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ANIMAL SCIENCE

In silico development microsatellite marker panel and characterization for the endangered neotropical species *Brycon orbignyanus* (Characiformes, Bryconidae): a short communication

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Abstract: This short communication presents novel molecular tools designed for the endangered species *Brycon orbignyanus*. In conservation studies, the development of population-specific markers tailored to particular geographic regions is essential. In this study, the prevalence of imperfect microsatellites highlights a evolution of this type of microsatellite in a population of Pelotas, Rio Grande do Sul. Therefore, the methodology used can serve as a guideline for genetic variability analysis even before conducting *in vitro* or *in situ* tests. These findings contribute significantly to technological prospecting and hold promise for applications in conservation programs. Additionally, they provide a basis for *in vitro* assays, such as PCR, to support species monitoring efforts.

Key words: Molecular markers, Neotropical fish, Next generation sequencing, Non-model species.

INTRODUCTION

Brycon orbignyanus (Ethnospecies: *Piracanjuba*) has historically been the focus of intense sport and industrial fishing activities. As a rheophilic species, it undertakes extensive migrations for reproduction. Furthermore, it exhibits seasonal spawning, external fertilization, and lacks parental care behavior. These biological characteristics render it particularly vulnerable to environmental changes, particularly those induced by human activities such as overfishing, habitat fragmentation, and the degradation of riparian forests (Macedo et al. 2024, Marques et al. 2021, Santos et al. 2022).

In recent decades, natural populations of *B. orbignyanus* have undergone a significant reduction in their distribution range. Presently, the species is listed in the "Red Book of Brazilian Fauna Threatened with Extinction," categorized as "Endangered" due to a population decline of greater than or equal to 50% (MMA 2018).

The development of genomic tools for *B. orbignyanus* is paramount for implementing advanced techniques in both *in situ* and *ex situ* genetic management of the species. Despite the availability of molecular markers previously developed for the species (Arias et al. 2016, Yazbeck et al. 2018) and focused on populations

from Minas Gerais, Brazil, the present study reports the first microsatellite markers developed for specimens from populations occurring in the state of Rio Grande do Sul, Brazil. These tools are crucial not only for maintaining natural stocks in the region but also for facilitating their sustainable commercial exploitation (Carvalho et al. 2019, Fazzi-Gomes et al. 2021). Thus, the objective of this study was to develop and characterize SSR-type markers for *B. orbignyanus*, employing Next Generation Sequencing technology.

MATERIALS AND METHODS

Molecular DNA processing data

The DNA was extracted from caudal fin samples of *Brycon orbignyanus* collected in the Uruguay River basin and maintained in captivity at the Panama Piscicultura Aquaculture Station, Santa Catarina, Brazil, using the commercial Wizard® Genomic DNA Purification Kit (Promega), followed by next-generation sequencing using the Illumina platform, specifically employing the Illumina Universal Adapter, Illumina Small RNA 3' Adapter, Illumina Small RNA 5' Adapter, and Nextera adapters on the MiSeq™ system. Quality control of the sequencing data was performed using Trimmomatic (Bolger et al. 2014) and FastQC (Andrews 2010). The *de novo* assembly was made using the SPAdes program (Bankevich et al. 2012) based on a k-mer length of 23.

SSRs identification and primer design

To identify microsatellites, both the MISA program (Beier et al. 2017) and the EasySSR program (Alves et al. 2023) were utilized, each employing default parameters to assess their effectiveness in characterizing microsatellite markers within the genome of *B. orbignyanus*. Subsequently, the BatchPrimer3 program (You et al. 2008) was employed for the *in silico* prediction and design of microsatellite marker primers specific to *B. orbignyanus*. Customized parameters included a primer length range of 15-30 bp, a GC content of 40-50%, a primer melting temperature (T_m) of 55 °C, and a maximum T_m difference of 3 °C.

RESULTS

Illumina sequencing and *de novo* assembly

A total of 794,299 reads were obtained, with lengths ranging from 36 to 151 bp. These reads met the filtering criteria, exhibiting Q scores ≥ 20, an average quality per read of 38, and a GC content of 42%.

The SPAdes program produced 594 contigs longer than 1,000 bp, including 27 contigs exceeding 5,000 bp in length. Additionally, SPAdes achieved an N50 value of 714. In total, the assembly generated 227,565 contigs with a cumulative length of 73,215,541 bp.

