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Genetic Diversity of *Chanos Chanos* (Forsskål, 1775) from Natural Populations in Vietnam

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Abstract

The genetic diversity of five natural populations of milkfish (*Chanos chanos*) collected in Nghe An, Quang Binh, Binh Dinh, Phu Yen, Khanh Hoa provinces in Vietnam was examined using COI gene sequence analysis. Twelve haplotypes were noted from a total of 50 sequences along with 12 variable sites and 6 parsimony informative sites. The Quang Binh milkfish population had the highest haplotype (0.889 ± 0.060) and nucleotide diversities (0.00301 ± 0.00049). Overall, haplotype and nucleotide diversities were 0.804 ± 0.036 and 0.00212 ± 0.00026, respectively. Genetic differentiation (F_{ST}) was high between the milkfish populations of Nghe An – Quang Binh (0.21744) and Nghe An - Phu Yen (0.26215). Haplotype network analysis indicated that milkfish populations shared common haplotypes and each population had its own private haplotypes. Population structure and demographic expansion were not evident for all populations except for Quang Binh. This is the first principal endeavor to understand genetic information of milkfish in Vietnam, thereby providing information for scientists, managers, and the general public to establish timely strategies to explore, protect, and develop milkfish genetic resources in the future.

Keywords

COI sequences, milkfish, natural populations, genetic diversity, genetic relationship

Introduction

The milkfish, *Chanos chanos* (Forsskål, 1775), is the only living species of the family Chanidae (Bagarinao, 1991; Leis & Reader, 1991). They are widely distributed in the Indo-Pacific region and inhabit subtropical and tropical areas (Beveridge & Haylor, 1998). *C. chanos* is one of the most important fish species cultured in Asian

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countries such as the Philippines, Indonesia, and Taiwan. The total milkfish production in these countries accounts for 99.8% of the global milkfish production (Santos *et al*., 2019). Vietnam is a country with a high potential to develop milkfish aquaculture. However, studies on milkfish in Vietnam have only begun in recent years, and these studies have mainly focused on the collection, storage, and preliminary understanding of some biological characteristics of milkfish (Le Van Sinh *et al*., 2005; Ta Thi Binh, 2015; Nguyen Thi My Dung *et al*., 2020). Moreover, there are no records of genetic information for milkfish in Vietnam prior to this study.

There are many different methods that enable the assessment of genetic diversity of a species. In particular, molecular markers are commonly used for genetic diversity studies. Microsatellite markers are widely used and are effective in studying the genetic structures of fish (Abdul-Muneer, 2014). On the other hand, mitochondrial DNA cytochrome oxidase I (COI) sequence information also plays an important role in identifying and assessing genetic diversity (Bingpeng *et al*., 2018). In animals, the COI gene is frequently used to identify species through the use of the polymerase chain reaction (PCR) technique and universal primers to facilitate amplification (Folmer *et al*., 1994). This gene sequence is always preserved within a species, and the rate of mutation is fast enough to distinguish between closely related species (Rebijith *et al*., 2016). For milkfish conservation and aquaculture development in Vietnam, it is important to gain a better understanding of the genetic variability in milkfish stocks. Therefore, we conducted this study to investigate the genetic diversity of natural milkfish populations in Vietnam using mitochondrial COI gene sequence analysis.

Materials and Methods

Sample collection

Fifty milkfish (*C. chanos*) samples were randomly collected from five provinces in Central Vietnam, with ten specimens per province, from April 2017 to March 2018 (**Table 1**). The fin samples for DNA extraction were clipped from the caudal fin and preserved in 96% ethanol at 4°C until further use.

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted using the ethanol precipitation of DNA method according to Sambrook & Russell (2001). The cytochrome c oxidase subunit I (COI) sequences were amplified using the primers FishF1 [5′-TCAACCAACCACAAAGACATTGGCAC-3′] and FishR1 [5′-TAGACTTCTGGTGGCCAAGAATCA-3′] (Ward *et al*., 2005). PCR was conducted using an Eppendorf Mastercycler® pro. The PCR reaction mixture in a 50-µL reaction contained 200 ng μL^{-1} of DNA, 20µM of each primer (1µL), 25µL of MyTaq™ Mix 2x (Bioline), and water to complete the 50µL volume. Amplification was performed with a two-minute denaturation step at 94°C, followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing at 56°C for 30 seconds, and extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. A UVITEC Gel Documentation System was used to check the quality of the PCR products on a 2% agarose gel. The PCR products were sequenced following the forward direction.

