Molecular and histopathological insights of *Amyloodinium ocellatum* infection in Maroon Clownfish

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Journal of Livestock Science (ISSN online 2277-6214) 17: 515-523
Received on 26/1/26; Accepted on 16/7/26; Published on 19/7/26
doi. 10.33259/JLivestSci.2026.515-523

Abstract

In marine hatchery with clinical signs of distress, including lethargy, in appetence, and rapid respiration were noted in Maroon Clownfish (*Premnas biaculeatus*). Gross examination revealed a characteristic fine, dusty coating on the skin. Microscopic analysis confirmed the presence of trophonts in gill biopsies, with wet mounts also revealing tomont stages (2-, 4-, and 8-cell stages) indicative of active parasitic reproduction. This indicates the presence of *Amyloodinium* spp. The infection significantly impaired the respiratory efficiency of the fish due to heavy parasitic load on the gill lamellae. Parasitic Frequency Index (PFI) analysis revealed a prevalence of 50% among the sampled population (n=4), with a high severity score (1), classifying the infection as "common." Infections were localized to the gills, body surface, and fins. Histopathology of the tissues from moribund specimens revealed epithelial hyperplasia, mucus hypersecretion, mechanical tissue disruption, immune cell infiltration, lamellar fusion, and necrosis. Molecular detection via 18S rRNA gene-based PCR amplification confirmed species level molecular identification as *Amyloodinium ocellatum*, yielding amplicons of 336 bp and 225 bp in a two-step process. Sequences were submitted to NCBI GenBank under accession numbers PQ932024 and PQ932025. This incident underscores the need for prompt diagnosis and management of parasitic infections in aquaculture settings to minimize mortality and economic loss.

Key words: *Premnas biaculeatus, A. ocellatum, PFI,* Histopathology, Molecular detection

Introduction

Fish farming (both inland and marine) is an important part of socio-economic condition of substantial population in Indian subcontinent (Hridoy et al., 2024). The Maroon Clownfish (*Premnas biaculeatus*), a distinctive member of the Pomacentridae family, is renowned for its vibrant coloration and ecological importance within the marine environments. This species, identifiable by its deep maroon hue and prominent white or yellow bars, is endemic to coral reef habitats in the Indo-Pacific region (Veron 2000). It's striking appearance and unique behaviours, such as symbiotic relationships with sea anemones, make it a favourite among aquarists and marine enthusiasts. The maroon clownfish holds significant ecological and economic importance. Ecologically, it contributes to the biodiversity and stability of coral reef systems, particularly through its symbiosis with anemones, which provides mutual protection and enhances nutrient cycling. Economically, the species is highly valued in the ornamental fish trade due to its vivid coloration and resilience in captivity (Colin, McDermott & Brian, Palmeiro 2013). This demand underscores the need for sustainable harvesting practices and aquaculture development to mitigate pressures on wild populations. Despite the ecological and economic benefits of marine ornamental fishes, globally various diseases such as marine ich, fin rot, and brooklynellosis have been reported, affecting wild and captive populations alike (Shinn et al. 2015). In India, disease outbreaks in marine ornamentals have been linked to bacterial infections, parasitic infestations due to poor aquaculture practices (Andrew et al. 2024).

Premnas biaculeatus, commonly known as the maroon clownfish, is susceptible to several parasitic and bacterial diseases, particularly in captive and ornamental aquaculture settings. Among the most significant is amyloodiniosis, caused by the dinoflagellate *Amyloodinium ocellatum*, which manifests as respiratory distress and a dusty skin appearance (Beraldo & Paola 2026). Cryptocaryoniasis, or marine white spot disease caused by *Cryptocaryon irritans*, has also been reported, leading to visible cysts and increased mortality (Pedro Henrique MC et al, 2019) Additionally, brooklynellosis, often referred to as clownfish disease and caused by *Brooklynella hostilis*, is particularly severe in clownfish, including *P. biaculeatus*, and results in heavy mucus production and rapid mortality (Roger Sie-Maen Chong (2022). Opportunistic bacterial infections from pathogens like *Vibrio* spp. and other vibrio bacteria has also been noted, especially under stress conditions, further complicating health management in maroon clownfish (Triga et al. 2023). Although less frequently documented, viral infections such as lymphocystis and nodaviruses may pose emerging threats in ornamental systems (Hoitsy et al. 2025).

