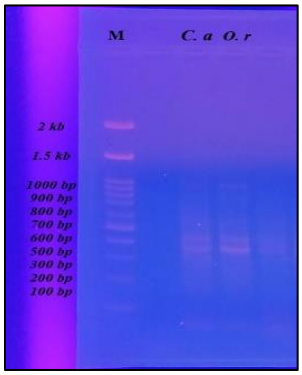

Figure 5. PCR amplified mtCO1 gene products of two collected fish species on 1.5% agarose gel [*C. a* - *O. aureus* isolate DANU-1; *O. r* - *O. ruber* isolate DANU-2].

Sequence Analysis

Biologics Corp server was used to determine the characteristics of mtCO1 gene sequence including nucleotide composition and GC percentage from the two collected fish species. The total number of

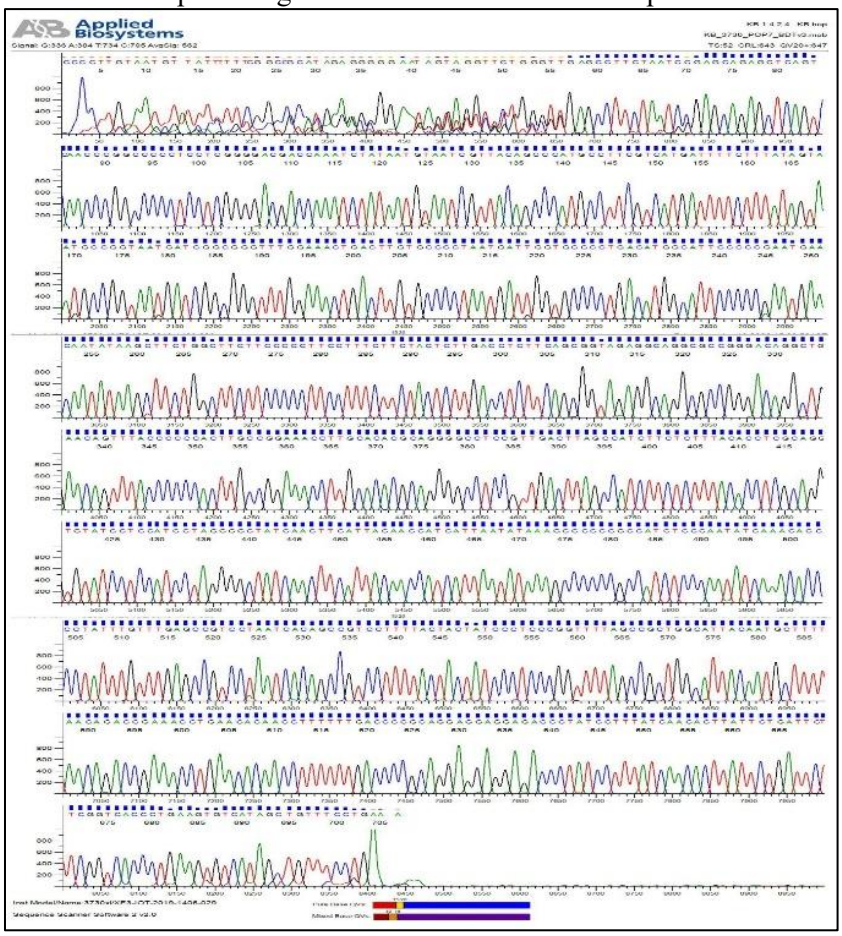

Figure 6. Sequencing chromatogram of mtCO1 gene from *C. aureus* isolate DANU-1.

