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# Transoceanic genetic diversity of milkfish, *Chanos chanos* (Fabricius, 1775), based on cyt b sequence analysis

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Original Article

## Abstract

The genetic structure of milkfish (*Chanos chanos*), an economically important aquaculture species, was examined across four populations in the Indo-Pacific region: French Polynesia, India, Indonesia, and the Philippines. The mitochondrial cytochrome *b* sequences of 99 individuals from India were compared with data from 268 individuals from French Polynesia, Indonesia, and the Philippines, providing a broad representation of the species across the region. Within these four populations, 73 unique haplotypes were identified, with the Indian populations exhibiting the highest levels of gene diversity (0.798) and low nucleotide diversity (0.003). In contrast, the Indonesian populations showed the lowest levels, with values of 0.520 and 0.001, respectively. An analysis of molecular variance (AMOVA) unveiled that 95.53% of the genetic variation resided within populations, while only 4.46% was attributed to differences among populations. Hierarchical AMOVA and pairwise $F_{ST}$ analyses indicated significant genetic differences among Group I, II and III populations in the Indo-Pacific region. These findings highlight the importance of coordinated regional strategies for the effective and location-specific management of milkfish stocks across this region. The haplotype network exhibited a star-like genealogical pattern, indicative of a demographic expansion within the milkfishpopulations across these locations. These findings were further corroborated by Tajima's D statistics. With its significant findings, this study not only delineates the genetic structure of this important species in the Indo-Pacific region but also provides a valuable resource for fishery stock identification, facilitating effective management strategies and conservation efforts.

***Keywords:*** *Cyt b, genetic stock structure, Indo-Pacific, mitochondrial markers*

## Introduction

The milkfish is a valuable edible fish species with significant economic importance and belongs to the Chanidae family (Chang *et al*., 2022). This species is naturally distributed in the Indian Ocean and has extended its range to the Pacific Ocean. It spreads from South Africa to Hawaii and the Marquesas Islands, passing through the Galapagos region of California, North Japan, and South Australia. Along the Indian coastlines, substantial populations of their seeds can be found, particularly on the southeast coast of India, as well as the southwest coast (Lal Mohan, 1984; Lazarus and Nandakumaran, 1987). Milkfish constitutes 1.9% of global finfish aquaculture, placing it in the fifth position (FAO, 2024).

Genetic diversity is essential for species survival, adaptation, and sustainable fishery management (Gu *et al*., 2021; Rumisha *et al*., 2017). Molecular markers, particularly the mitochondrial cyt b gene, are widely used to assess genetic variation and population structure due to their conserved and variable regions that support both phylogenetic and demographic studies (Lydeard *et al*., 1997; Rocha-Olivares *et al*., 1999; Kirchman *et al*., 2000). Connectivity, encompassing the exchange of larvae, juveniles, or adults across a habitat range of species, holds immense significance in metapopulation dynamics and fisheries ecology (Palumbi, 2003; Kool *et al*., 2013). This is particularly relevant for migratory species like milkfish, which traverse vast marine areas and international borders. Over the past two decades, molecular tools have become indispensable for studying marine connectivity, informing fisheries management, and guiding conservation decisions (Jones *et al*., 2009).

Several studies have explored the utility of the cyt b gene in investigating milkfish genetic populations (Adiputra *et al.*, 2010; Delrieu-Trottin *et al.*, 2018; Santos *et al.*, 2019). This examination aimed to shed light on their genetic structure in the Indo-Pacific region using the cyt b gene. Understanding genetic differentiation is vital for maintaining genetic health in hatchery programs and ensuring resilience in aquaculture systems. Additionally, a comprehensive transoceanic exploration of milkfish genetic diversity becomes instrumental in the prudent management of the genetic resources of this species.