SSRs loci *in silico* mapping

The MISA program identified a total of 123,228 sequences containing microsatellites within the *B. orbignyanus* genome, yielding a collective count of 211,025 SSRs (Table I). These SSRs were categorized majority into the perfect class. Pentanucleotide repeats emerged as the most prevalent, accounting for 51.58% of the total SSRs. In contrast, the EasySSR program identified a total of 133,989 microsatellite markers, corresponding to an SSR density of 18% per bp across the genome. The majority of these markers were imperfect comprising 92.66% of the total.

Further examination of perfect microsatellite markers revealed tetranucleotide repeats 44.63% as the most prevalent. Conversely, imperfect microsatellite markers were predominantly composed of dinucleotide 32.58%.

Interestingly, the distribution of SSR motifs varied between the two marker search

Table I. General statistics of microsatellites identified in *Brycon orbignyanus* samples.

Parameters	MISA	EasySSR
Perfect	93,35%	7,34%
Imperfect	4,89%	92,66%
Compound	1,76%	0
Mononucleotide	4.62%	4.19%
Dinonucleotide	13.96%	30.71%
Trinonucleotide	2.62%	24.54%
Tetranonucleotide	5.23%	28.62%
Pentanonucleotide	51.58%	6.73%
Hexanonucleotide	21.99%	5.21%

programs. While the MISA program (Figure 1) primarily identified pentanucleotide repeats, the EasySSR program (Figure 2) predominantly highlighted dinucleotide motifs. Specifically, the AC motif was the most frequently observed motif according to MISA, occurring 17,910 times. Conversely, the TG motif was the most common according to the EasySSR program, with 7,656 occurrences.

SSR primers

Out of the 501 input sequences, successful primer pairs were obtained for 254 sequences, resulting in a total of 511 primer pairs selected. These primer pairs were distributed across various classes of repeat motifs, including di-, tri-, tetra-, penta-, and hexanucleotides, with 178, 125, 189, 40, and 30 primers developed for each respective class. Notably, primer development was most successful for tetranucleotide markers, resulting in a greater number of markers from this class being generated. The complete set of generated primers is available for reference in the supplementary material (DOI: 10.17632/2sz349gtvm.1).

DISCUSSION

Our findings present a novel panel of microsatellite markers for *Brycon orbignyanus*, characterized through a rapid and cost-effective methodology, encompassing perfect, imperfect, and compound microsatellites, a molecular tool wich can serve in studies investigating genetic variability and evaluating population structures (Herkenhoff et al. 2023). While Yazbeck et al. (2018) previously developed a panel of microsatellite markers for this species, their focus was solely on perfect microsatellites. This approach is essential for achieving higher resolution in species traceability at the population level, as accounting for population-specific characteristics is critical for informing conservation and fisheries management strategies (Herkenhoff et al. 2023).

The distribution of microsatellite markers identified by the MISA output, particularly the prevalence of pentanucleotide motifs, deviates from the typical pattern observed in fish species. In species such as *Danio rerio*, *Takifugu rubripes*, *Ictalurus punctatus*, *Esox lucius*, *Oncorhynchus mykiss*, and *Astyanax mexicanus* of the Characiformes Order, the distribution pattern commonly follows di- > mono- > tetra- > tri- >