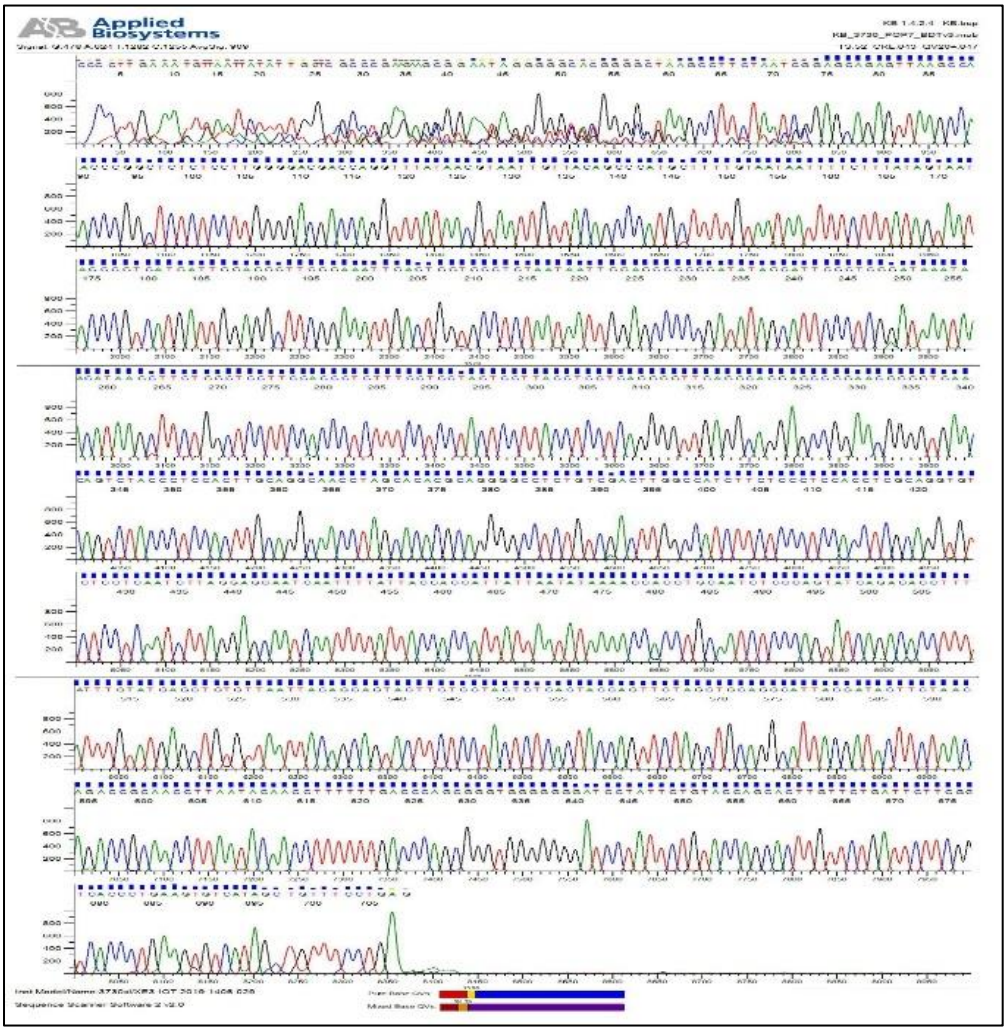

Figure 7. Sequencing chromatogram of mtCO1 gene from *O. ruber* isolate DANU-2.

nucleotides in the mtCO1 gene from the *C. aureus* DANU-1 and *O. ruber* DANU-2 were found to be 706 and 708 respectively. As well as, the percentage of GC in the mtCO1 gene for the corresponding fish species were calculated as 49.29 and 47.74. Table 1 shows the nucleotide composition of the PCR amplified mtCO1 gene from two fish species. Figure 6 and 7 showed the sequencing chromatograms of mtCO1 gene from *C. aureus* DANU-1 and *O. ruber* ANU-2.

Table 1. Nucleotide composition of mtCO1 gene from the collected two fish species.

S. No.	Parameter	*C. aureus* isolate DANU-1		*O. ruber* isolate DANU-2	
		No. of Ntd.	%. of Ntd.	No. of Ntd.	%. of Ntd.
1	Total bases	706	-	708	-
2	Adenine	158	22	168	23
3	Thymine	200	30	202	30
4	Guanine	137	19	144	20
5	Cytosine	211	29	194	27
6	% GC	348	49.29	338	47.74

CCCCTTGTAATGTTATTTTTTCGGCCGCATAGAGGGGGAATAGTAGGTTCTGGGTTG
AGCCTTCTAATCCGAGCAGAGCTCAGTCAACCCGGCCCCCTCCTCGGGGACGACC
AAATCTATAATGTAATCGTTACAGCCCATGCCTTCGTCATGATTTTCTTTATAGTAAT
GCCGGTAATGATCGGCGGGTTTGGAAACTGACTTGTGCCCCTAATGATTGGTGCCC
CTGACATGGCATTCCCCCGAATGAACAATATAAGCTTCTGGCTTCTTCCCCCTTCCT
TTCTTCTACTCTTGACCTCTTCAGCGGTAGAGGCAGGCGCCGGGACAGGCTGAAC
AGTTTACCCCCCACTTGCCGGAAACCTTGCACACGCAGGGGCCTCCGTTGACTTA
GCCATCTTCTCTTTACACCTCGCAGGTGTATCCTCCATCCTAGGGGCTATCAACTTC
ATTACAACCATCATTAATATAAAGCCCCCCGCCATCTCCCAATATCAAACACCCCTA
TTTGTTTGAGCCGTCCTAATCACAGCCGTCCTTTTACTACTATCCCTCCCGGTTTTA
GCCGCTGGCATTACAATGCTTTTAACAGACCGAAACCTGAACACAACCTTTTTTGA
CCCCGCAGGAGGAGGAGACCCTATCCTTTATCAACACTTATTCTGATTCTTCGGTC
ACCCTGAAGTGTCATAGCTGTTTCCTGAAA

Figure 8. Nucleotide sequence of *C. aureus* isolate DANU-1 mtCO1 gene (706 bp).

