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# Development and characterization of microsatellite *loci* of *Microglanis cottoides* (Siluriformes: Pseudopimelodidae) and cross-species amplification

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Thirteen microsatellite *loci* were isolated and characterized in *Microglanis cottoides*. Of these, two were monomorphic and 11 were polymorphic. These polymorphic *loci* tested on 24 individuals from a wild population produced a total of 108 different alleles, with levels of variability high, ranging from 2 to 20, with an average of 8.3 alleles per *locus*. The observed and expected heterozygosity ranged from 0.125 to 0.958 and from 0.119 to 0.931, respectively. A high combined probability of paternity exclusion value and a low probability combined genetic identity value obtained show that the set of *loci* described herein displays good suitability for paternity studies and differentiation of *M. cottoides*. Additionally, all thirteen microsatellite primers developed for *M. cottoides* were tested in four other Pseudopimelodidae species and successful cross-species amplification was achieved for the majority of *loci*.

Treze *loci* microssatélites foram isolados e caracterizados em *Microglanis cottoides*. Destes, dois foram monomórficos e 11 foram polimórficos. Estes *loci* polimórficos foram testados em 24 indivíduos de uma população selvagem e produziram um total de 108 alelos diferentes, com níveis de variabilidade alta, variando de 2 a 20, com uma média de 8,3 alelos por *locus*. A heterozigosidade observada e esperada variou de 0,125 a 0,958 e 0,119 a 0,931, respectivamente. Um elevado valor de exclusão de paternidade e um baixo valor de identidade genética foram obtidos, demostrando que o conjunto de *loci* descritos no presente trabalho exibe boa aplicabilidade no estudo de parentesco e diferenciação populacional em *M. cottoides*. Adicionalmente, os treze primers de microssatélites desenvolvidos para *M. cottoides* foram testados em outras quatro espécies de Pseudopimelodidae e a transferabilidade foi obtida para a maioria dos *loci*.

**Key words:** Brazilian Eastern basin, Catfish, Enriched genomic library, Genetic marker, SSR.

## Introduction

*Microglanis* Eigenmann 1912, also known as bumblebee catfishes (Shibatta, 2003a), is a genus of small South American catfishes that do not exceed over 110 mm in standard length (Shibatta, 2003a; Shibatta & Benine, 2005). Initially regarded as a member of the pimelodids (Eigenmann, 1912), this genus was subsequently included in the small Pseudopimelodidae family (Shibatta, 2003b), which is considered a monophyletic group (Lundberg *et al*., 1991).

With 21 valid species described to date (Ruiz & Shibatta, 2011), the genus *Microglanis* is also characterized by its wide mouth (gape width same as head width), short maxillary barbel (occasionally reaching pectoral-fin origin) and small eye without free orbital margin, among other particular characters (Shibatta, 2003a, 2003b). These small catfishes are widely distributed across South America, occurring from Guyana to the Rio de La Plata basin in Argentina (Shibatta & Benine, 2005; Sarmento-Soares *et al*., 2006).

Although our knowledge of the number of *Microglanis* species has increased in the last decade (Shibatta, 2003a, 2003b; Bertaco & Cardoso, 2005; Shibatta & Benine, 2005; Sarmento-Soares *et al*., 2006; Alcaraz *et al*., 2008; Ottoni *et al*., 2010; Shibatta & Ruiz, 2010; Shibatta & Ruiz, 2011), the

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biology of *Microglanis* still remains poorly known (Shibatta, 2003a). Recently, it has been shown that some species can occur in small populations patchily distributed along the river basins where they are found (Esguícero & Arcifa, 2010). According to these authors, such conditions which may be associated with the progressive environmental degradation of freshwater habitats could represent threats to populations of some *Microglanis* species. Despite this fact, up to this moment population genetic studies involving *Microglanis* species are still lacking.

Among the known species, *Microglanis cottoides* (Boulenger 1891) is especially interesting for phylogeographic and genetic population studies, owing to its geographical distribution in the Brazilian Atlantic Forest, being present in the Uruguay River basin and along the eastern coast of Brazil, with records of their occurrence in drainages across the southern and southeastern regions (Malabarba & Mahler, 1998; Shibatta, 2003b, 2007; Mori & Shibatta, 2006). These regions can be considered of great biogeographical significance, not only for the high degree of endemism of its fish fauna (Bizerril, 1994, 1995), but also for being a very populated area with high environmental degradation due to human activities.

Studies on geographic distribution of genealogical lineages have been widely used to describe historical events, such as habitat fragmentation or expansion of the range of species and populations. These studies also serve to understand migration events, vicariance and extinction of gene lineages, and other processes that affect the population structure or generate speciation in a spatial and temporal context (Avise, 2001, 2009; Hardy *et al*., 2002).

A good way to study the genetic structure of natural fish populations is through the use of molecular markers such as microsatellite (SSR-Simple Sequence Repeats) (O'Connell & Wright, 1997). In fact, SSR are considered a class of molecular markers highly polymorphic and useful for population studies (Zane *et al*., 2002). Therefore, the current work describes 13 SSR markers developed for *Microglanis cottoides* and tests their applicability for four other Pseudopimelodidae species.