## Material and methods

In the current research, we conducted a comparative analysis of cyt b gene sequences from milkfish samples collected in various regions, including Indonesia (Sulawesi, Medan) (samples collected between 2012 and 2014), French Polynesia (Niau, Kauehi) (samples collected in 2011), India (Mandapam, Cochin, Tuticorin, Karwar, Calicut, Chilika) (samples collected between 2016 and 2018), and the Philippines (Antique; Claveria, Cagayan; Currimao, Ilocos Norte; Glan, Sarangani; Guindulman, Bohol; Sacol Is, Zamboanga City; Camiguin; Sagnay, Camarines) (samples collected between 2012 to 2014) (Fig. 1). Cyt b sequences from India (Jose *et al.,* 2023) is compared with sequence data (Table 1 for downloaded NCBI accession numbers) of cyt b haplotypes from Santos *et al.* (2019) and Delrieu-Trottin *et al.* (2018) were aligned using the BioEdit sequence alignment editor v.7.0.5.2 (Hall, 1999). Subsequently, complete sequences from all four locations were aligned and edited for molecular analyses. Genetic variability indices, including nucleotide diversity ($\pi$) and haplotype diversity (h), across samples in the Indo-Pacific region, were calculated using DnaSP5.0 (Librado and Rozas, 2009). Nucleotide diversity ($\pi$) and gene diversity (h), which represent the probability of two randomly chosen haplotypes being different, were estimated to evaluate intrapopulation diversity (Tajima, 1989; Nei and Kumar, 2000).

### *Population genetic structure and demography*

The degree of divergence between four populations in the Indian and Pacific oceans from one another was determined through molecular variance analysis (AMOVA) using Arlequin ver 3.5. The coefficient of population differentiation ($F_{ST}$), which measures the degree of genetic divergence between distinct populations, was evaluated using the Arlequin 3.5 (Excoffier *et al.*, 1992). The Kimura two-parameter model was used to calculate pairwise sequencing divergence among populations using MEGA 11 (Tamura *et al.*, 2021). Hierarchical genetic differentiations were tested using AMOVA. For this, populations

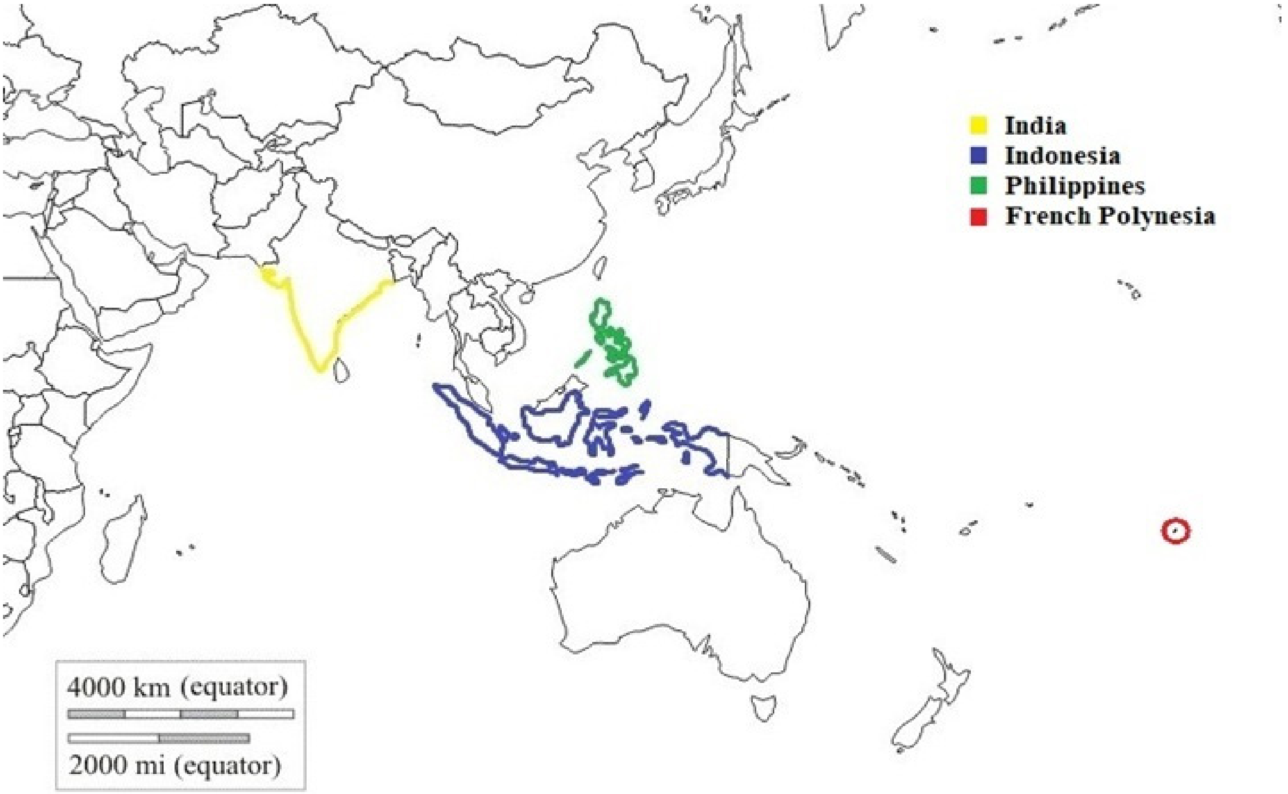

Fig. 1. Map showing the locations of milkfish sampling sites in the Indo-Pacific region for stock delineation based on genetic analysis