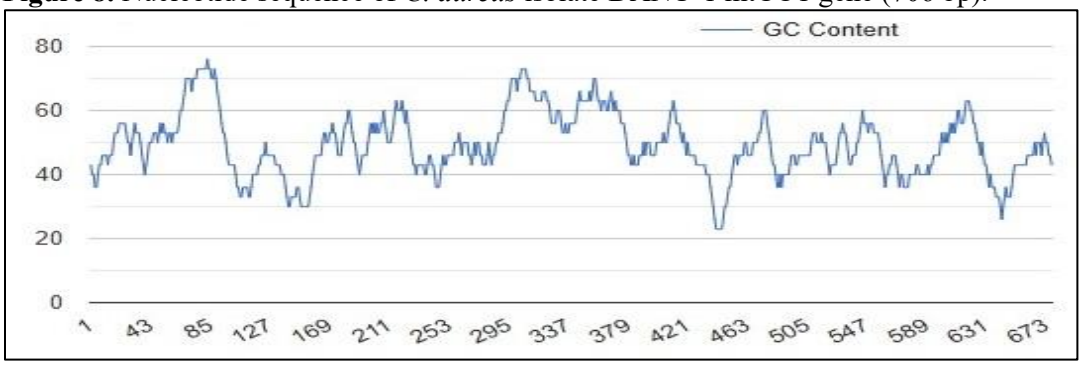

Figure 9. GC distribution over the amplified mtCO1 gene sequence of *C. aureus* isolate DANU-1.

CCCCTTGAAATGTTAATTATATTGGTCGCCCGAAGAAGCGGAATAGGGGGCACGG
GGCTAAGCCTTCTAATCGGAGCAGAGTTAAGCCAACCCGGCTCTCTCCTTGGGGA
CGACCAGGTTTATAACGTAATTGTTACAGCCCATGCTTTTGTAATAATTTTCTTTATA
GTAATACCCGTCATGATTGGAGGCTTCGGAAATTGACTGGTGCCTCTAATAATTGG
AGCCCCCGATATAGCATTCCCTCGGATAAATAACATAAGCTTCTGGCTCCTTCCACC
CTCTTTCCTCCTACTCCTTACCTCCTCAGGGGTTGAGGCAGGAGCCGGAACGGGG
TGAACAGTCTACCCTCCACTTGCAGGCAACCTAGCACACGCAGGGGCCTCTGTCG
ACTTGGCCATCTTCTCCCTCCACCTCGCAGGTGTCTCCTCAATCTTAGGAGCAATC
AATTTTATTACCACCATTATTAATATAAAACCACCTGCAATCTCCCAGTATCAGACA
CCTTTATTTGTATGAGCTGTGTTAATTACAGCAGTACTTCTCCTACTCTCACTACCA
GTTCTAGCTGCAGGCATTACGATACTTCTAACAGACCGCAACCTTAATACAACCTT
TTTTGACCCAGCGGGTGGGGGGGATCCTATTCTGTACCAGCACTTGTTCTGATTCT
TCGGTCACCCTGAAGTGTCATAGCTGTTTCCTGAG

Figure 10. Nucleotide sequence of *O. ruber* isolate DANU-2 mtCO1 gene (708 bp).

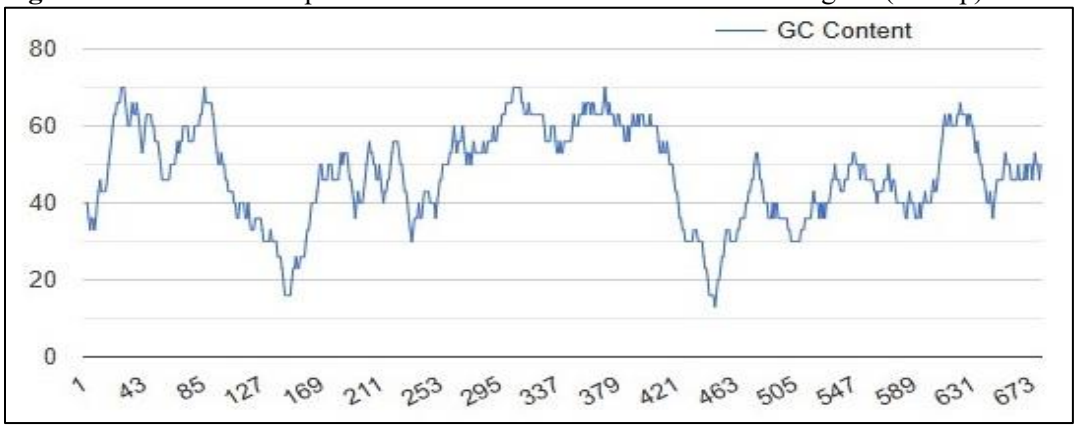

Figure 11. GC distribution over the amplified mtCO1 gene sequence of *O. ruber* isolate DANU-2.

