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Development and Characterization of 12 Microsatellite Markers for an Economically Important Fish, *Caranx ignobilis*, in the Philippines

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Abstract

The giant trevally (*Caranx ignobilis*) is an important food fish that faces increasing demand due to human population growth, necessitating genetic monitoring. This study intends to spearhead the development of microsatellite markers to assess the genetic variation of *C. ignobilis* populations. A total of 150 *C. ignobilis* specimens from six localities in the Philippines were analyzed. Based on next-generation sequencing data, 12 primer pairs were developed with high amplification rates and polymorphism across different loci. This study introduces the first microsatellite markers exclusively for *C. ignobilis*, which can be used for monitoring and management purposes.

Introduction

Genetic monitoring of various aquaculturally significant species is more critical nowadays because of the increased demand for these resources due to a rapid increase in the human population (Toth & Szigeti, 2016). Numerous management and conservation efforts are being made to maintain their genetic variation which helps to ensure the long-term stability of these species in the face of increasing anthropogenic activities (Abdul-Muneer, 2014). Examples of such efforts include DNA barcoding studies that seek to document and assess the biodiversity of a particular location (Aquilino et al., 2011). Other studies include the use of molecular markers (such as mitochondrial DNA and nuclear DNA) in order to assess the demographic history of particular species, which helps determine the stability and adaptive potential of those populations in changing environments (Martinez et al., 2018). Molecular markers have also been utilized to assess the genetic structure of a particular population or species. This is instrumental in conservation initiatives as it helps identify distinct management units within a population (Reiss et al., 2009). Thus, generating genetic information has become an invaluable part of monitoring and conservation efforts, in the hope of better understanding the biological traits of a particular species (Beger et al., 2014).

One of the emerging aquaculture species is the giant trevally, *Caranx ignobilis*, due to its relatively large body size, excellent meat quality, and high market value (Alaira & Rebancos 2014). *Caranx ignobilis* is a marine

migrant teleost fish that is widely distributed across the Indo-Pacific regions (Lédée et al., 2015). Due to its aquaculture potential, several countries, such as the Philippines, have started culturing this species in captivity (Mutia et al., 2020).

To date, there have been relatively few studies focused on the genetic analysis of *C. ignobilis*. Specifically, these studies have exclusively relied on mitochondrial DNA as their molecular marker (Santos et al., 2010; Aquilino et al., 2011; Willete & Padin, 2014; Torres & Santos, 2021). No extensive studies have utilized nuclear DNA, in particular microsatellite markers, in assessing the genetic variation of *C. ignobilis* populations. Microsatellites have become a popular molecular marker used for investigating genetic variation and structure in the field of fisheries. This popularity stems from their even distribution in the genome, high level of polymorphism, codominant mode of inheritance, and relatively small size (Liu & Cordes, 2004). Traditionally, generating markers for microsatellite loci is known to be expensive and labor-intensive. However, through the advent of more advanced technologies, such as next-generation sequencing (NGS), microsatellite marker generation has been increasingly cost-effective and time-efficient over the past few years (Ariede et al., 2018). With this information into consideration, this study intends to develop microsatellite markers for *C. ignobilis* populations using data from next-generation sequencing.

Materials and Methods

A total of 150 *C. ignobilis* specimens were collected from six localities in the Philippines (Figure 1). Sample tissue was excised from the epaxial muscle behind the operculum on the right side of the fish and stored in absolute ethanol at -20°C before DNA extraction. 20 to 25 milligrams of the preserved muscle tissue were used for DNA extraction using the ISOLATE II Genomic DNA Extraction Kit (Meridian Bioscience, Australia).

The genome contigs of *C. ignobilis* (Santos, 2014) were downloaded from the Data Dryad website (Santos, 2014) and were used for partial assembly, microsatellite marker search, and primer design. These procedures were carried out at the Philippine Genome Center, Quezon City, Philippines. The microsatellite loci were predicted using MIcroSAtellite Identification Tool (Misa) version 2.1 (Beier et al., 2017), mreps version 2.6 (Kolpakov et al., 2003), and SciRoKoCo version 3.4 (Kofler et al. 2007). The Polymerase Chain Reaction (PCR) primer pairs were designed using Primer3 version 0.4.0. The microsatellite loci in the dataset were then filtered according to the following criteria: (1) loci must be perfect, (2) loci must be within the di, tri, and tetra period, and (3) loci must not be a mononucleotide repeating unit. For all the resulting branches, another three filtering steps (primer length, amplicon length, and annealing temperature) were done to identify loci with a good likelihood of successful amplification. In terms of primer length, primer pairs with lengths outside of 20 bp to 22 bp for both forward and reverse primers were excluded. In terms of amplicon length, primer pairs with expected output outside of 100 bp to 450 bp were removed. Lastly, in terms of annealing temperature, primer pairs with annealing temperatures outside of 57 to 58°C were removed. All the criteria used were based on previous literature (Renshaw et al.. 2016; Premachandra et al., 2017) that performed microsatellite analysis for species belonging to the subfamily Caranginae, which is a taxon to which *C. ignobilis* belongs.