Table 1. Sampling details of milkfish from Indian waters and other sequences obtained from NCBI database

| Location (Code) | Accession numbers | Sample size |
|---|---|---|
| **India** | | **99** |
| Mandapam (MM) | MW962888–MW962905 | 19 |
| Cochin (PV) | MW962945–MW962963, MZ323080 | 20 |
| Tuticorin (TT) | MW962908–MW962925 | 19 |
| Karwar (CK) | OQ867187-OQ867203 | 21 |
| Chilika (CL) | MW962928–MW962944, MZ323081 | 20 |
| **Indonesia** | | **84** |
| Sulawesi (IoSw) | MF412090- MF412119 | 36 |
| Medan (IoMn) | MF411690- MF411738 | 48 |
| **Philippines** | | **94** |
| Antique (PhAq) | MF411520- MF411537 | 19 |
| Claveria (PhCl) | MF411590- MF411607 | 18 |
| Camarines Sur (PhCm) | MF411540- MF411557 | 18 |
| Sarangani (PhSg) | MF412039- MF412059 | 21 |
| General Santos City (PhGs) | MF411939- MF411956 | 18 |
| **French Polynesia** | | **90** |
| Kauehi (FpKa) | MF59222- MF592239 | 38 |
| Niau (FpNa) | MF592173- MF592225 | 52 |

were grouped into three. Group 1: French Polynesia, Group 2: India, and Group 3: Indonesia and the Philippines. The analysis involved assessing $F_{CT}$ (variation among groups), $F_{SC}$ (variation among samples within groups), and $F_{ST}$ (variation among all samples in populations) values. The AMOVA calculations were performed with 10,000 permutations to assess statistical significance.

Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) were computed to identify potential population bottlenecks or expansions and deviations from the polymorphism pattern anticipated under the neutral model of evolution. A mismatch graph was plotted to examine the number of pairwise mutational changes between haplotypes and their respective frequencies (Rogers and Harpending, 1992; Slatkin and Hudson, 1991). Estimating the time since the most recent population expansion was achieved by fitting the population parameter $\gamma$ (Rogers and Harpending, 1992) for the sampled regions. We calculated the Harpending raggedness index, $\gamma$ (Harpending, 1994), and the sum of squared deviations (SSD) for the goodness-of-fit test to evaluate the agreement between the observed and expected haplotype distributions. All the analyses were conducted using Arlequin 3.5 (Excoffier and Lischer, 2010).

To estimate the time of divergence of the species, we used BEAST v1.10.4 (Bouckaert *et al.*, 2014). An .xml file was created using BEAUti v1.10.4, and the .tre file generated from BEAST was used as an input file for Tree Annotator v1.10.4. The results were visualised in FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/http://tree.bio.ed.ac.uk/software/ figtree/) (Fig. 2).

## Haplotype network analysis

Haplotype networks were employed to depict the evolutionary relationships among distinct haplotypes visually. A Minimum Spanning Haplotype Network showing genetic relationships among the haplotypes was constructed using PopART Ver. 1.7 (Leigh and Bryant, 2015) (Fig. 3).