Multiple Sequence Alignment (MSA)

The mtCO1 gene sequences from the *C. aureus* isolate DANU-1 and *O. ruber* isolate DANU-2 were used as query sequences for nucleotide BLAST to determine the similarities with other species' mtCO1 gene sequences which are available in the GenBank database. The BLASTn report demonstrates the similar sequences with the mtCO1 sequences of three query sequences, along with their percentage of identity. CLUSTAL was used to produce a multiple sequence alignment of the mtCO1 region (Higgins *et al.,* 1992). The multiple sequence alignment indicated that there are a variable number of deletions and insertions in the mtCO1 gene.

MSA of O. aureus Isolate DANU-1

The BLASTn search of the 706 bp length mtCO1 gene sequence of *C. aureus* isolate DANU-1 against the GenBank database revealed that it has the maximum resemblance to *Chrysochir aurea* voucher ML20, with an identity of 100% and an E value of 0.0. From the matches, a total of 13 sequences from different species were retrieved from the GenBank database and employed for multiple sequence alignment. The list of selected gene mtCO1 sequences from GenBank database for the construction of multiple sequence alignment and phylogenetic tree of *C. aureus* isolate DANU-1 were listed in Table 2. The multiple sequence alignment of *C. aureus* isolate DANU-1 with the selected homologous sequences was shown in Figure 12.

Table 2. Selected species sequences used for MSA and phylogenetic study of *C. aureus* isolate DANU-1.

Species	Max score	Total score	Query cover	E value	% Ident.	Length
KY118817.1 *Chrysochir aurea* voucher N4	1173	1173	94%	0.0	98.50	681
MN083162.1 *Chrysochir aurea* isolate 11451	1155	1155	90%	0.0	99.37	637
MH230982.1 *Chrysochir aurea* voucher DUZMMF258C	1146	1146	89%	0.0	99.68	642
KY802040.1 *Chrysochir aurea* voucher ML20	1142	1142	88%	0.0	100%	621
KY118817.1 *Chrysochir aurea* voucher N4	1061	1061	90%	0.0	98.05	652
OP120943.1 *Chrysochir aurea* isolate CIFRI.CA1	1133	1133	91%	0.0	98.45	655
KP722708.1 *Chrysochir aurea* isolate WJC451	1098	1098	92%	0.0	97.09	654
PV830202.1 *Chrysochir aurea* voucher KTS_05580	937	937	76%	0.0	98.31	534
ON695928.1 *Protonibea diacanthus* voucher S8	697	697	87%	0.0	87.15	646
KP722735.1 *Megalonibea fusca* isolate WJC1818	695	695	88%	0.0	86.91	652
KP722760.1 *Pennahia pawak* isolate WJC448	693	693	92%	0.0	85.89	654
PQ060355.1 *Larimus breviceps* isolate LbrSC08	647	647	87%	0.0	85.58	624
OQ843562.1 *Nebris occidentalis* voucher hap1	645	645	86%	3e-180	85.76	614
EU266377.1 *Miichthys miiuy* isolate CO1A13	645	645	95%	3e-180	84.13	712

The MSA of *C. aureus* isolate DANU-1 with the selected species indicates the phylogenetic parameters for the mtCO1 as follows: conserved sites 461, variable sites 230, parsimony sites 144, and singleton sites 80, with an overall mean distance of 0.12. The phylogenetic parameters of mtCO1 alignments for *C. aureus* isolate DANU-1 is presented in Table 3. The evolutionary variation in sequences of selected strains at the generic level ranges from 0.0000 to 0.24286. Collected *C. aureus* isolate DANU-1 species has the greatest evolutionary divergence with *Miichthys miiuy* (0.24286) and the lowest with *Chrysochir aurea* voucher ML20 (0.00324). The evolutionary distances of mtCO1 gene sequence from *C. aureus* isolate DANU-1 with the selected sequences were presented in Table 4.

Table 3. Phylogenetic parameters of collected *C. aureus* isolate DANU-1 with their homologous species.

S. No.	Parameter	No. of sites	% of sites
1	Conserved sites	461/733	62.89
2	Variable sites	230/733	31.38
3	Parsimony sites	144/733	19.65
4	Singleton sites	80/733	10.91
5	Overall mean distance	0.12	-

MSA of O. ruber Isolate DANU-2

The BLASTn search of the 708 bp length mtCO1 gene sequence of *O. ruber* isolate DANU-2 against the GenBank database revealed that *O. ruber* DANU-2 has the maximum resemblance to *Otolithes ruber* isolate FGB48, with an identity of 99.52% and an E value of 0.0. From the matches, a total of 11 sequences from different species were retrieved from the GenBank database and employed for multiple sequence alignment. The list of selected gene mtCO1 sequences from GenBank database for the construction of multiple sequence alignment and phylogenetic tree of *O. ruber* isolate DANU-2 were listed in Table 5. The multiple sequence alignment of *O. ruber* isolate DANU-2 with the selected homologous sequences was shown in Figure 13.

