Turk. J. Fish.& Aquat. Sci. 23(8), TRJFAS20982
https://doi.org/10.4194/TRJFAS20982

Turkish Journal of FISHERIES and AQUATIC SCIENCES

SHORT PAPER

Twenty Polymorphic Microsatellite Markers for *Trachurus trecae* Cadenat, 1950 (Perciformes: Carangidae): An Important Fishing Genetic Resource Along the West African Atlantic Coast

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How to Cite

Mota, K.G., Vaini, J.O., Velho, F.V., Sardinha, M.L., Hilsdorf, A.W.S. (2023). Twenty Polymorphic Microsatellite Markers for *Trachurus trecae* Cadenat, 1950 (Perciformes: Carangidae): An Important Fishing Genetic Resource Along the West African Atlantic Coast. *Turkish Journal of Fisheries and Aquatic Sciences*, *23(8)*, *TRJFAS20982.* https://doi.org/10.4194/TRJFAS20982

Article History
Received 24 November 2021
Accepted 27 January 2023
First Online 02 February 2023

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Keywords
Short tandem repeat
Population genetics
Conservation
Kunene Horse mackerel
Marine resource

Abstract

This study provides the first set of 20 species-specific microsatellite loci for genetic population studies of *Trachurus trecae,* an important marine fishing resource for the African Atlantic coast. Twenty microsatellite loci were developed by next generation sequencing and tested by genotyping 48 animals collected along the Angolan Atlantic coast. The number of alleles per locus varied from 6 to 19, while the expected and observed heterozygosities varied from 0.73 to 0.94 and 0.16 to 0.86, respectively. These microsatellites are a valuable tool for further genetics monitoring of this species.

Introduction

Trachurus trecae, known as the Cunene horse mackerel, is an important marine genetic resource in the African Atlantic, mainly off the coast of Angola. A decrease in fished pelagic fish species led to an increase in *T. trecae* fishing during the 1980s, reaching a peak of more than 500,000 tonnes. However, the stock capture in the 1990s showed substantial reductions, ranging from 250,000 to 300,000 tonnes (Vaz Velho, 2010; Roux *et al*., 2013; FAO, 2020). Pelagic fishing is the third most profitable economic sector in Angola, accounting for 3–5% of GDP (Gross Domestic Product) and 80% of total catches. It is a primary source of animal protein (Vaz Velho, 2010; Roux *et al*., 2013). Thus, Cunene horse mackerel fisheries play an essential part in Angola´s fisheries and, therefore, the food protein supply.

Trachurus trecae is benthopelagic with a range extending to Namibia and Morocco (Bianchi *et al*., 1999) and an average body weight of 500 g at sexual maturity. Shoals of immature *T. trecae* can occur at depths of 20–300 m, whereas sexually mature fish usually dwell at depths of 100–300 m (Ndjaula *et al*., 2013; Carpenter & De Angelis, 2016). Adult individuals can reach up to 80 cm in length and weigh 2 kg (Carpenter, 1992; Ly *et al*., 1996). Globally, fishing resources are under constant pressure, with capture efforts for some species exceeding their recovery capacities. It is estimated that

overfishing efforts have caused natural stocks to decline, with annual losses of 50 billion dollars. Therefore, conservation and fishery resource management strategies must be undertaken (Anticamara *et al*., 2011; Melnychuk *et al*., 2017). The efficient planning and management of fishery resources require, among other things, knowledge of their inter- and intrapopulation genetic diversity (Zhou *et al*., 2010; Domingues *et al*., 2018). Microsatellite markers (Simple Sequence Repeats - SSR) are regarded as one of the most valuable tools to assess a species' genetic diversity (Liu *et al*., 2021) and are often used in marine fish (Cuéllar-Pinzón *et al*., 2016; Vaini *et al*., 2019).