Premnas biaculeatus is susceptible to various diseases, notably *A. ocellatum*, a parasitic dinoflagellate responsible for marine velvet disease. This pathogen infects the gills, skin, and fins of fish, leading to respiratory distress, lethargy, and high mortality rates if untreated (Roberts 2012). Outbreaks of *A. ocellatum* pose significant challenges to both wild populations (brood stocks) in hatchery and aquaculture operations (grow out), as the parasite's rapid life cycle and environmental persistence complicate control measures. An outbreak affecting captive-bred percula clownfish (*Amphiprion percula*), was reported, with the fish exhibiting signs of discoloration, erratic swimming, and labored breathing due to heavy infestation by trophonts, the parasitic stage of *Amyloodinium ocellatum*. Economic losses due to marine velvet disease are substantial, particularly in the ornamental fish industry. Mortality rates during outbreaks can decimate stocks, resulting in financial strain for breeders and exporters. Moreover, the disease's impact on wild populations could disrupt local ecosystems and compromise the sustainability of the ornamental trade (Colin, McDermott & Brian, Palmeiro 2013).

A. ocellatum infestations initially cause stress, which is frequently followed by mortalities. Therefore, studying parasitic infestations and histological changes in the vital organs of parasitized fishes is crucial. Histological alterations serve as an important indicator of the overall health of populations within an ecosystem. Therefore, the objective of the present study was the molecular identification of *A. ocellatum*, understand its prevalence and evaluate the histological changes in vital organs for the effective management of the parasitic diseases.

Therefore, the present study aimed to investigate a suspected parasitic disease outbreak in maroon clownfish (*Premnas biaculeatus*) through clinical examination, morphological characterization, histopathological assessment, and molecular analyses to identify the etiological agent, characterize the associated pathological alterations, and document the first occurrence of *Amyloodinium ocellatum* infecting *P. biaculeatus* in India. Early and accurate diagnosis, coupled with appropriate management strategies, is essential for reducing fish mortality and minimizing economic losses in hatchery and aquaculture systems.

Materials and methods

Sample collection and microscopic examination:

Samples of Maroon Clownfish (*Premnas biaculeatus*) were collected during monsoon in live condition from the marine hatchery, Kodibag (14°49'44.0"N 74°07'39.0"E) and transported to the laboratory (Microbiology laboratory, Karwar RS of ICAR-CMFRI, Karwar) in aerated buckets. Scrapings from external body surface and gills, and tissue squantsofvital organs examined under a microscope for the presence of parasites. Parasitic stages of *A. ocellatum*, including tomonts at the reproductive 2-cell, 4-cell, and 8-cell stages, were observed on the gills.

Determination of Parasitic Frequency Index (PFI)

The Parasitic Frequency Index (PFI) was calculated as the percentage of hosts infected by a specific parasite species relative to the total number of hosts examined within the study area. The frequency index were further classified into rare (0.1 – 9.9%), occasional (10-29.9%), common (30 – 69.9%) and abundant (70-100%) as per Ramudu and Dash 2013; Ramudu and Rathod 2024.

Determination of Severity of Infection/Infestation

To assign a numerical qualitative value to the severity grade of infections, surface infestations, and disease syndrome severity, a generalized scheme outlined by Ramudu and Dash 2013; Kurva et al. 2025 was followed.

Histopathology

Representative gill tissues were fixed in 10% neutral buffered formalin (NBF) for 24 hours, then transferred to a fresh tube with 10% NBF for one hour before being moved to 70% ethyl alcohol for storage and further analysis. Histopathological procedures followed the method described by Roberts 2012. The final slides were examined and screened, and color microphotographs at various magnifications were captured using a binocular phase-contrast microscope with a built-in digital camera (Carl Zeiss MicroImaging GmbH 37081, Axio-Germany), documented the observed pathological changes.