Table 4. Selected species sequences used for MSA and phylogenetic study of *O. ruber* isolate DANU-2.

Species	Max score	Total score	Query cover	E value	% Ident.	Length
PX247922.1 *Otolithes ruber* isolate FGB48	1142	1142	89%	0.0	99.52	628
OQ730275.1 *Otolithes ruber* voucher MAHN118	1044	1044	90%	0.0	96.23	641
JN312884.1 *Otolithes ruber* voucher BW-A10197	1022	1022	88%	0.0	96.16	652
PX376343.1 *Otolithes ruber* isolate FGB21	985	985	83%	0.0	96.78	590
KX778040.1 *Otolithes ruber* isolate WJC2779	972	972	84%	0.0	95.99	598
OQ730276.1 *Otolithes ruber* voucher PAR1545	950	950	78%	0.0	97.82	550
KP641499.1 *Pseudotolithus* sp. JKK-2015 isolate PKU_10785	732	732	85%	0.0	88.56	608
KR632715.1 *Micropogonias megalops* dev_stage larva_pre1	682	682	90%	0.0	86.01	683
PX312740.1 *Otolithes ruber* isolate FGB14	656	656	82%	0.0	87.07	582
PV837840.1 *Micropogonias furnieri* voucher STRI-6482	649	649	89%	0.0	85.33	655
GU225144.1 *Bairdiella chrysoura* voucher MX671	645	645	88%	3e-180	85.28	652

Table 5. Phylogenetic parameters of collected *O. ruber* isolate DANU-2 with their homologous species.

S. No.	Parameter	No. of sites	% of sites
1	Conserved sites	499/713	69.99
2	Variable sites	188/713	26.38
3	Parsimony sites	106/713	14.87
4	Singleton sites	76/713	10.66
5	Overall mean distance	0.12	-

Table 6. Estimates of evolutionary distances for collected *C. aureus* isolate DANU-1 with its homologous species.

S. No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*C. aureus* DANU-1	1													
2	KY118817.1	0.04871	1												
3	MN083162.1	0.00634	0.00000	1											
4	MH230982.1	0.01265	0.00628	0.00632	1										
5	KY802040.1	0.00324	0.00000	0.00000	0.00161	1									
6	OP120943.1	0.04219	0.01238	0.00160	0.00482	0.00164	1								
7	KP722708.1	0.04012	0.01867	0.01915	0.01908	0.01799	0.01747	1							
8	PV830202.1	0.01707	0.01707	0.01707	0.01707	0.01707	0.01900	0.00188	1						
9	ON695928.1	0.17244	0.15661	0.15686	0.15683	0.15498	0.15796	0.15652	0.15921	1					
10	KP722735.1	0.18285	0.15289	0.15477	0.16304	0.15495	0.15827	0.16089	0.16166	0.00779	1				
11	KP722760.1	0.18197	0.15592	0.16055	0.16834	0.16085	0.16202	0.15541	0.17136	0.18261	0.17847	1			
12	PQ060355.1	0.18101	0.17345	0.17345	0.17126	0.17195	0.17646	0.17592	0.18192	0.19414	0.20083	0.18372	1		
13	OQ843562.1	0.17891	0.18488	0.18440	0.18759	0.18592	0.19183	0.19584	0.20042	0.21821	0.21567	0.18811	0.15137	1	
14	EU266377.1	0.24286	0.18792	0.17977	0.18703	0.18280	0.18009	0.17592	0.18344	0.18037	0.17835	0.14976	0.18758	0.18681	1

Table 7. Estimates of evolutionary distances for collected *O. ruber* isolate DANU-2 with its homologous species.

S. No	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	*O. ruber* DANU-2	1											
2	PX247922.1	0.00642											
3	OQ730275.1	0.04289	0.03537										
4	JN312884.1	0.07116	0.03434	0.00000									
5	PX376343.1	0.03349	0.03349	0.00000	0.00000								
6	KX778040.1	0.04229	0.03726	0.00167	0.00167	0.00179							
7	OQ730276.1	0.02253	0.01896	0.01289	0.01289	0.01183	0.01475						
8	KP641499.1	0.13597	0.14356	0.12840	0.13148	0.12546	0.13155	0.12231					
9	KR632715.1	0.23178	0.16754	0.15788	0.15899	0.14705	0.16675	0.17124	0.23055				
10	PX312740.1	0.15426	0.15197	0.15567	0.15722	0.15371	0.15556	0.14617	0.19491	0.19411			
11	PV837840.1	0.23088	0.18164	0.17218	0.17011	0.16414	0.17909	0.18219	0.22921	0.01392	0.19878		
12	GU225144.1	0.22984	0.18038	0.17095	0.17011	0.16278	0.17909	0.18219	0.22809	0.01242	0.19752	0.00000	1