The conservation of genetic fisheries resources is of paramount importance for their long-term sustainable use. The genetic diversity extant within and among fisheries stocks dictates the adaptability and resilience of a species to future environmental changes (FAO, 2020). Regarding this, multiyear climatic variation in the Benguela upwelling region, designated the "Benguela Niño," has been reported to strongly affect regional fisheries (Shannon *et al*., 1986; Shillington *et al*., 2006). The 1995 Benguela Niño event brought a high mortality rate to diverse pelagic marine species, including *T. trecae*.

These and others anthropic threats have jeopardized the genetic integrity of many fisheries species worldwide (Davies & Baum, 2012). In recent decades, genetics has been regarded as an essential biological component of the complex interaction between fish and their ecosystems. Therefore, diverse DNA-based molecular markers have been developed to unveil the underlying mechanisms of organisms' adaptation and evolution (Cuéllar-Pinzón *et al*., 2016). Domingues *et al*. (2018) have emphasized that fisheries management and conservation policies must consider genetic diversity information to manage and conserve fisheries stocks.

Microsatellite markers are a class of molecular markers extensively used in marine fisheries assessments (Abdul-Muneer, 2014). Over the years, SSR loci have been easily identified using large-scale DNA sequencing technology dubbed next-generation sequencing (NGS) (Andrés & Bogdanowicz, 2012; Kumar & Kocour, 2017). For the *Trachurus* genus, SSR panels have been developed and reported on for *T. trachurus* (12 loci; Kasapidis & Magoulas, 2008), *T. murphy (*13 loci; Cárdenas *et al*., 2009; Galleguillos *et al*., 2012), and *T. japonicus* (11 loci: Chang *et al*., 2009; 33 loci: Niu *et al.*, 2021).

The use of molecular markers to assess genetic resources by characterizations within and among fisheries stocks has focused on the species *T. murphyi* and *T. picturatus*, with the cross-species amplification of SSR loci (Cárdenas *et al*., 2009; Moreira *et al*., 2020) as well as species-specific SSR loci for *T. murphyi* (Galleguillos *et al*., 2012). Regarding *T. trecae*, a unique study was conducted on its genetic stock structuring using isoenzymes (Sardinha & Naevdal, 2012).

Thus far, however, there are no published species-specific SSR loci for *T. trecae*. Species-specific SSR loci have the advantage of avoiding a lack of polymerase chain reaction (PCR) amplification due to mutations in the amplicon flanking regions, which can occur even among phylogenetically close species (Primmer *et al*., 2005; Maduna *et al*., 2014). The use of non-specific primers can also cause changes in average allele lengths because the loci lengths may differ in related species, substantially affecting the quality of genotyping (Crawford *et al*., 1998; Barbará *et al*., 2007; Li & Kimmel, 2013). With this background in mind, we used the 454-pyrosequencing platform (ROCHE) to develop a SSR panel for *T. trecae*, from which a set of 20 species-specific polymorphic SSR markers are proposed for use in assessing its genetic diversity.

Materials and Methods

Development of Microsatellite Markers

Genomic DNA was extracted from the muscle tissue of 8 specimens of *T. trecae* using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). The 8 DNA samples were pooled together and sent to GenoScreen (Lille, France) to build the SSR library. The DNA was fragmented by sonication (~1500 bp; S220 Focused-Ultrasonicator; Covaris, Newtown, CT, USA) and used to build a shotgun library (kit GS-FLX Titanium; Roche Diagnostics Corporation, Branford, CT, USA), which was then submitted for NGS on the 454 GS-FLX Titanium pyrosequencer platform (Roche Diagnostics Corporation). The probes used to enrich the fragments were TG, CT, AAC, AAG, AGG, ACG, ACAT, and ACTC. The software QDD (Meglécz *et al*., 2010) was used to identify the SSR regions within the obtained sequences. The software identified 6591 sequences with microsatellite regions (Supplementary Material 1).