Molecular identification

DNA extraction

Genomic DNA was extracted from approximately 20 mg of infested gill tissue using a modified lysis buffer–phenol–chloroform method following Sambrook et al. (2006). The tissue was homogenized in lysis buffer containing proteinase K, incubated at 56 °C, heat-treated at 95 °C, and purified by sequential phenol–chloroform and chloroform–isoamyl alcohol extractions. DNA was precipitated with absolute ethanol, washed with 70% ethanol, air-dried, and resuspended in 50 µL of nuclease-free water. The extracted DNA was stored at −20 °C until further molecular analysis.

18SrRNA gene polymerase chain reaction

The 18S rRNA gene was amplified from extracted genomic DNA using AmyF1/AmyR1 and AmyF2/AmyR2 primer sets following the protocols of Levy et al. (2007a) and Marques et al. (2019). PCR was performed in 25 µL reactions using EmeraldAmp® Max PCR Master Mix under published cycling conditions, followed by a second-round (nested) PCR. Amplified products were resolved on a 1.2% agarose gel stained with ethidium bromide and visualized alongside a 100 bp DNA ladder. Purified amplicons were sequenced commercially at GeneSpec Pvt. Ltd., Kochi, India.

Sequencing and phylogenetic analysis

The obtained 18S rRNA gene sequences were edited and aligned using BioEdit 7.2 (Hall, 1999), and sequence identities were confirmed through BLAST searches against the NCBI database. Phylogenetic analysis was performed in MEGA X (v10.1.8) using representative *Amyloodinium* isolates, with Piscinoodinium sequences as the outgroup. A Maximum Likelihood tree was constructed under the Kimura 2-parameter model with gamma distribution (K2 + G), and node support was evaluated using 1000 bootstrap replicates. Molecular analysis confirmed *Amyloodinium ocellatum* based on 18S rRNA gene amplification, and the sequences were deposited in GenBank under accession numbers PQ932024 and PQ932025.

Results

Gross signs and microscopic examination

The incident was first noticed when clownfish exhibited signs of distress. These signs included lethargy, loss of appetite, and rapid respiration. Upon closer inspection, a fine, dusty coating was observed on the skin of the clownfish (Fig.1), indicative of *A. ocellatum* infection. Further microscopic examination of gill biopsies from affected fish revealed the presence of both *A. Ocellatum* trophonts and tomonts (reproductive stage-2, 4 and 8-cell stages) (Fig.2 & 3).

The combined effect of these parasites on the gill tissues severely compromised the respiratory efficiency of the fish. The gill lamellae were heavily infested (Fig.4), leading to reduced oxygen uptake, significant stress, and an increased susceptibility to secondary bacterial infections. Mortality rates began to rise sharply, causing alarm among the hatchery staff and prompting immediate action.

Prevalence

Out of four fish investigated, two were infected by *A. ocellatum*, resulting in a Parasitic Frequency Index (PFI) of 50%. This indicates the presence of the parasite at a "common" level based on the PFI classification scale. The site of infection included the gills, body surface, and fins. The severity of infection was categorized as high (severity score = 1). Gross clinical signs observed in the infected fish included lethargy, loss of appetite, and rapid respiration, reflecting the impact of the parasite on the health of the fish (Table 1).