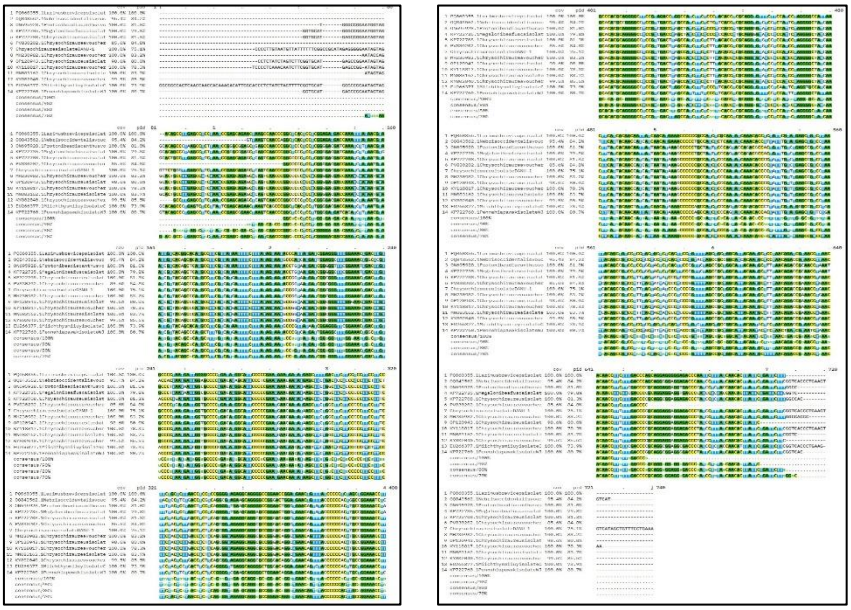

Figure 12. Multiple sequence alignment of *C. aureus* isolate DANU-1 with the selected homologous sequences.

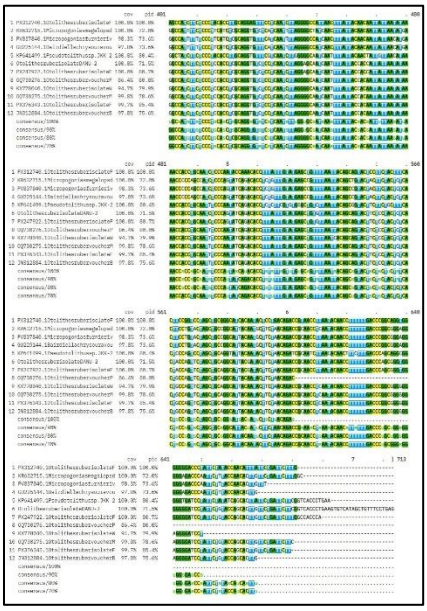

Figure 13. Multiple sequence alignment of *O. ruber* isolate DANU-2 with the selected homologous sequences.

The MSA of *O. ruber* isolate DANU-2 with the selected species indicates the phylogenetic parameters for the mtCO1 as follows: conserved sites 499, variable sites 188, parsimony sites 106, and singleton sites 76, with an overall mean distance of 0.12. The phylogenetic parameters of mtCO1 alignments for *O. ruber* isolate DANU-2 is presented in Table 6. The evolutionary variation in sequences of selected strains at the generic level ranges from 0.0000 to 0.23178. Collected *O. ruber* isolate DANU-2 has the greatest evolutionary divergence with *Otolithes ruber* isolate FGB48 (0.23178) and the lowest with *Micropogonias megalops* dev_stage larva_pre1 (0.00642). The evolutionary distances of mtCO1 gene sequence from *O. ruber* isolate DANU-2 with the selected sequences were presented in Table 7.

Phylogenetic Tree Analysis

The selected homologous sequences of mtCO1 gene against *C. aureus* isolate DANU-1 and *O. ruber* isolate DANU-2 were extracted from the GenBank database and used to construct a phylogenetic tree. The distance matrix was used to determine the differences between the selected sequences. Depending on the expressed differences in the distance matrix, the maximum-parsimony phylogenetic tree was built using MEGAX and the topologies of the phylogenetic tree were evaluated using the bootstrap method with 1000 replicates for all nodes (Felsenstein, 1985). A satisfactory result was established by the use of the mtCO1 gene as a marker to evaluate the phylogenetic relationship.