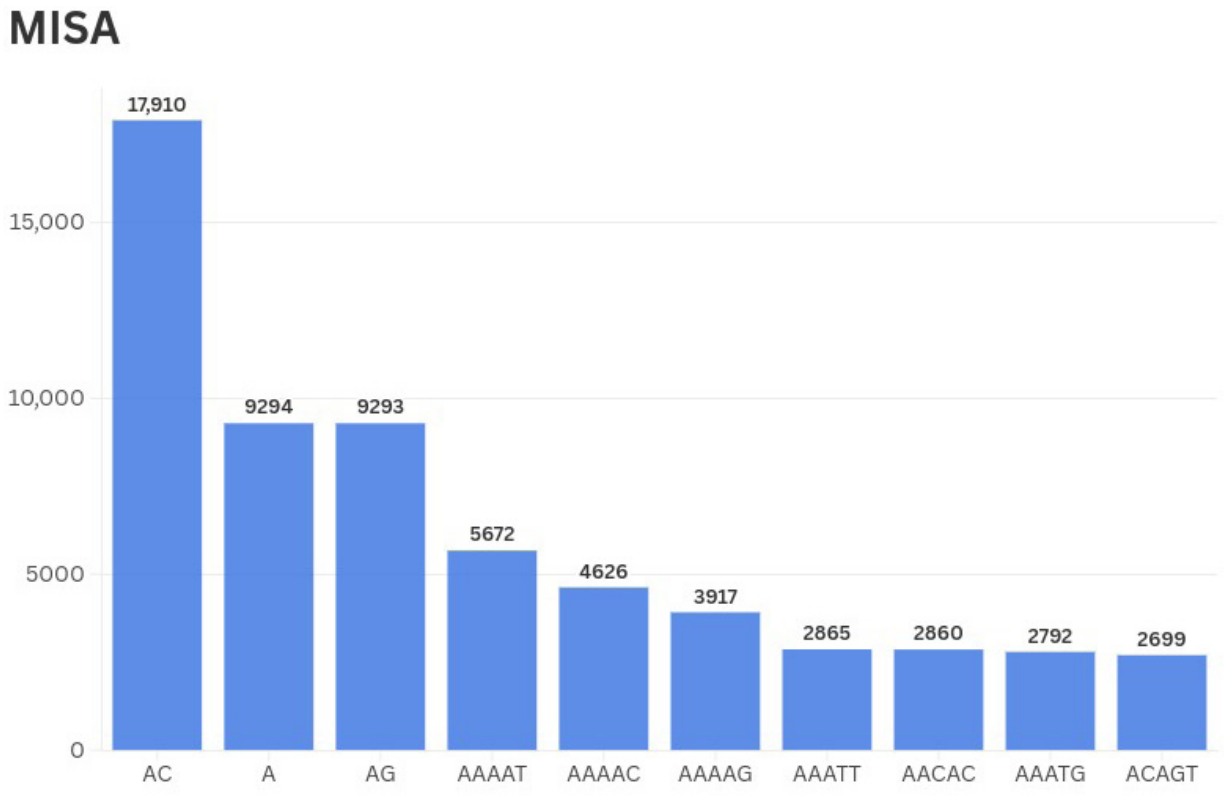

Figure 1. Distribution of the 10 most abundant microsatellite motifs identified using the MISA program.

penta- > hexanucleotides (Lei et al. 2021). This discrepancy is further highlighted by the higher frequency of pentanucleotide markers observed in the *B. orbignyanus* genome, contrasting with the findings of Yazbeck et al. (2018). In their study, microsatellites of the dinucleotide class accounted for 74.22% of the total, nearly three times more abundant than all other motif classes combined. However, it is important to note that Yazbeck et al. (2018) solely screened for perfect microsatellite motifs, using the SSRIT program (Temnykh et al. 2001), thus overlooking the imperfect class of microsatellites. As a result, the proportion of SSR motif classes reported by the authors failed to account for imperfect microsatellites.

The MISA program was designed for the identification of perfect simple microsatellites and compound non-redundant motifs within DNA sequences, albeit at the cost of sensitivity to degenerate (imperfect) microsatellites (Beier et al. 2017). In contrast, the EasySSR program utilizes IMEX command lines (Mudunuri & Nagarajaram 2007), offering greater sensitivity towards imperfect microsatellites. Additionally, it allows for flexible parameterization of imperfection thresholds, enabling its use for the identification of perfect microsatellites as well. Hence, the prevalence of imperfect markers observed in the *B. orbignyanus* genome, as indicated by the EasySSR program, is plausible, as its results align more closely with the expected microsatellite distribution for this species.

The characteristics of imperfect microsatellite markers are linked to significant implications for species conservation (Bhargava & Fuentes 2010). In evolutionary contexts or within populations under impact, imperfect motifs exhibit a lower mutation rate and, consequently, are associated with a reduction in genetic variability (Zhu et