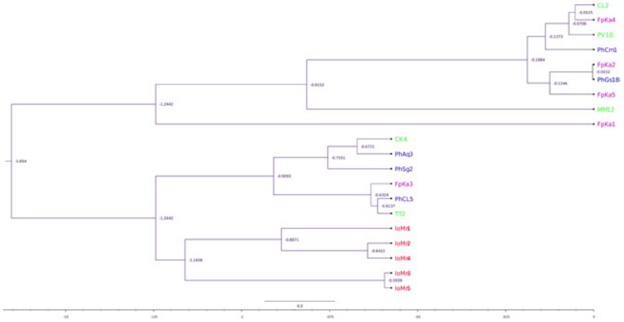

Fig. 2. Bayesian phylogenetic tree for milkfish based on mtDNA sequences (cyt b) haplotypes

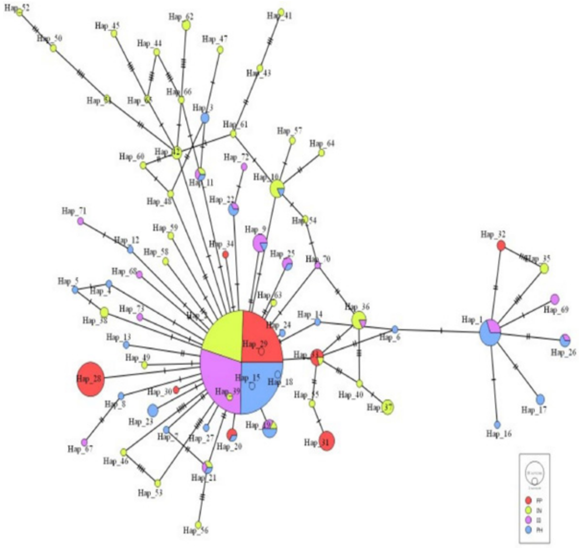

Fig. 3. Haplotype network tree of milkfish using cyt b mtDNA sequences in the Indo-Pacific region. Circles represent haplotypes, mutational steps by vertical stripes and colours indicate geographical locations. FP: French Polynesia, IN: India, IO: Indonesia, PH: Philippines

# Results

## Population genetic structure and demography

An extensive analysis was conducted that aligned and edited 367 cyt b sequences of milkfish from the Indo-Pacific, yielding a total sequence length of 987 base pairs. The examination of genetic parameters within these four populations unveiled significant diversity trends. Notably, India exhibited a high haplotype diversity (h) of 0.798, while Indonesia displayed a considerably lower value of 0.520. In terms of nucleotide diversity (P), values ranged from 0.001(French Polynesia) to 0.003 (India) across the four populations, as outlined in Table 2. A high level of haplotype diversity (H), averaging 0.688 and a low level of nucleotide diversity ($\pi$), with an average of 0.002, were observed within these four populations.

According to Santos *et al.* (2019), the bottleneck could arise from the impact of purifying selection, which is a restrictive force on the evolutionary changes within the cytochrome b gene. We identified 82 variable (polymorphic) sites, defining 73 distinct haplotypes. Within these 82 polymorphic sites, 34 were singleton variable sites, and 48 were parsimony informative sites. The average frequencies of the four nucleotides were as follows: A: 26.11%, T: 25.72%, C: 32.56%, and G: 15.61%. Furthermore, we observed a total of 99 transitions and 26 transversions. The estimated Transition/Transversion bias (R) was calculated to be 3.80. Pairwise $F_{ST}$ values, computed between different groups, and genetic divergence estimates among sampling locations exhibited a range of values from 0.009 to 0.06 (Table 3).

Table 2. Sequence characteristics of milkfish based on cyt b sequences

| Locations | No. of haplotypes | Nucleotide diversity | Haplotype diversity |
|---|---|---|---|
| French Polynesia (FP) | 9 | 0.629 +/- 0.044 | 0.00143+/- 0.000 |
| India (IN) | 39 | 0.798 +/- 0.042 | 0.003 +/- 0.001 |
| Indonesia (IO) | 17 | 0.520 +/- 0.066 | 0.001 +/- 0.001 |
| Philippines (PH) | 27 | 0.697 +/- 0.052 | 0.002 +/- 0.001 |

Table 3. Pairwise $F_{ST}$ comparisons between sampling regions of milkfish based on cyt b gene (below diagonal) and associated *P values* (above diagonal)

| Locations | French Polynesia (FP) | India (IN) | Indonesia (IO) | Philippines (PH) |
|---|---|---|---|---|
| French Polynesia (FP) | | 0.000 | 0.000 | 0.000 |
| India (IN) | 0.055* | | 0.000 | 0.000 |
| Indonesia (IO) | 0.056* | 0.030* | | 0.100 |
| Philippines (PH) | 0.066* | 0.045* | 0.009 | |