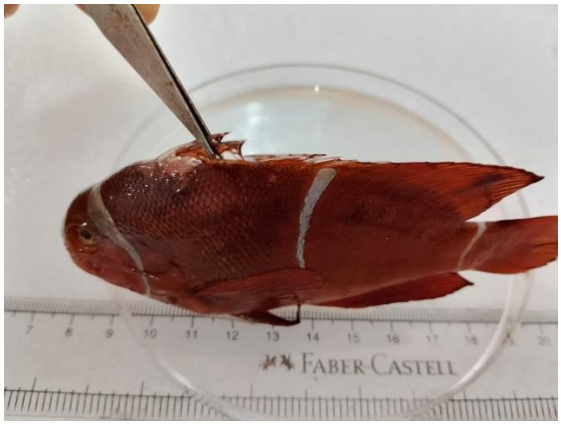

Fig.1 A fine, dusty coating accompanied by an excessive layer of mucus observed on the surface of the sample

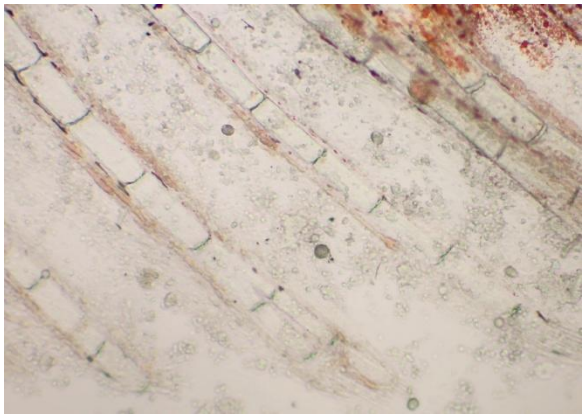

Fig.2 Trophonts attached to the fins (50x)

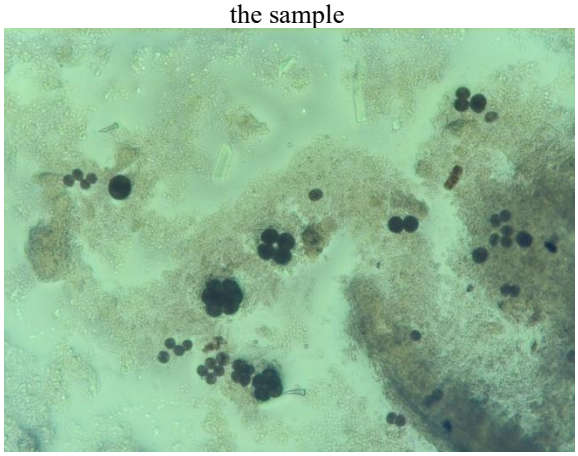

Fig.3 Wet mounts of gills showing presence of Tomonts stages of *A. ocellatum* (100x)

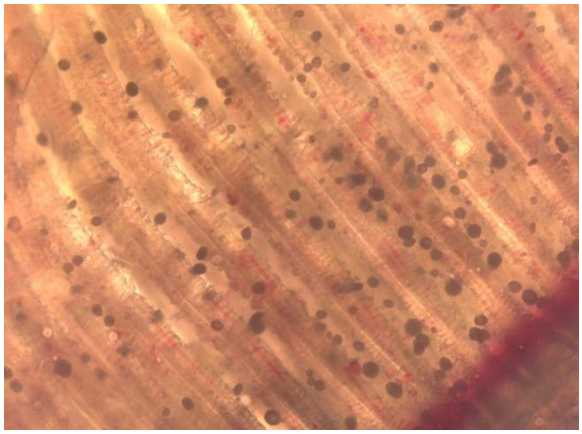

Fig.4 Wet mount slide showed that the presence of Tomonts (reproductive stage) stages of *A. ocellatum* in the gill lamellae (100x)

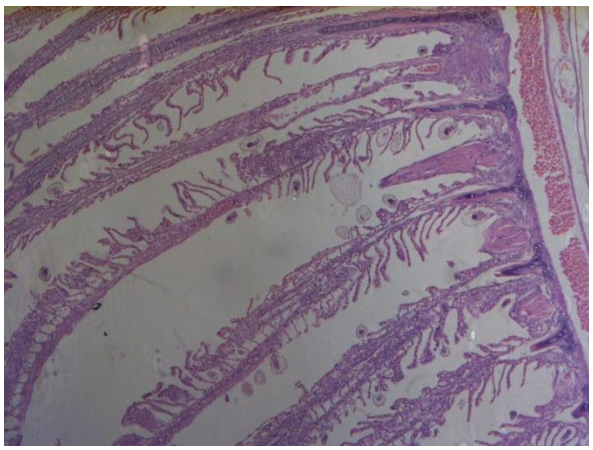

Fig. 5 Histology section showed that the presence of Tomonts (reproductive stage) stages of *A. ocellatum* with lamellar fusion (100x)