Phylogenetic Tree of C. aureus Isolate DANU-1

The maximum-parsimony phylogenetic tree of *C. aureus* isolate DANU-1 with the selected homologues sequences was shown in Figure 14. *Chrysochir aureus* isolate DANU-1 is the focal point of a phylogenetic tree that, using the available genetic sequences, shows the evolutionary relationships among different species within the Sciaenidae family. The tree shows that *C. aureus* isolate DANU-1 is closely related to other reference sequences of the same species, such as KY118817.1 (voucher N4), MN083162.1 (isolate 11451), and KY802040.1 (voucher ML20). A high bootstrap score (93%), which indicates significant confidence in this grouping, supports this cluster.

Apart from other closely similar *Chrysochir aureus* isolates like MH230982.1 (voucher DUZMMF258C), OP120943.1 (isolate CIFRI.CA1), KP722708.1 (isolate WJC451), and PV830202.1 (voucher KTS 05580), *Chrysochir aureus* isolate DANU-1 also forms its own clade. In addition to *Chrysochir aureus*, the tree contains other Sciaenidae genera such as *Protonibea*, *Megalonibea*, *Pennahia*, *Miichthys*, *Larimus*, and *Nebris*, demonstrating the genetic uniqueness of the species. Its taxonomic identity has been validated by the phylogenetic analysis, which shows that *C. aureus* isolate DANU-1 is closely related to other *Chrysochir aureus* isolates and forms a well-supported clade within the genus. The inferred links among the included taxa are supported by the robust bootstrap values across the tree.

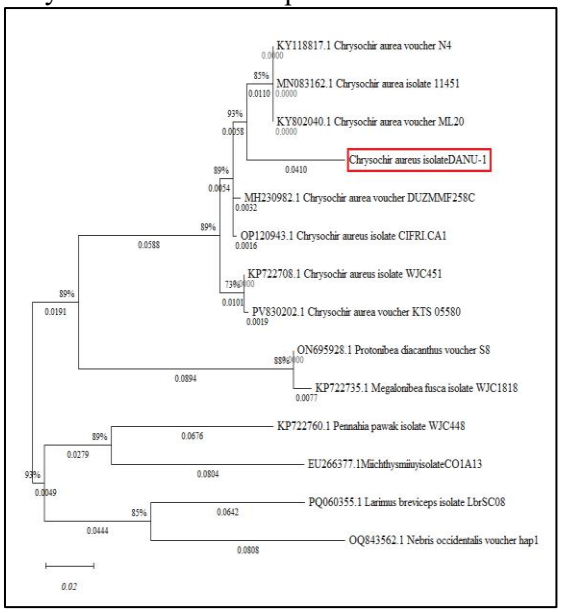

Figure 14. Maximum Parsimony tree of *Chrysochir aureus* isolate DANU-1 and other relative species based on the mtCO1 gene.

Phylogenetic Tree of O. ruber Isolate DANU-2

Figure 15 illustrates the maximum-parsimony phylogenetic tree of *O. ruber* isolate DANU-2, utilizing the selected homologous sequences. The phylogenetic tree demonstrates the evolutionary connections among several isolates and voucher specimens within the genus *Otolithes*, as well as outgroups from allied genera

like *Pseudotolithus*, *Micropogonias*, and *Bairdiella*. The *Otolithes ruber* group constitutes a robust clade (bootstrap value 100), signifying substantial genetic coherence among the analyzed sequences.

The isolate designated *Otolithes ruber* DANU-2 closely clusters with the *Otolithes ruber* isolate FGB48, creating a subclade with a bootstrap value of 100, indicating a high level of confidence in this association. This subclade is strongly linked with another group that includes OQ730276.1 *Otolithes ruber* voucher PAR1545. All these sequences belong to the primary *Otolithes ruber* cluster, demonstrating that DANU-2 is genetically congruent with other *O. ruber* isolates and vouchers. Additionally, the phylogenetic tree is established, with Bairdiella chrysoura and *Micropogonias* species functioning as outgroups, substantiating the larger classification of *Otolithes* within the Sciaenidae family.

The *O. ruber* clade is clearly delineated from the outgroup species, such as *Pseudotolithus*, *Micropogonias*, and *Bairdiella*, thereby affirming the genetic integrity of the *O. ruber* group. These results validate the molecular identification of DANU-2 as *Otolithes ruber* and illustrate the phylogenetic integrity of the species, with robust support for the evolutionary relationships represented in the tree.