Microsatellite Loci Characterization

Seventy-eight SSR loci were initially selected for the polymorphism tests, and of these, 20 amplified adequately and demonstrated evidence of polymorphism (Table 1). Using the QDD software (Méglecz *et al*., 2010), primer sets were designed based on the SSR flanking sequences. Forty-eight *T. trecae* individuals originating from different locations along the Angolan coast (12 northern, 12 central, and 24 southern) were assessed to validate the specific SSR marker panel for the species. For each primer set, the forward primer was designed with the addition of the universal M13 sequence (5'- TGTAAAACGACGGCCAGT-3') as a 5' tail (Schuelke, 2000). Polymerase chain reactions were performed at a final volume of 20 µL (~100 ng of genomic DNA, 1× PCR buffer, 0.25 mM dNTPs, 1.5–3.0 mM $MgCl_2$, 0.5 U Taq DNA polymerase, 0.2 µM of fluorescently labeled (IRDye700) universal M13 primer, and 10 µM each of the forward and reverse

Table 1. Genetic characteristic of 20 microsatellite loci in *Trachurus trecae.*

Locus	GenBank acession	Primer Sequence (5´ - 3´)	Repeat motif	Ta (°C)	N	A	Size Range (bp)	Ho	He	PIC	HWE
TTR01	MW026705	F: GATGAATGGCTCCATCACCTA R: TTGTCTTGCATGGCTGTGAT	$(AC)_2$	58	34	15	135-167	0,21	0,89	0,868	0,0000*
TTR02	MW026706	F: CCGGCTTACTACTCCCACAT R: AGAGCGAGGAAATGGACAAA	$(CA)_2$	58	30	9	204-262	0,70	0,85	0,817	0,0231*
TTR03	MW026707	F: ATCAGTCATGGTGGCCTCTC R: AATTCAAAGCTTGGCTGCTC	$(GAG)_3$	58	36	9	139-160	0,69	0,85	0,814	0,0135*
TTR04	MW026708	F: TGTCCAAACCCAACTGCTCT R: ACGATGCAGGGAGTGTCTTC	$(GGA)_3$	58	34	9	135-159	0,74	0,79	0,746	0,27
TTR05	MW026709	F: GGCAGTTACGTACACCAGCA R: GGAGGGCAGTGTTGTAATTTG	$(AC)_2$	58	28	9	190-208	0,18	0,83	0,794	0,0000*
TTR06	MW026710	F: CAGCTGGCACTTCGACAATA R: AACGCAGTGTGGTCTGAAAA	$(TG)_2$	58	31	19	189-225	0,74	0,94	0,921	0,0003*
TTR15	MW026711	F: TTTATGTGGAGCTTCCCTGC R: ACTGGTGTCGGAAACAAACC	$(CA)_2$	58	31	8	122-136	0,16	0,79	0,750	0,0000*
TTR30	MW026712	F: ACAGCCACTGTGACACTGAA R: AATCCTCCACTCGTAGCAGC	$(AC)_2$	52	36	11	115-135	0,56	0,90	0,876	0,0000*
TTR35	MW026713	F: TCGGGGAAAATCAAACAGAG R: CTCGTCCCAGGATGCACT	$(GAG)_3$	58	28	7	141-165	0,36	0,81	0,764	0,0000*
TTR40	MW026714	F: GCCTGCTGTGTCATCAGTGT R: TGCAGTGAGACGGATGAATG	$(TTCA)_4$	56	33	11	102-154	0,70	0,88	0,848	0,0052*
TTR41	MW026715	F: CCCACCAGCAGAGGAAATAA R: TTAATTGTCTGTGCCCATGC	$(CAG)_3$	50	35	16	233-263	0,77	0,91	0,889	0,0094*
TTR43	MW026716	F: AGACGTCTTCTGAGCGAAGG R: CCCAACAGTGCTCATTCAGA	$(GGA)_3$	50	35	18	215-266	0,71	0,93	0,916	0,0000*
TTR44	MW026717	F: GCCTTGTTGCCGTAATCCTA R: GAATAGCAAATGATGCCACA	$(ATGG)_4$	50	36	7	262-290	0,53	0,73	0,687	0,0023*
TTR48	MW026718	F: GGGTCAATCCTGTTAGTGTGC R: AGTCACCGATGATCAAACCG	$(ATCC)_4$	56	36	9	112-136	0,69	0,83	0,795	0,0266*
TTR51	MW026719	F: TGTTGAGTGTTTTGTTGTGCTG R: TTTGTTGAATCACAAGAAATAGATG	$(TCAA)_4$	56	35	8	156-184	0,86	0,87	0,845	0,46
TTR53	MW026720	F: CCACCCACCCATCAATCTAT R: GCTTTGGAGGAGATAAAAGCA	$(CATC)_4$	50	34	9	148-168	0,32	0,87	0,841	0,0000*
TTR58	MW026721	F: TGTGATGAGCTGTTTCGAGG R: CCTGAACCTCCCACGTCC	$(AGTG)_4$	60	33	7	141-149	0,61	0,76	0,708	0,0257*
TTR64	MW026722	F: TCTGAACATTGCGTCGTAGC R: ATGAACTCGTTTCCAGACGG	$(GTTT)_4$	58	35	6	156-179	0,86	0,77	0,729	0,15
TTR69	MW026723	F: GATCCACCATCACCGTGTAA R: GCAGTTGGAAGTTGGGGTTA	$(TC)_2$	60	23	8	194-202	0,48	0,80	0,760	0,0002*
TTR70	MW026724	F: TGCTCCTGTCTTTCAGTCCC R: GTTTTTGTTTGCCTCCGTGT	$(CA)_2$	60	24	11	194-206	0,58	0,87	0,832	0,0001*