**P value* < 0.05

Samples from the Philippines and French Polynesia exhibited greater genetic differentiation compared to other locations, whereas the Philippines and Indonesia showed low genetic divergence. So, they were considered a single genetic group. The Analysis of Molecular Variance (AMOVA) revealed a substantial degree of variation within populations (94.86%) and relatively low variation between populations (4.51%) (Table 4). These pairwise $F_{ST}$ estimates, which fell within the range of 0 to 0.05, were consistently found to be statistically significant ($P < 0.05$) when comparing the populations across the Indo-Pacific region. The results of the two-way AMOVA showed that the fixation index among groups ($F_{CT}$ = 0.0451) did not illustrate statistical significance (*P* = 0.168). Likewise, no statistically significant variation was observed among populations within groups ($F_{SC}$ = 0.0065, (Table 4).

Tajima's D statistic revealed a mean value that was considerably different from zero (-1.96; P<0.05). The Fu's Fs, which is explicitly devised to detect population expansion and is more sensitive to the presence of singletons in the samples, showed significant values in four populations with a mean significantly negative value except for French Polynesia (Fu's Fs = -15.08; P<0.05). The distribution of haplotypes in all populations resulted in negative values in Fu's Fs test, indicating an excess of rare haplotypes compared to what would be expected under neutrality. This strongly rejects the hypothesis of neutral evolution across all populations. The mean raggedness index and SSD (0.024 and 0.074), were not significantly different from zero (P>0·05) (Table 5).

Table 4. Hierarchical AMOVA results, including values for $F_{CT}$ (difference among groups), $F_{SC}$ (differences among populations within groups), and $F_{ST}$ (differences among all individuals within populations). AMOVA analyses using cyt b in milkfish

| Source of variation | d.f. | Sum of squares | Variance components | % of variation | F Statistics | *P* |
|---|---|---|---|---|---|---|
| Among populations | 2 | 14.476 | 0.048 Va | 4.52 | $F_{CT}$: 0.045* | 0.04 |
| Among populations within groups | 1 | 1.608 | 0.006 Vb | 0.62 | $F_{SC}$: 0.006 | 0.08 |
| Within populations | 363 | 368.45 | 1.015 Vc | 94.86 | $F_{ST}$: 0.051* | 0.00 |
| Total | 366 | 384.53 | 1.070 | 100 | | |

Table 5. Values of neutrality tests (Fs and D), sums of squared deviation (SSD) and Harpending raggedness index ($\gamma$) for milkfish populations using cyt b gene

| | Neutrality tests | | Sudden expansion model | |
|---|---|---|---|---|
| Locations | Tajima's *D* (p value) | Fu's FS (p value) | SSD (p value) | Raggedness index ($\gamma$) (p value) |
| French Polynesia (FP) | -1.467* (0.046) | -1.648 (0.241) | 0.0217* (0.023) | 0.107* (0.037) |
| India (IN) | -2.253* (0.0003) | -26.414* (0.000) | 0.002 (0.947) | 0.010 (0.993) |
| Indonesia (IO) | -2.096* (0.002) | -10.376* (0.000) | 0.327 (0.612) | 0.149 (0.998) |
| Philippines (PH) | -2.058* (0.002) | -21.890*` (0.000) | 0.006 (0.823) | 0.032 (0.926) |

* *P value* < 0.05

### *Haplotype network analysis*

The minimum spanning haplotype network illustrating genetic relationships among the haplotypes was generated using PopART v.1.7, which depicted a star-like genealogy (Fig. 2). The absence of separate clades implies that there has not been substantial genetic isolation or differentiation among these populations. This suggests that these populations are currently undergoing population expansion. Each circle's size is proportional to the actual haplotype frequency and includes the number of individuals associated with that specific haplotype. This mtDNA gene tree type represents a network showcasing the minimal mutational distances between cytochrome b haplotypes distributed throughout the Indo-Pacific region. Evolutionary analysis using BEAST v1.10.4 estimated that milkfish populations in the Indo-Pacific region reached a stable population size approximately 1.65 million years ago.