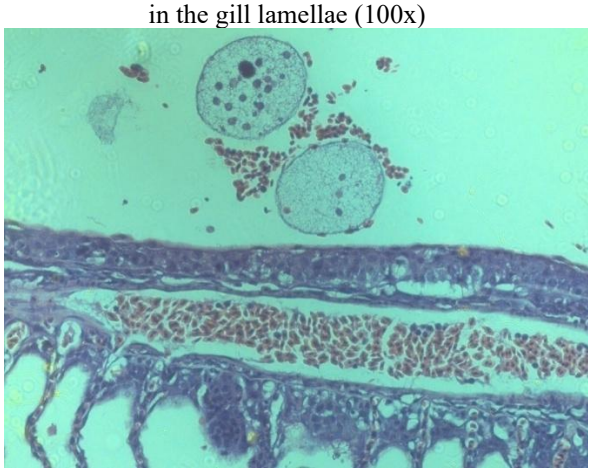

Fig.6 Evidenced by the infiltration of immune cells, including lymphocytes and macrophages (400x)

Table 1: Prevalence (PFI %) and Severity of infection of parasites in Maroon Clownfish (*P. biaculeatus*)

No. of Fish investigated	Parasites present	No. of Infected fishes	PFI (%)	Site of infection	Severity of infection	Gross signs
4	*Amyloodinium ocellatum*	2	50[c]	Gills, body surface & fins	1	lethargy, loss of appetite, and rapid respiration

[*]PFI: Parasitic frequency index & severity score: 0 = Absent, 0.5 = Present, low grade, 1=present, High grade, 2= present, Very high grade
[*]PFI=Parasitic Frequency Index (%). a=rare (0 – 9.9%); b=occasional (10 – 29.9%); c = common (30 – 69.9%); d = abundant (70 – 100%).

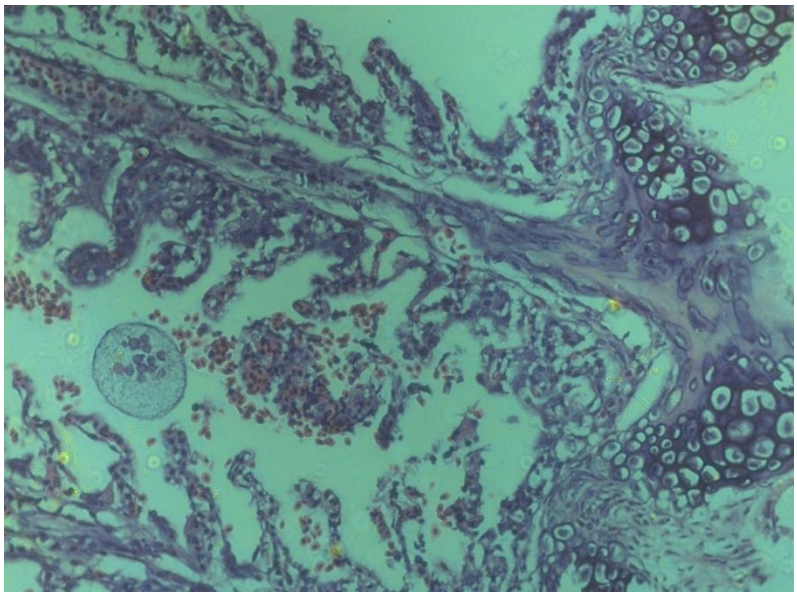

Fig.7 Epithelial hyperplasia showed clear evident in gill histology-red arrow (400x)