Ta: Annealing temperature; N: number of individuals; A: number of alleles; Bp: allele size in base pairs; Ho: observed heterozygosity; He: expected heterozygosity; PIC: polymorphic information content; HWE: Hardy-Weinberg Equilibrium *p*-values; *p<0.05 significant departure from Hardy-Weinberg Equilibrium; *Primer* 5' com cauda M13 (5'TGTAAAACGACGGCCAGT 3') (Schuelke, 2000).

primers) in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Foster City, California, USA).

The amplification protocol for all markers consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 40 s, 1 min at the annealing temperature (see Table 1), and 72°C for 40 s, ending with a final extension at 72°C for 10 min. The resulting amplicons were separated on a 6.5% polyacrylamide denaturing gel in an automatic DNA sequencer (DNA Analyzer 4300; LI-COR Biosciences, Lincoln, NE, USA). Alleles were identified using a 50–350 bp standard (LI-COR Biosciences), and genotypes were scored using SAGA 3.3 software (LI-COR Biosciences). In order to minimize any scoring errors associated with automated allele calling, microsatellite allele sizing for every individual was carried out by visual checking by two independent individuals.

Genetic Analysis

The number of alleles per locus, observed (Ho) and expected (He) heterozygosities, and deviations from Hardy–Weinberg equilibrium were determined with the randomized Markov-chain test (Guo & Thompson, 1992) using HW-Quickcheck software (Kalinowski, 2006). Linkage disequilibrium was assessed with GENEPOP software v.4.2 (Rousset, 2008), and Cervus software v.3.0.7 (Kalinowski *et al*., 2007) was used to evaluate the polymorphic information content (PIC).

Results and Discussion

A total of 6594 sequences were found to have SSR regions; of these, 940 presented simple and perfect repeats with a minimum of 5 repeat motifs, including 706 dinucleotides, 159 trinucleotides, 65 tetranucleotides, 9 pentanucleotides, and 1 hexanucleotide. Of these, 20 were tested and showed consistent evidence of polymorphism. It is worth mentioning that the complete panel of 6594 sequences is shown in the Supplement file table (S.F. Table 1) in the supplementary material, which can be explored for the further assessment of other microsatellites provided they are not considered neutral and located within genes and gene promoters (Chistiakov *et al*., 2006; De-Santis & Jerry, 2007).