Data analysis

The COI sequences were verified, aligned, and trimmed to the same length using BioEdit 7.2.5 with the ClustalW function under default settings (Thompson *et al*., 1994). The BioEdit software was also used to determine the degree of sequence similarity. The software DnaSP version 5.0 was used to estimate the molecular diversity indices such as haplotype diversity (Hd), nucleotide diversity (π), the number of private haplotypes (Hp), and demographic patterns using Tajima's D test and Fu's Fs test (Librado & Rozas, 2009). Hierarchical analyses of the molecular variance (AMOVA) were performed using Arlequin 3.5 to calculate the level of genetic differentiation among the different populations (Excoffier & Lischer, 2010). A haplotype network was constructed using NETWORK version 5.0.0.3 (Forster *et al*., 2007).

Table 1. Milkfish sampling sites and the genetic variability parameters of the examined populations

Sampling site	Coordinates	Collection date	n	n_H	H_p	Hd (Mean ± SD)	π (Mean ± SD)
Nghe An	18°52'34.7"N, 105°44'47.9"E	12-Apr-2017	10	5	2	0.756 ± 0.130	0.00147 ± 0.00037
Quang Binh	17°29'48.9"N, 106°44'45.4"E	4-Apr-2018	10	5	2	0.889 ± 0.060	0.00301 ± 0.00049
Binh Dinh	13°59'44.2"N, 109°18'36.0"E	10-Oct-2017	10	5	1	0.822 ± 0.097	0.00171 ± 0.00035
Phu Yen	13°07'14.1"N, 109°24'29.7"E	6-Mar-2018	10	4	1	0.644 ± 0.152	0.00144 ± 0.00040
Khanh Hoa	12°04'06.5"N, 109°16'34.8"E	12-Mar-2018	10	5	2	0.800 ± 0.100	0.00236 ± 0.00073
Total			50	12	8	0.804 ± 0.036	0.00212 ± 0.00026

Note: n: sample size; n_H: number of haplotypes; Hp: number of private haplotypes; Hd: haplotype diversity; π: nucleotide diversity.

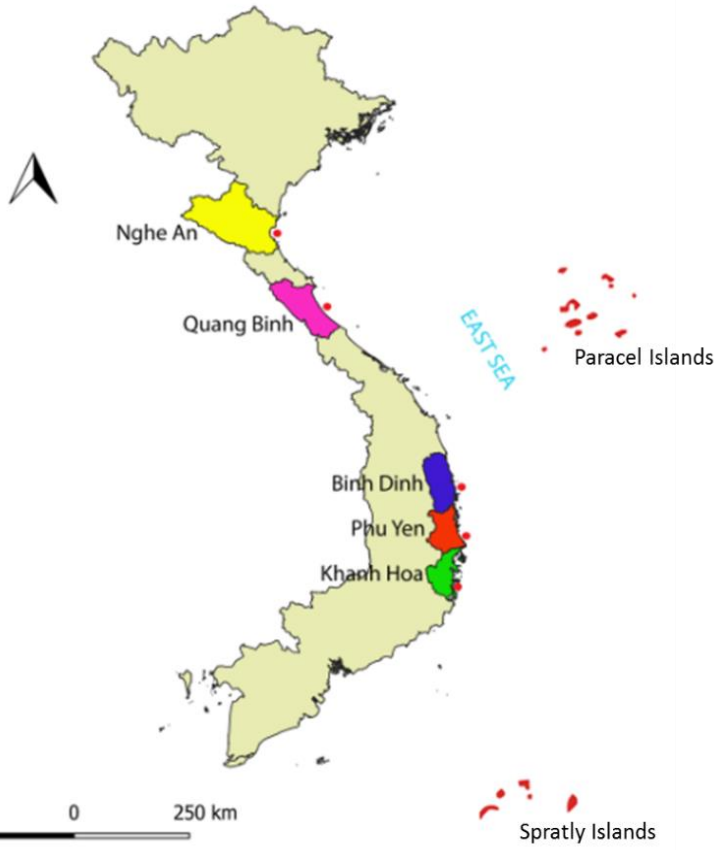

Figure 1. Sampling locations map with the five sampling sites indicated by red dots