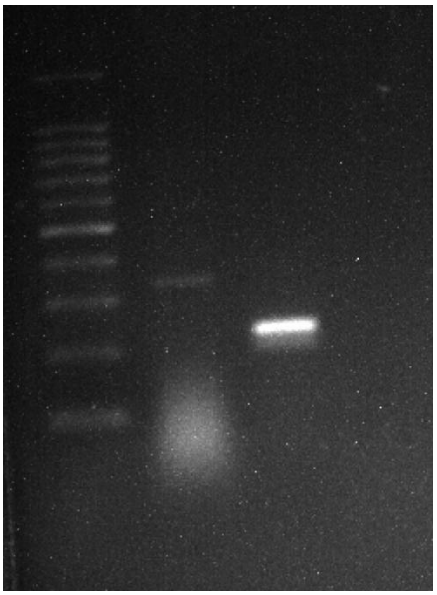

Fig 8: Gel electrophoresis image showing PCR amplicons from *A.ocellatum*-. Lane 1: 100 bp DNA ladder; Lane 2: First-step PCR amplification product (336 bp); Lane 3: Second-step PCR amplification product (225 bp).

Molecular detection using 18SrRNA gene based PCR

DNA extracted from *A.ocellatum*-infested gill tissue was amplified using 18S rRNA gene primers, producing a 336 base pair (bp) amplicon in the first step and a 225 bpamplicon in the second step (Fig.8). The PCR products were sequenced, and the resulting sequences was deposited in the NCBI GenBank® database with accession number PQ932024 and PQ932025 (methods).

BLAST analysis and phylogenetic tree

BLASTN analysis revealed that the 18S rRNA gene sequenceobtained from *A.ocellatum* (PQ932024.1)in this studywas 60% identical to an *A.ocellatum* isolated from the Java rabbit fish India (KY474336.1). A phylogenetic tree constructed using 18S rRNA sequences from seven*A. ocellatum* isolates, demonstrated that the isolate obtained in this study clustered within a distinct clade of *Amyloodinium* isolates with bootstrap support value of 99%at key nodes. This clade encompassed *A. ocellatum* isolates from India,Gulf of Mexico, the USA, the Mediterranean Sea, and Japan. The phylogenetic placement of *Piscinoodinium* sp. (EF016917.1) and *Protodinium simplex* (KY980125.1) suggests a close evolutionary relationship with *Amyloodinium*, although they remain distinct from the primary *A. ocellatum* clade. Other dinoflagellates such as *Gyrodiniumrubrum* and *Alexandriumfragae*appear as more distant relatives, while *Paramecium tetraurelia* and *Toxoplasma gondii*served as the outgroups (Fig. 9).