Table 1 shows the primer sequences, GenBank accession number, repetition motif, annealing temperature, and size range of the alleles in base pairs (bp). Table 1 also summarizes the characteristics found at each locus, including the number of alleles, PIC, and diversity parameters such as the expected (He) and observed (Ho) heterozygosities and Hardy–Weinberg equilibrium. Most deviations from HWE were due to heterozygote deficiencies. This homozygosis excess in the tested individuals may not be because of null alleles generated by failing in single-locus amplification since they succeed at more than 90% of the samples. However, such events must be considered carefully in future population genetics assessments of the *T. trecae*.

Among the 20 polymorphic loci, the number of alleles per locus varied from 6 (Ttr64) to 19 (Ttr06), with a mean of 10.3 alleles per locus; the observed heterozygosity ranged from 0.16 (Ttr15) to 0.86 (Ttr51 and Ttr64), with a mean of 0.57; and the expected heterozygosity ranged from 0.73 (Ttr44) to 0.94 (Ttr06), with a mean of 0.84 (Table 1). Three loci were in Hardy-Weinberg equilibrium: Ttr04, Ttr51, and Ttr64. The others showed significant deviation from Hardy–Weinberg equilibrium ($p<0.05$). The PIC values ranged from 0.687 (Ttr44) to 0.921 (Ttr06), with an average of 0.810. When separated by repetition motif, the dinucleotide, trinucleotide, and tetranucleotide loci had average PIC values of 0.810, 0.832, and 0.806, respectively, indicating the trinucleotide loci had the highest levels of polymorphism.

In this study, twenty polymorphic microsatellite loci for *T. trecae* were described. All 20 loci were highly informative, as their PIC values were above 0.5 (Botstein *et al*., 1980). They were also highly polymorphic, having greater power for population differentiation tests and thus allowing genetic differences to be detected that are not apparent with the use of less informative loci (Ward, 2000). There was statistical evidence of linkage disequilibrium between the loci Ttr02, Ttr03, and Ttr43 and loci Ttr05 and Ttr35 ($p<0.05$). Linkage disequilibrium can be found when species suffer from inbreeding, genetic drift, or natural selection, and these effects are most observed in overexploited, fragmented, and threatened populations (Aguiar *et al*., 2019).

Overfishing impacts have been reported around the world over recent decades; however, except for concerns about selective changes in exploited fish stocks, the loss of genetic diversity in marine fish stocks seems not to be eligible as a parameter for fisheries management. According to the FAO (2011), molecular markers have become more relevant for characterizing the genetic resources of animal breeds since the 1990s. Although a new class of molecular markers (single nucleotide polymorphisms or SNPs) has arisen recently, SSRs have a well-known methodological framework and low cost when used to characterize marine genetic resources. Therefore, in this study, a panel of 20 polymorphic SSRs was developed specifically for *T. trecae* and is provided to assist in further population genetic assessments for the temporal monitoring, conservation, and sustainable use of this important fishery genetic resource along the Angola-Benguela Front.

Ethical Statement

All sample collections and animal handling protocols in the present study adhere to the guidelines for the use of animals in research of the National Council for Animal Experimentation Control to ensure compliance with international guidelines for animal welfare.

Funding Information

This study was supported by the FAPESP (São Paulo Research Foundation # 2018/24559-0) and by Benguela Current Commission (#LMR/PEL/09/09).

Author Contribution

Mota, KG was responsible for the project where the set of loci was developed and wrote most of the manuscript. Vaini, JO helped with experiments and writing. Vaz Velho, F and Sardinha, ML provided the samples and the logistic information for this study. Hilsdorf, AWS revised and edited the manuscript and guided throughout the development of the work.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

KGM was supported by a Master of Science grant from FAPESP (São Paulo Research Foundation # 2018/24559-0). INIP- Instituto Nacional de Investigação Pesqueira de Angola (*National Fisheries Research Institute*) for collecting the samples and provide the funds for the microsatellite development through Benguela Current Commission. AWSH is the recipient of a Research Fellowship from CNPq (304662/2017-8).

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