Twelve (12) primer pairs were chosen and synthesized for further testing, each of which targeted the microsatellite loci. Validation of the 12 microsatellite primer pairs was performed using the DNA extracts of *C. ignobilis* collected from all the aforementioned sites. All collected specimens were amplified using a total volume of 25 µl of PCR mix, which consisted of 12.375 µL ultra-pure water, 6.875 µL reaction buffer (5x) containing 5 mM of dNTPs, 1 µL MgCl2 (25mM), 1.25 µL of forward primer (10 µM), 1.25 µL of reverse primer (10 µM), 0.25 µL of Taq polymerase, and 2.00 µL of purified DNA. The PCR condition optimized for all the primers was set at 95°C for 4 minutes, followed by 35 cycles of 95°C for 0.50 minutes, 58°C for 0.50 minutes, and 72°C for 0.50 minutes. Afterward, samples were subjected to the final extension step at 72°C for 10 minutes and then kept at 4°C.

The 12 primer pairs were screened for amplification and polymorphism using 10% polyacrylamide agarose gels. Analysis of the gel images and molecular weight detection was done using GelAnalyzer 2010a (Lazar & Lazar, 2010). This was done to validate the expected band size and identify whether the specimens were homozygote or heterozygote to a specific microsatellite locus. GenaAlex package (Peakall & Smouse, 2012), which ran in Microsoft Excel 2016, was used to calculate molecular diversity indices such as the number of alleles (N_A), effective number of alleles (N_e) observed heterozygosity (H_o), and expected heterozygosity (H_e).

Results

The three microsatellite marker search tools used in the study predicted a total of 965,474 microsatellite repeat loci. Among these, 821,104 (85.05%) were perfect repeats, while 144,370 (14.95%) were ambiguous repeats. A total of 2,399,740 primer pairs were generated from the perfect microsatellite loci predicted. Further classification of these primers was done using the mentioned filtering criteria in the materials and methods and yielded 34 primer pairs for dinucleotide repeats, 33 primer pairs for trinucleotide repeats, and 36 primer pairs for tetranucleotide repeats.

Out of the 103 primer pairs that had met these criteria, 12 primer pairs were amplified first using