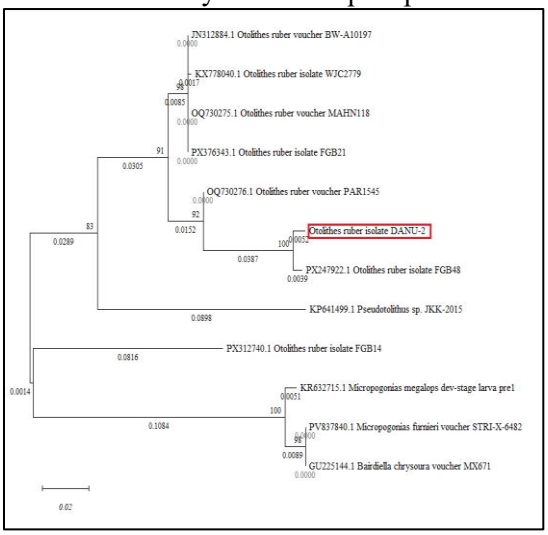

Figure 15. Maximum Parsimony tree of *Otolithes ruber* DANU-2 and other relative species based on the mtCO1 gene.

DISCUSSION

Phylogeny and Taxonomy

Phylogeny and taxonomy are essential for biodiversity assessment, conservation, and environmental management. Although traditional taxonomy has been widely used for species classification, it is often constrained by morphological similarity, specimen preservation, and the expertise required to distinguish closely related or cryptic species. Molecular approaches based on DNA provide a more accurate understanding of phylogenetic relationships and evolutionary history.

DNA sequencing has revolutionized molecular systematics and species identification over the past two decades (Clark *et al.,* 1995). Among molecular techniques, DNA barcoding has emerged as a rapid, reliable, and cost-effective approach with applications in ecology, conservation, food safety, and environmental monitoring (Hollingsworth, 2007; Li *et al.,* 2015). In addition to Sanger sequencing, advanced methods such as NGS, SNPs, and Real-Time PCR have further enhanced fish authentication (Manel and Holderegger, 2013; Fernandes *et al.,* 2020).

The mitochondrial cytochrome c oxidase subunit I (COI) gene is widely recognized as an ideal molecular marker because of its maternal inheritance, rapid evolutionary rate, limited recombination, and conserved structure (Filosto *et al.,* 2003; Javonillo *et al.,* 2010; Paz *et al.,* 2014; Ruan *et al.,* 2020). It effectively discriminates species, resolves phylogenetic relationships, and supports biodiversity assessments and

conservation studies (Sun *et al.,* 2011; Mohindra *et al.,* 2015; Desiandura *et al.,* 2017; Garg *et al.,* 2017). COI barcoding has successfully identified marine fishes, detected cryptic taxa, and supported fisheries management, although its resolution may decrease at higher taxonomic levels (Ward *et al.,* 2005; Hajibabaei *et al.,* 2007; Ratnasingham and Hebert, 2007; Bucklin *et al.,* 2011). Combining COI with additional mitochondrial or nuclear markers further improves phylogenetic resolution (Xing *et al.,* 2025). Recent studies have expanded COI reference databases for marine fishes from the Red Sea, Taiwan, the South China Sea, and Australia (Santana *et al.,* 2023; Jiang *et al.,* 2024; Solami, 2024; Appleyard *et al.,* 2025).
In the present study, genomic DNA of both fish species showed high purity (A260/A280 = 1.7–1.9), and universal mtCOI primers successfully amplified the target region, confirming the suitability of COI for molecular identification (Willian *et al.,* 1997; Hebert *et al.,* 2003; Xue *et al.,* 2009). Other mitochondrial markers, including cytochrome *b* and 16S rRNA, have also proven useful for phylogenetic studies (Castresana, 2001; Guo *et al.,* 2004; Kartavtsev *et al.,* 2007). The moderate GC content observed in both species agrees with previous reports and reflects evolutionary and environmental influences on mitochondrial genomes (Sinden, 1994; Clare *et al.,* 2008; Mann and Chen, 2010; Zhang and Gao, 2017). Sequence identities greater than 95% further support reliable species identification and emphasize the value of regional DNA barcode databases (de Groot *et al.,* 2011).
BLASTn analysis revealed that *Chrysochir aureus* isolate DANU-1 shared 100% identity with *Chrysochir aurea* voucher ML20, whereas *Otolithes ruber* isolate DANU-2 showed 99.52% identity with *Otolithes ruber* isolate FGB48. Multiple sequence alignment using CLUSTAL identified conserved and variable regions useful for assessing genetic divergence (Higgins *et al.,* 1992; Kaleshkumar *et al.,* 2015). The use of alignment tools such as CLUSTAL W, MUSCLE, and MAFFT remains fundamental for evaluating genetic diversity and phylogenetic relationships (Thompson *et al.,* 1994; Miya and Nishida, 2000; Ward *et al.,* 2005; Katoh and Standley, 2013). Maximum-parsimony analysis in MEGA X with 1,000 bootstrap replicates confirmed that *C. aureus* DANU-1 clustered with other *Chrysochir aureus* isolates, while *O. ruber* DANU-2 formed a strongly supported subclade with *O. ruber* isolate FGB48 (bootstrap = 100), demonstrating the effectiveness of the mtCOI gene as a robust marker for molecular identification and phylogenetic analysis (Felsenstein, 1985).

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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