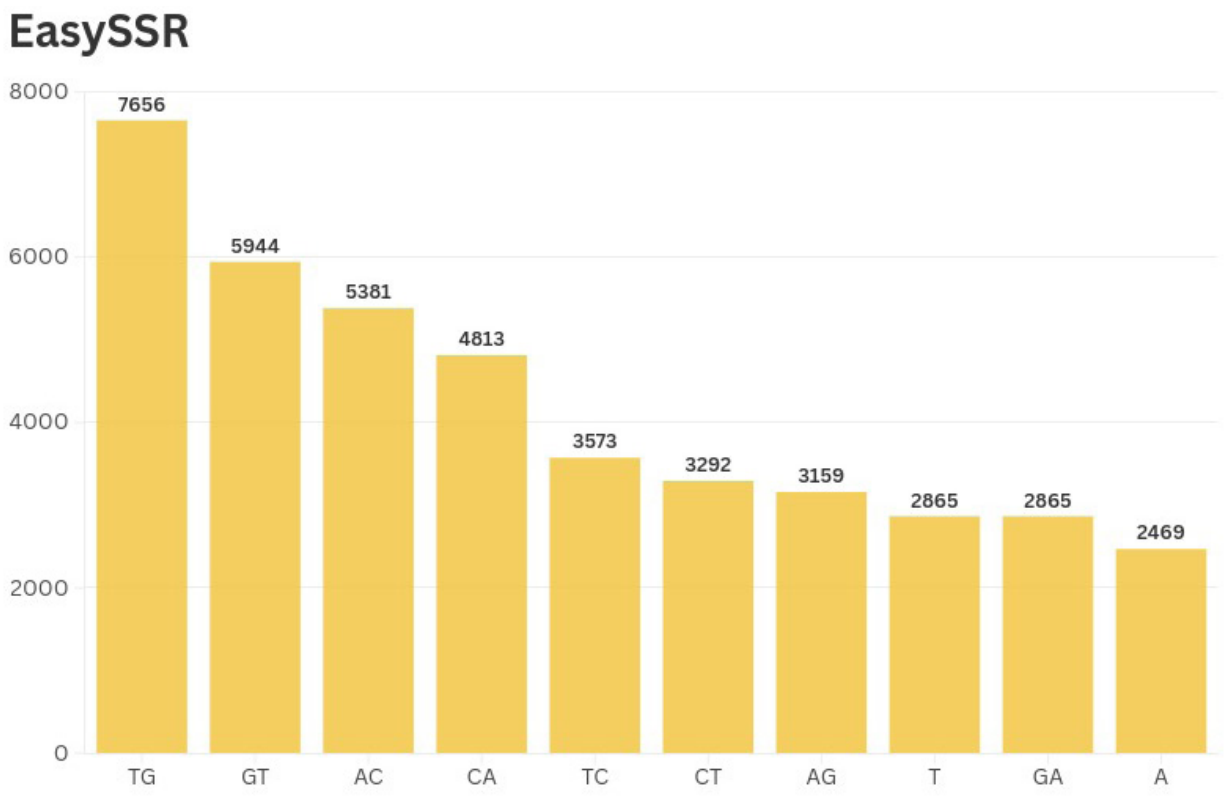

Figure 2. Distribution of the 10 most abundant microsatellite motifs identified using the EasySSR program.

al. 2000). Moreover, imperfect sequences have a tendency to persist and accumulate within a population, leading to fewer instances of polymerase slippage during replication and even the occurrence of perfect repeats. This phenomenon is referred to as "microsatellite death" (Kelkar et al. 2011). In this context, the characterization of microsatellite markers in the *Brycon orbignyanus* genome underscores a evolution of this type of microsatellite. This aligns with the significant decline in stocks of the species in the southern region of Brazil. The 189 tetranucleotide markers generated can serve as efficient tools for high-resolution analyses of the structure of wild populations or allele tracking of the species. Tetranucleotide markers are preferred in polymorphism analyses due to their high mutation rate and ability to minimize genotyping errors (Kelkar et al. 2008).

CONCLUSIONS

In conclusion, this study presents viable alternatives to facilitate the development of molecular markers for *Brycon orbignyanus*. By rapidly obtaining and characterizing microsatellite markers specific to this species, as well as developing a comprehensive panel of primers, our findings offer valuable insights. In this study, the prevalence of imperfect microsatellites highlights a evolution of this type of microsatellite in a population. Therefore, the methodology used can serve as a guideline for genetic variability analysis even before conducting *in vitro* or *in situ* tests. This work holds promise for enhancing the exploration of genetic resources and their applications in conservation programs targeting the endangered species *Brycon orbignyanus* and potentially other species within the Bryconidae family as well.

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Data availability

All data used in this study are available within the manuscript, either in tables or in the main text, and may be requested from the corresponding author via email.