Results

Haplotype diversity and nucleotide diversity

The lengths of the COI gene sequences were 649 bp after alignment. The 50 COI gene sequences obtained without indels had 12 variable sites and 6 parsimony informative sites, resulting in 12 haplotypes, 50% of which were singletons (represented by a single sequence in the sample). The number of haplotypes per

population ranged from four to five. Milkfish populations in Nghe An, Quang Binh, and Khanh Hoa had the highest number of private haplotypes (Hp = 2), followed by Binh Dinh and Phu Yen (Hp = 1). Quang Binh showed the highest values of haplotype diversity (0.889 ± 0.060) and nucleotide diversity (0.00301 ± 0.00049). The COI gene haplotype and nucleotide diversities had overall values of 0.804 ± 0.036 and 0.00212 ± 0.00026, respectively (**Table 1**).

Based on the COI sequences, a haplotype network was formed (**Figure 2**). The radial network had a number of distinct haplotypes linked to a central haplotype. **Figure 2** shows that the haplotype H_1 held the middle position in the network, one step distant from the other 11 haplotypes, accounting for 34% (17/50) of all the milkfish samples. This indicated that H_1 was the ancestral haplotype of the *C. chanos* populations in this study. Haplotype H_2 appeared in all the populations but at lower frequencies than H_1 (7/50). Haplotype H_7 was found in most of the studied populations except that of Nghe An, and the frequency of haplotype H_7 in Phu Yen accounted for nearly 50%. Haplotype H_8 for Quang Binh and haplotype H_11 for Khanh Hoa was the most distant from the others. Furthermore, each population had its own set of haplotypes that were unique to that population. All the haplotype sequences were deposited in the GenBank database (accession numbers: MK241873 - MK241884).

Genetic differentiation

Pairwise F_{ST} values among the populations are shown in **Table 2**. The milkfish population in Nghe An province was found to be significantly different from those of Quang Binh and Phu Yen provinces. Other milkfish populations had no genetic differences (P >0.05).

Population structure

Hierarchical analysis of AMOVA (**Table 3**) indicated that the majority of the overall genetic variation (92.96%, P <0.05) was from differences within populations, and only 7.04% (P <0.05) of the variation was found among populations. These results demonstrated that the total genetic variation occurred within populations, and

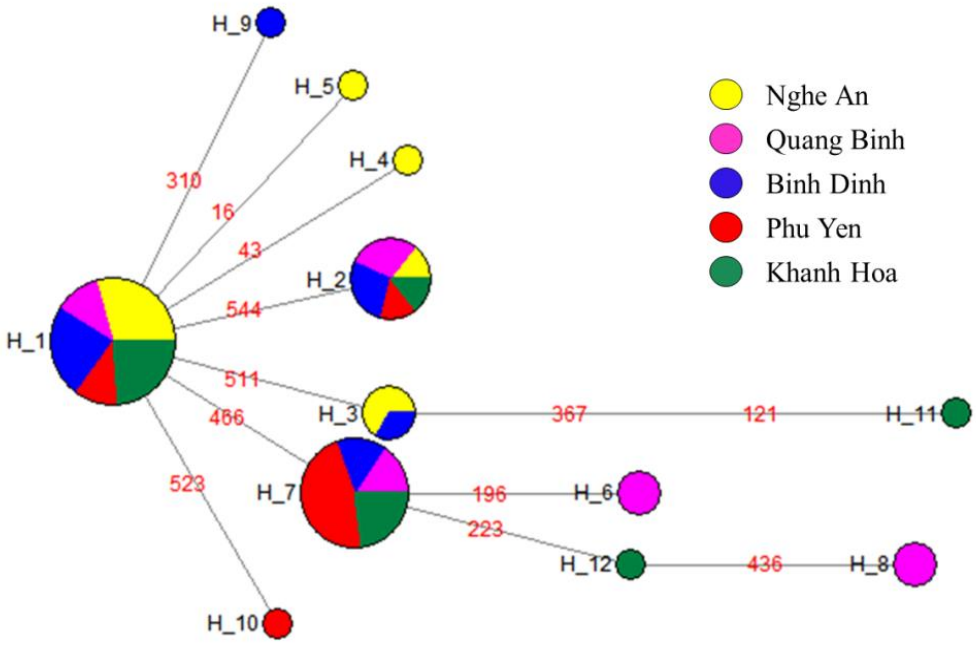

Figure 2. Haplotype network of the studied milkfish populations in Central Vietnam