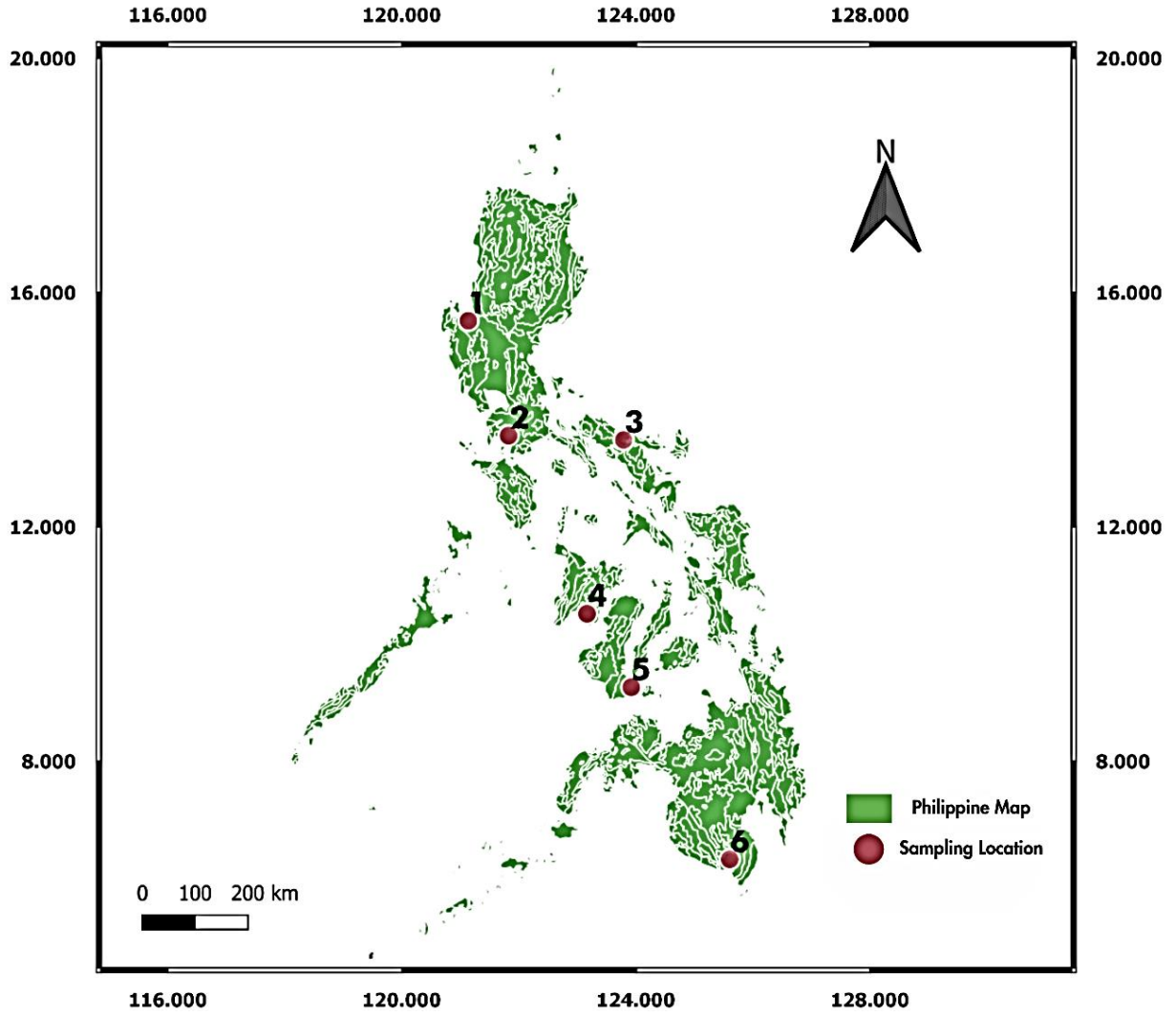

Figure 1. Sampling sites for *Caranx ignobilis* specimens: (1) Dagupan, Pangasinan (Lingayen Gulf); (2) San Nicholas and Talisay, Batangas (Taal Lake); (3) Calabanga, Camarines Sur (San Miguel Bay); (4) Iloilo City, Iloilo (Iloilo Strait); (5) Dumaguete, Negros Oriental (Bohol Sea); (6) Alabel, Sarangani (Sarangani Bay)

random stratified sampling; four (4) pairs were obtained from dinucleotide repeats, four (4) from trinucleotide, and four (4) from tetranucleotide repeats. All 12 primer pairs showed high amplification rates and exhibited high polymorphism among the different loci, as evidenced by the high expected heterozygosity levels and the number of alleles. The heterozygosity levels ranged from 0.915 to 0.946 while the number of alleles ranged from 17 to 26. On the other hand, the calculated observed heterozygosity values ranged from 0.067 (Ci07) to 0.928 (Ci01) while values for expected heterozygosity ranged from 0.915 (Ci04) to 0.946 (Ci11). The observed heterozygosity for all loci was lower than the expected heterozygosity (Table 1).

Discussion

The use of microsatellites, also known as simple sequence repeats (SSR), as a molecular marker of choice in the field of fish population genetics has become more widely accepted as a result of its hypervariability and high abundance in the genome. (Abdul-Muneer, 2014). The present study provides the first record of microsatellite primers that can be used specifically for *C. ignobilis* species. All the 12 primer pairs tested in this study exhibited a high degree of polymorphism having an expected heterozygosity level above 0.90. Microsatellites with a calculated expected heterozygosity level of more than 70% can be said to be polymorphically informative molecular markers that can be used to infer genetic variability in a population (Ott, 1992). Likewise, the generated primer in this study recorded the maximum number of alleles, which is noted to be 26 on three loci, which is higher in comparison with other studies of microsatellite variations in species belonging to the subfamily Carangidae (Renshaw et al., 2016; Premachandra et al., 2017). The high number of alleles recorded in this study may suggest that the chosen loci region has a high mutation rate or positive selection (Fonteque et al., 2014) which is one of the good characteristics of a molecular marker that is intended to be used for

Table 1. Description of 12 microsatellite loci developed and validated in the study.