225

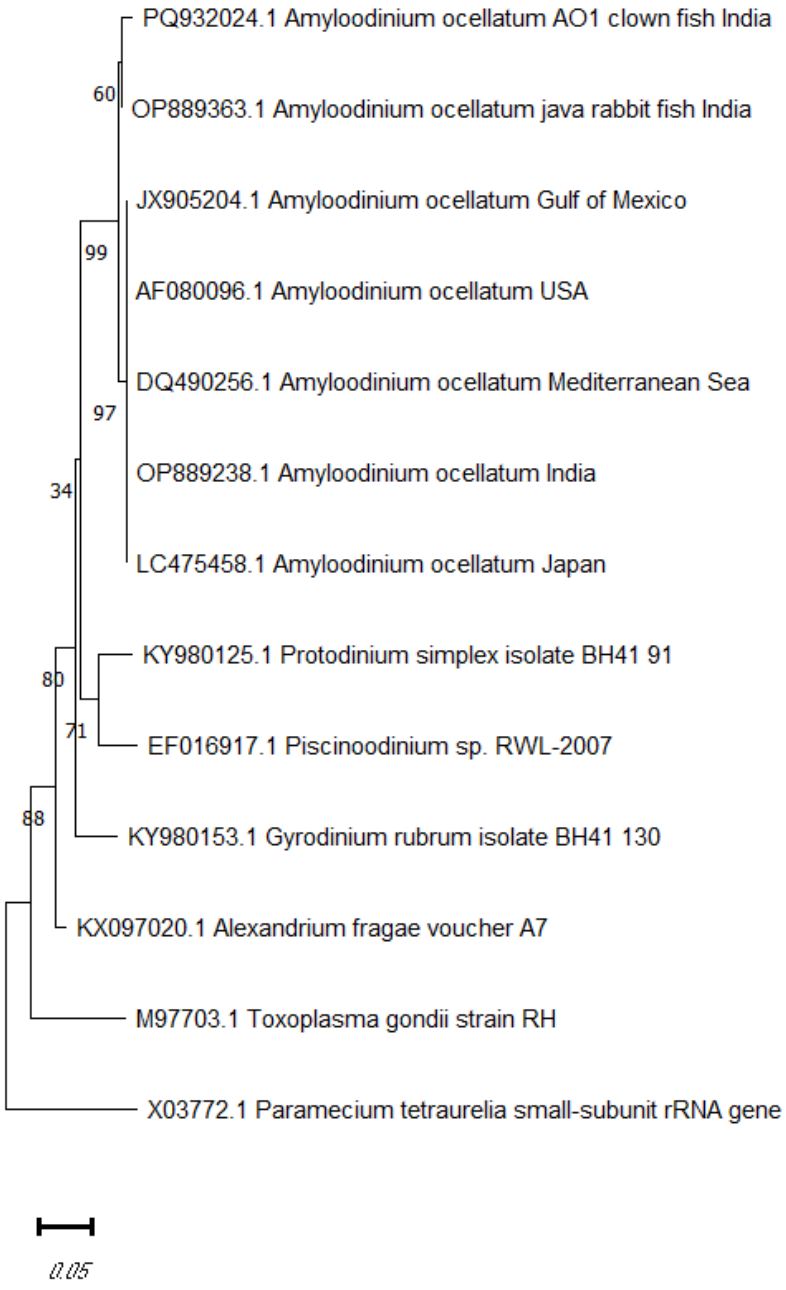

Fig 9: The phylogenetic tree was inferred using the Neighbor-Joining method (Tamura et al., 2021). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. This analysis involved 13 nucleotide sequences. There were total of 196 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (11.0.13)

Discussion

The present study documents a case of *A. ocellatum* infection in Maroon Clownfish (*P. biaculeatus*), marked by classical clinical signs, including lethargy, inappetence, and rapid respiration. The combined effect of these parasites on the gill tissues severely compromised the respiratory efficiency of the fish; these manifestations are consistent with previously reported signs of amyloodiniosis in marine teleosts (Marques, et al. 2019; Madhu et al. 2023). The observation of a fine, dusty coating on the skin often referred to as "velvet disease" further corroborates the presence of this parasitic dinoflagellate, which primarily targets epithelial tissues of the gills and skin (Sudhagar et al. 2022)

The gill lamellae showed heavy infestation, resulting in impaired oxygen uptake, heightened stress, and greater vulnerability to secondary bacterial infections. (Yue & Guo 2025) A sudden spike in mortality raised concern among hatchery personnel, prompting urgent intervention.

Microscopic and histopathological analyses revealed extensive colonization of gill lamellae by trophonts and reproductive tomont stages of *A. ocellatum*, severely compromising the gill structure and respiratory function. Epithelial hyperplasia, lamellar fusion, and necrosis observed in this study align with earlier findings that describe how *A. ocellatum* induces mechanical damage and inflammation in host tissues, resulting in impaired gas exchange and increased susceptibility to opportunistic bacterial infections (Paladini et al. 2017). These histological changes, combined with mucus hypersecretion and immune cell infiltration, highlight a robust pathological response to parasitic infestation.