## Discussion

In this study, we used cyt b to assess connectivity patterns among wild populations of milkfish in the Indo-Pacific region. This marker has been proven to be a potent tool for evaluating intraspecific genetic diversity in aquatic species (Kocher *et al*., 1989; Irwin *et al*., 1991). Specifically, we analysed cyt b sequences of milkfish across India, Indonesia, the Philippines, and French Polynesia to investigate their population structure. Our findings revealed low but significant genetic differentiation between Groups 1, 2 and 3. The nucleotide and haplotype diversities observed in these studies offer valuable insights into the historical dynamics of the species across the Indo-Pacific region. They exhibit genetic heterogeneity, characterised by high haplotype diversity (0.520–0.798) and low nucleotide diversity (0.001–0.003). Among the four populations analysed, haplotype diversity was highest in India (0.798) and lowest in Indonesia (0.520). Notably, all populations-except those from French Polynesia—showed this pattern of high haplotype and low nucleotide diversity, which is typical of marine species such as *Eleutheronema tetradactylum* (Wang *et al*., 2014), *Trichiurus nanhaiensis* (Gu *et al*., 2022), and *Pampus argenteus* (Sun *et al.,* 2012).

These diversity indices show that despite having a greater number of analysed samples (n=90), the French Polynesia population had a lower number of haplotypes (9) and low nucleotide diversity (0.001). The low diversity can be attributed to inbreeding, fixation of alleles, or the founder effect. This refers to the loss of genetic variation that occurs when a new population is established by a very small number of individuals from a larger population. The sequences obtained from French Polynesia are from Niau and Kauehi. Niau's atoll has an enclosed lagoon that has a milkfish population whose origin is enigmatic and is expected to be human-derived from the surrounding ocean population. The Indian population has high haplotype diversity with 39 haplotypes when 99 samples were analysed.

The neutrality test, utilising Tajima's D showed negative values for all populations, signifying statistical significance ($P<0.05$). Furthermore, Fu's test revealed negative values of Fs, which were statistically significant for all populations. This indicates an excess number of alleles, which is expected during a population expansion in the Indo-Pacific region. Fu's F-test produces negative Fs values as it is sensitive to changes in population demographics. Additional evidence supporting the ongoing population expansion includes the low and statistically insignificant sums of squared deviation (SSD) and raggedness index ($\gamma$) values. Pairwise mismatch distribution plots for all four populations of milkfish are given in Fig. 4. The unimodal progression observed in the mismatch distribution analysis also suggests the same observation. Using the Sum of Squares Deviations (SSD) and raggedness index ($\gamma$), the goodness of fit between the observed and expected distributions under the sudden expansion models was calculated. No statistically significant differences were detected except in French Polynesia. Remarkably, the haplotype network exhibited star-shaped phylogenies, with shared haplotypes predominantly at the centre representing Indo-Pacific origin. The oldest haplotypes naturally occupy this central position, characterised by many connections to other haplotypes. Samples from both Indian and French Polynesia show an extended distribution. The haplotype network tree indicates that populations across the Indo-Pacific region do not form distinct clades and suggests that these populations are in an early stage of differentiation and demographic expansion, where lineage divergence has begun but has not yet progressed to the formation of clearly separate clades.