Primer Label	Primer Sequences	Annealing Temperature (°C)	Repeat Motif	Expected Product Size (bp)	Size Range	Number of Alleles (N_a)	Effective Number of Alleles (N_e)	Observed Heterozygosity (H_o)	Expected Heterozygosity (H_e)
Ci01	F: 5'-AGCTGATTCACACCTTCACC-3' R: 5'-AGTGTTGAGTCAAAGGAGTTCA-3'	58	$(CA)_{10}$	128	90-163	26	19	0.928	0.939
Ci02	F: 5'-CACACACACACACACACATT-3' R: 5'-AGAGAGAGAGAGAACAACAGGT-3'	58	$(AC)_6$	141	104-197	26	18	0.856	0.943
Ci03	F: 5'-AACACACACATGAACACAATGG-3' R: 5'-GCTGATTTCACGGCCAACAG-3'	58	$(AC)_6$	345	300-453	24	18	0.331	0.943
Ci04	F: 5'-ACTCCATTCTCCCACTCCC-3' R: 5'-TTTCTGTCTGTCCACGGCTC-3'	58	$(CT)_8$	301	250-365	18	13	0.270	0.915
Ci05	F: 5'-CACACACACACTCCCTGTAG-3' R: 5'-TTCAGCTGCCTCAATTCACT-3'	58	$(AGG)_6$	132	96-203	20	14	0.289	0.924
Ci06	F: 5'-TCAGTGGTCATGCATGTCTTT-3' R: 5'-CCCTCATTATCACATCAAATCCC-3'	58	$(AGT)_5$	148	101-198	18	14	0.155	0.921
Ci07	F: 5'-AAGCCAGTACAGTCAGAGAGA-3' R: 5'-TCACCACCGAGTTAAGTGGC-3'	58	$(ATT)_5$	316	307-440	17	14	0.067	0.920
Ci08	F: 5'-TCCTCCTCTTCTTCTTCTGTTCT-3' R: 5'-GAGGTCTCGGTGGGTTTCTG-3'	58	$(TCT)_5$	303	268-387	22	16	0.277	0.925
Ci09	F: 5'-AGGTACCTACCACTTCTCGT-3' R: 5'-GTGCGATGAACACTTCATTACT-3'	58	$(TAGA)_5$	118	98-201	22	17	0.528	0.935
Ci10	F: 5'-TGGATTCCTCACTACTTCCTGA-3' R: 5'-ATGAGGTCTCTCAGTCCTTCT-3'	58	$(TTTA)_5$	179	115-238	20	14	0.206	0.925
Ci11	F: 5'-ATGTGCGCGCTACAAATAGA-3'	58	$(TGAA)_5$	353	253-	26	20	0.425	0.946

investigation of genetic diversity and population structure (Li et al., 2009).

In terms of genetic variability, the calculated observed heterozygosity for the 10 microsatellite loci resulted in low values, which ranged from 0.067 (Ci07) to 0.528 (Ci09). On the other hand, only two (2) microsatellite loci resulted in high observed heterozygosity, with values of 0.856 (Ci02) and 0.928 (Ci01). A study conducted by Martinez et al. (2018) estimated the heterozygosity levels across fishes and reported that marine fishes that have a recorded observed heterozygosity value above 0.67 should be considered to have a high heterozygosity level. Although the study revealed a relatively low value for observed heterozygosity, its calculated value was congruent with the results of the study conducted by Glass et al. (2021), which measures the genetic divergence levels in *C. ignobilis* populations in the Indo-Pacific region using SNP as the molecular marker. The study also recorded low heterozygosity levels due to a possible population bottleneck experienced by the *C. ignobilis* population, especially in the Central Pacific region.

The general low observed heterozygosity recorded for *C. ignobilis* populations in this study can also be correlated with the impact of anthropogenic activities. *Caranx ignobilis* is regarded to possess high economic significance owing to its high impact on the global aquaculture sector (Alaira & Rebancos 2014) making them prone to overfishing. Improved general fishing management policies and targeted regulations for *C. ignobilis* must be drafted and implemented to help the wild population recover from the effects of overharvesting. Maintenance of high genetic variation is important to ensure the stability of a population since it helps them adapt to a changing environment.

Conclusion

This study is the first to design and report the microsatellite markers that can be used for *C. ignobilis* populations. The tested 12 microsatellite primers showed high polymorphic quality, as seen by their high values for expected heterozygosity and allele numbers. Overall, the results of the study may be used for formulating appropriate fishing strategies and policies in order to conserve the local stock of *C. ignobilis*.

Ethical Statement

No ethical statement to declare.

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Author Contribution

Shenna Kate M. Torres: Writing - Original draft, Laboratory Work, Data Curation, Visualization, Formal Analysis.

Verinna Charisse B. Mangonon: Laboratory Work, Writing- editing.

Maria Theresa T. Tengco: Laboratory Work, Writing- editing.

Brian S. Santos: Supervision, Conceptualization, Funding Acquisition, Writing - review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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