Note: The lengths of the connecting lines are related to the number of mutational steps between haplotypes, and the sizes of the circles are proportional to the haplotype frequency. The authors assigned external information related to the genotypes (Nghe An, Quang Binh, Binh Dinh, Phu Yen, Khanh Hoa) to replace the circles with colors that represented the frequency of each group. Colors are used in the haplotype network to show the distribution of groups or populations within each haplotype.

Table 2. Pairwise F_{ST} values based on the COI sequences (below diagonal) and associated P values (above diagonal)

Milkfish populations	Nghe An	Quang Binh	Binh Dinh	Phu Yen	Khanh Hoa
Nghe An		0.00000	0.66667	0.01802	0.14414
Quang Binh	0.21744		0.17117	0.46847	0.52252
Binh Dinh	-0.01307	0.08730		0.23423	0.79279
Phu Yen	0.26215	-0.00309	*0.08730*		0.82883
Khanh Hoa	*0.07131*	-0.00255	-0.03299	-0.02778	

Note: Bolded values are significant (P <0.05).

Table 3. Results from the analysis of molecular variance (AMOVA) of the population structure

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation
Among populations	4	4.560	0.04911	7.04
Within populations	45	29.200	0.64889	92.96
Total	49	33.760	0.69800	

population structure was not well defined among the studied populations.

Demographic history

Tajima's D and Fu's Fs values were negative for all five populations except that of Quang Binh (Tajima's D value). These values were not significant for most of the populations except those of Nghe An and Binh Dinh (Fu's Fs values) (**Table 4**). Tajima's D and Fu's Fs tests having negative values indicate that population demographic expansion is occurring (Tajima, 1989; Fu, 1997).

Discussion

Milkfish genetic variations have been previously investigated in the Pacific Ocean from the Philippines to Hawaii using isozymes and PCR-RFLP (Winans, 1980; Ravago-Gotanco & Juinio-Meñez, 2004). Other studies utilized, namely, the use of: (a) mitochondrial cytochrome b sequences to determine the genetic relationships among milkfish from Indonesia, the Philippines, and Taiwan (Adiputra *et al*., 2011); (b) amplified fragment length polymorphism (AFLP) in Indonesian stocks (Adiputra *et al*., 2012); (c) microsatellite markers of milkfish in the Philippines which were characterized using novel short tandem repeats (Santos *et al*., 2015; Romana-Eguia *et al*., 2018); (d) the mtDNA control region and cytochrome *b* genes in the Philippines and Indonesia (Santos *et al*., 2019); and (e) ATPase 6/8 genes in India (SriHari *et al*., 2019). This research is the first attempt to compare the genetic diversity of milkfish in Vietnam and also is the first study to use the COI marker for genetic assessment in milkfish.

Previously reported values of milkfish genetic diversity were considerably lower than this preliminary study. Winans (1980) reported

Table 4. The COI sequence-based neutrality measures and demographic estimates for the five wild milkfish populations

Populations	Fu's Fs	Tajima's D	Fu and Li's D	Fu and Li's F
Nghe An	-2.377*	-1.24468	-1.12706	-1.29372
Quang Binh	-0.653	0.42681	1.30011*	1.21749
Binh Dinh	-1.993*	-0.82229	-0.33833	-0.51090
Phu Yen	-1.020	-0.43130	-0.80490	-0.79808
Khanh Hoa	-1.215	-1.14612	-1.51001	-1.59382