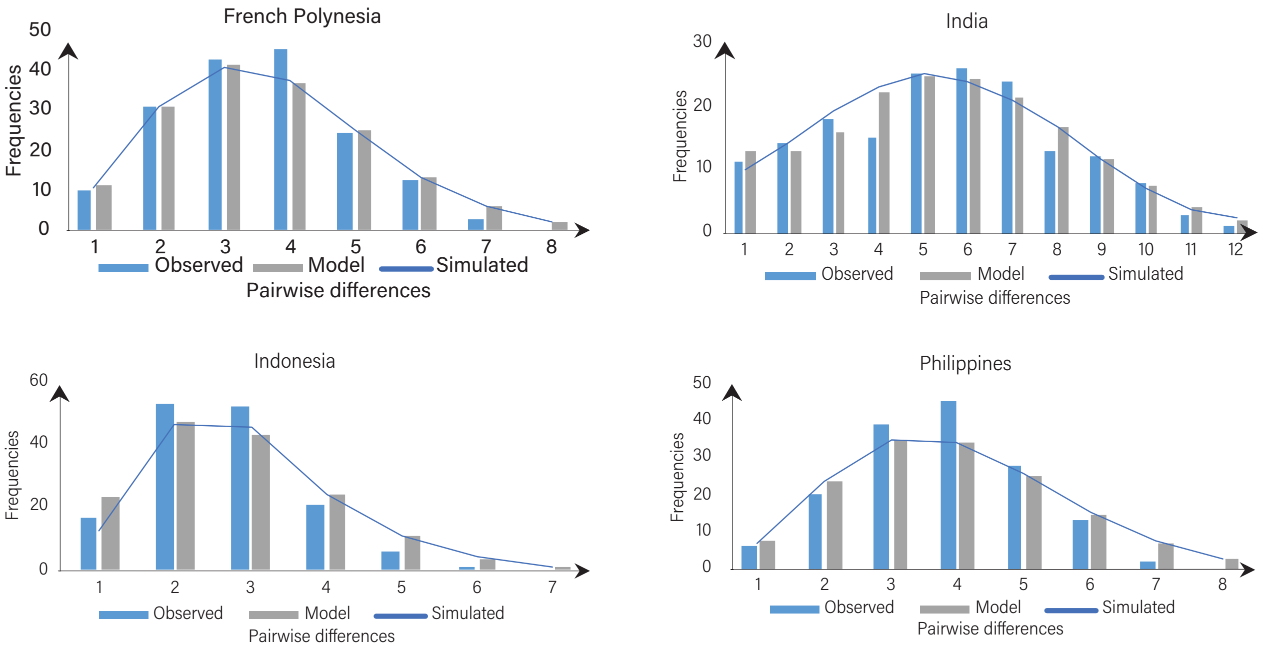

Fig. 4. Pairwise mismatch distribution curve using the sudden expansion model for each population of milkfish

Our study, employing AMOVA and F-statistics, unveiled statistically significant genetic differentiation within the Indo-Pacific region. Among the four Indo-Pacific locations, Indonesia and the Philippines showed no genetic differentiation, while the most significant genetic distance (0.067) was observed between the Philippines and French Polynesia. As one-way AMOVA revealed no significant difference between Indonesia and the Philippines, a hierarchical AMOVA was conducted to assess genetic variation at multiple levels, $F_{CT}$ (difference among groups), $F_{ST}$ (difference among samples within groups) and $F_{ST}$ (overall differences among all samples in a population). In the hierarchical AMOVA analysis, the population were divided into three distinct groups: Group 1 (French Polynesia), Group 2 (India), and Group 3 (Indonesia and the Philippines). Results from this study suggest a significant variation among the three groups. This is due to restricted gene flow and population differentiation, likely driven by geographic isolation, oceanographic barriers, and possibly human-mediated introductions. The genetic similarity between Indonesia and the Philippines suggests strong connectivity facilitated by marine currents, natural larval dispersal, or broodstock exchange through aquaculture. In contrast, the high genetic distance of French Polynesia-particularly from isolated atolls as Niau-points to long-term isolation and potential founder effects.

The Bayesian phylogenetic tree indicates that the milkfish population in the Indo-Pacific region reached a stable population size approximately 1.65 million years ago, during the late Pleistocene period. During the late Pleistocene, repeated glacial cycles caused major sea-level drops and environmental changes, which reshaped marine habitats and species distributions (Imbrie *et al.*, 1992; Lambeck *et al.*, 2002). These shifts led to genetic consequences that can now be explored using molecular markers (Hewitt, 2000). DNA polymorphism and haplotype patterns provide insights into population history and structure (Li, 1997), while statistical tools help estimate evolutionary parameters and test demographic hypotheses (Fu, 1993, 1997; Tajima, 1983, 1989). The undertaking of population studies is crucial for the effective conservation and management of the biodiversity of the species.

## Conclusion

This transoceanic study reveals that milkfish populations in the Indo-Pacific are not panmictic but exhibit clear regional genetic structuring. High connectivity is observed among Indian and Southeast Asian populations, while remote populations like those in French Polynesia are genetically distinct due to isolation. The observed genetic patterns indicate the demographic expansion of milkfish populations and their typical dispersal dynamics. These findings highlight the importance of region-specific conservation and management strategies to preserve the genetic diversity of species.

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## Author contributions

Conceptualisation: DPR; Data Collection: MC; Methodology: DMJ; Writing Original Draft: DMJ, DPR; Data Analysis: DMJ; Supervision: DPR.

## Data availability

The data are available and can be requested from the corresponding author

## Conflicts of interest

The authors declare that they have no conflict of financial or non-financial interests that could have influenced the outcome or interpretation of the results.

## Ethical statement

No ethical approval is required as the study does not include activities that require ethical approval or involve protected organisms/ human subjects/ collection of sensitive samples/ protected environments.

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## Publisher's note

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