Excessive mucus secretion is a notable response to parasitic infestation, which further impairs gill function (Mai Dang et al. 2020). *A. ocellatum* trophonts, along with infiltrating lymphocytes and macrophages, were detected in gill sections (Katharios & Karageorgou E. P. 2025). Frequent lamellar fusion was observed, contributing to a significant reduction in the functional respiratory surface (Zawisza et al. 2024). Moreover, necrotic alterations in the gill epithelium and compromised vascular integrity were evident, resulting in reduced oxygen transport capacity (Zawisza et al. 2024). Altogether, these pathological changes profoundly disrupt the respiratory efficiency and overall physiological stability of the affected fish

The Parasitic Frequency Index (PFI) of 50% observed in the present outbreak, although based on a limited sample size, categorizes the infection as “common” and underscores the potential of this parasite to establish itself rapidly under conducive conditions. The high severity score of infection further emphasizes the virulent nature of *A. ocellatum* and its potential to cause sudden and extensive fish mortality if not promptly managed.

Molecular identification using 18S rRNA gene-based PCR was crucial for confirming the presence of *A. ocellatum*, providing a reliable diagnostic method that complements morphological observations. Phylogenetic analysis clustered the study isolate within a distinct clade that includes other global isolates from regions such as the Gulf of Mexico, the Mediterranean Sea, and Japan, which supports the notion of wide geographic distribution and evolutionary divergence within this parasitic species (Levy et al. 2007b).

The phylogenetic analysis demonstrates a clear genetic relatedness among global *A. ocellatum* isolates, with Indian isolates clustering within a broader *Amyloodinium* clade that includes samples from geographically distant regions (Levy et al. 2007a; Francis-Floyd and Floyd 2011). The close grouping of Indian isolates with those from the USA, Mediterranean, and Japan suggests a possible global distribution of this pathogenic dinoflagellate, likely facilitated by marine fish trade, aquaculture practices, and oceanic currents.The tree also supports the distinctiveness of *Amyloodinium* from *Piscinoodinium*, reinforcing the taxonomic separation between these parasitic dinoflagellates (Levy et al. 2007b). The moderate bootstrap values at some internal nodes indicate minor genetic variability within the *Amyloodinium* clade, which could be attributed to local adaptation or minor genetic divergence over time.Additionally, the inclusion of other dinoflagellates and protozoans such as *Toxoplasma gondii* and *Paramecium tetraurelia* as distant relatives provides context for the evolutionary placement of *Amyloodinium* within the broader eukaryotic phylogeny.

Overall, this study reiterates the pathogenic potential of *A. ocellatum* in marine ornamental species and highlights the need for routine surveillance, early diagnosis, and the implementation of effective management practices in hatchery environments. Future studies should explore the efficacy of various treatment protocols, and genetic monitoring should be continued to better understand the parasite’s evolution and host adaptation patterns.

Conclusion

This study confirmed *Amyloodinium ocellatum* as the etiological agent responsible for a parasitic outbreak in maroon clownfish (*Premnas biaculeatus*) through integrated clinical, histopathological, and molecular analyses. The findings highlight the importance of early diagnosis and effective health management strategies for minimizing disease-associated mortality and economic losses in marine hatchery and aquaculture systems.

Author’s contributions

Conceptualization: KRR; Methodology: KRR, SR, RP; Data Collection: KRR, LMJ; design, execution: KRR, SR, RP, LMJ; Writing Original Draft: KRR, SR; Writing Review and Editing: KSSR, AA,TH; Supervision: KC

Acknowledgement

Research was supported by the lndian Council of Agricultural Research, Department of Agricultural Research and Education, Government of lndia The authors gratefully acknowledge the support and facilities provided by the Director, ICAR-CMFRI, Kochi; the Head, Marine Biotechnology, Fish Nutrition and Health Division, ICAR-CMFRI, Kochi; and the Scientist In-Charge, KRS, ICAR-CMFRI, Karwar, which enabled the successful completion of this research work.

Conflict of interest

The authors declare no conflicts of interest.

Ethical statement

Ethical approval was not required for this study, as it did not involve any activities necessitating such approval, nor did it include work with protected species, human subjects, sample collection, or activities conducted in protected environments.

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