*Note: * Significant at P <0.10 for related index.*

the average heterozygosity (H) was 0.075, Adiputra *et al*. (2012) presented the range of H from 0.041 to 0.187, Santos *et al*. (2019) showed that haplotype diversity (Hd) was 0.66 for cytochrome *b* gene, and SriHari *et al*. (2019) recorded that the Hd ranged from 0.5684 to 0.8053 and the nucleotide diversity (π) fluctuated from 0.001838 to 0.002519. Genetic diversity in marine fish is generally considered high when Hd > 0.5 and $\pi > 0.5\%$ (Grant & Bowen, 1998). In this study, the five populations had a high level of haplotype diversity (average of 0.804) and a low value of nucleotide diversity (average of 0.00212). According to Grant & Bowen (1998), high Hd and low π imply a bottleneck or founder effect followed by rapid expansion, whereas high values for both Hd and π indicate that the populations are large and stable. The bottleneck could be due to purifying selection, which has slowed the evolution of the COI gene. The value of Hd in this study was also noted for being at a high level when compared with other marine fish species (Grant & Bowen, 1998; Gaither *et al*., 2010; Winters *et al*., 2010). As presented by Bay *et al*. (2004), two fish populations of *Chlorurus sordidus* in Amirante and Papua New Guinea had the highest Hd (Hd = 1). The highest Hd values were also recorded by Hobbs *et al*. (2013) for fish populations of *Centropyge flavicauda* in Christmas Island and *Centropyge joculator* in the Cocos Islands. Both studies of Bay *et al*. (2004) and Hobbs *et al*. (2013) used the mitochondrial control region (D-loop) to investigate the genetic diversity of marine fish populations. The highest haplotype diversity in milkfish (Hd = 0.997) was reported in wild populations in the Philippines using the mitochondrial control region (Santos *et al*., 2019). This difference is likely due to the mitochondrial control region gene having higher levels of haplotype diversity than those of cytochrome *b* and COI.

According to Grant *et al*. (1987), the differences in allele frequencies or genotypes among marine fish populations are due to differences in migration, genetic drift, and natural selection. These authors also assumed that there would be little or no expected genetic differences between marine fish populations due to the high gene flow between populations. In this study, the Nghe An milkfish population was found to be significantly different from the Quang Binh and Phu Yen milkfish populations. This can be explained by the lack of migration or gene flow between these populations due to fishing activities and the collection of fry for rearing. Moreover, Nghe An, Quang Binh, and Phu Yen are geographically distant areas. An overabundance of low-frequency polymorphisms is indicated by a negative Tajima's D value and is usually interpreted as purifying selection, or as a signal of recent population expansion. On the other hand, a positive Tajima's D means low rates of both low and high-frequency polymorphisms, suggesting a reduction in population size. Except for Quang Binh, all populations in this study had negative Tajima's D values. None of these values were significant. This can be explained in that the number of samples per population was not large enough to assess significance or that these populations are neutrally evolving. Fu and Li's D and F statistics showed that all values were negative and non-significant except for the fish from Quang Binh. These negative values indicated an excess of singletons and can be assumed to mean that there is a population expansion in Quang Binh due to Fu and Li's statistics. In fact, Nghe An, Phu Yen, Khanh Hoa, and especially Binh Dinh are areas with a long tradition of catching milkfish fry from the wild for combined aquaculture with shrimp and crab. These activities have not been recorded in Quang Binh. This could be one of the reasons for the population expansion observed only in Quang Binh. The most important aspects that determine and affect the current status of fish species are biological factors such as breeding and feeding habits, seasonal migration behaviors, and environmental factors. Besides, overfishing also has a great influence on a fish population's genetic structure. Based on these research findings, we recommend that the authorities should complete the policy for the appropriate utilization and conservation of milkfish resources. Moreover, scientists need more studies to get further information about milkfish resources as well as investigating methods to

manage the milkfish genetic resources in Vietnam.

Conclusions

In this study, five milkfish populations generally exhibited a high value of haplotype diversity and a low value of nucleotide diversity based on mtDNA COI marker analysis. High indices were recorded for the Quang Binh population. In terms of genetic differentiation, the milkfish population in Nghe An was found to be significantly different from those of Quang Binh and Phu Yen. Population structure and demographic expansion were not evident for the populations except for Quang Binh. This study is the first major attempt to understand the genetic diversity of milkfish in Vietnam. Further studies on milkfish in Vietnam should focus on expanding sampling sites, screening more samples in both hatchery and other wild populations, and using other polymorphism molecular markers.

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