## Material and Methods

Microsatellites were isolated and characterized following the protocol of Billotte *et al*. (1999), with some minor modifications. Total genomic DNA was extracted from muscle tissue preserved in 95% ethanol. The DNA was isolated according to Almeida *et al.* (2001). Genomic DNA (5 µg) was digested with *Rsa*I and the blunt-ended fragments were ligated to the adaptors Rsa 21 and Rsa 25 (Edwards *et al.*, 1996). Fragments were selected with $(AGA)_5$, $(CT)_8$ and $(GT)_8$ probes, and then cloned into the pGem®-T (Promega) vector. Such recombinant plasmids were used to transform the *E. coli* DH5- α lineage. The recombinant clones were selected and sequenced using the BigDye Terminator™ kit (v. 3.1 - Applied Biosystems). Sequencing was performed on an ABI 3500 XL automated sequencer. Sequences were analyzed using BioEdit v.7.0 software (Hall, 1999) and primers were designed using Primer 3 software (Rozen & Skaletsky, 2000). The selected forward primers were labelled with the M13 sequence (5'-TGTAAAACGACGGCCAGT-3') at the 5' end (Schuelke, 2000). The AutoDimer software (Vallone & Butler, 2004) was used to test the potential presence of hairpin structures and primer-dimer problems. The individual genotyping was performed on an ABI 3500 XL automated sequencer.

PCR amplifications were carried out on 24 individuals of *M. cottoides* (MZUEL 7453), collected in the arroio Divisa (30°54'5.6"S 52°05'18.0"W), municipality of Cristal, Rio Grande do Sul State, southern Brazil. Cross-species amplification tests were performed using four other pseudopimelodid species, as follows: *Microglanis cibelae* Malabarba & Mahler, 1998 (MZUEL 7452), from the rio Maquiné (Rio Grande do Sul State, 29°37'2.1"S 50°15'51.8"W); *M. garavelloi* Shibatta & Benine, 2005 (MZUEL 7878), from the Couro de Boi Stream (Paraná State, 23º05'41.4"S 50º59'49.5"W); *M. parahybae* Steindachner, 1880 (LBP 10741), from the rio Paraíba do Sul (Rio de Janeiro State, 22º04'07.8"S 41º54'36.2"W) and *Pseudopimelodus pulcher* Boulenger, 1887 (MZUEL 6039), from rio Laranjinha (Paraná State, 23°24'52.98"S 50°27'8.55"W), using five individuals of each species.

Reactions were performed in 5 µL reaction volume containing 1× GoTaq Master Mix (Promega), 10 ng of template DNA, 1% glycerol, 0.125 µM forward labeled primer (FAM, HEX or NED, Applied Biosystems, CA), 0.125 µM reverse primer, 0.0125 µM forward primer. Amplifications were performed with an initial denaturation step at 94°C for 4 min, followed by 35 cycles at 94°C for 40 s, 48°C, 54°C or 60°C (according to Table 1) for 1 min, 72°C for 1 min, and a final extension at 72°C for 30 min. The PCR products were submitted to electrophoresis on an automated sequencer (Applied Biosystems 3500 Genetic Analyzer) and the GeneScan 600 Liz (Applied Biosystems) was used as the molecular weight standard.

The individuals were genotyped with the GeneMarker 1.85 software (SoftGenetics, State College, PA) followed by manual checking. Tests for Hardy-Weinberg Equilibrium (HWE) and the presence of linkage disequilibrium among the pairs of *loci* were calculated using GENEPOP 4.0.10 (Raymond & Rousset, 1995; Rousset, 2008); *P* values were subsequently adjusted applying the sequential Bonferroni correction (Rice, 1989). The software GenAlEx v.6.41 (Peakall & Smouse, 2006) was used to estimate the observed ($H_o$) and expected ($H_e$) heterozygosities and the average number of alleles per *locus*. The paternity exclusion probability (*Q*) (Weir, 1996), and the genetic identity probabilities (*I*) (Paetkau *et al*., 1995) were estimated using Identity 1.0 (Wagner & Sefc, 1999). Estimates of the polymorphic information content (PIC) and potential null alleles were obtained through Cervus v.3.0 (Marshall *et al*., 1998) and Micro-Checker v.2.2.3 (Van Oosterhout *et al*., 2004) software, respectively. Default settings were used for all tests.

## Results

Of 96 clones sequenced, 25 contained microsatellite repeats but only 18 were suitable for primer design and PCR reactions. After testing the different amplification conditions, 13 *loci* were successfully amplified. From these, two were monomorphic and 11 were polymorphic (Table 1).

The eleven polymorphic *loci* produced a total of 108 different alleles, with high levels of variability, ranging from 2 to 20, with an average of 8.3 alleles per *locus*. The observed and expected heterozygosity ranged from 0.125 to 0.958 and from 0.119 to 0.931, respectively. After Bonferroni correction for multiple comparisons, no evidence of linkage disequilibrium between any pair of *loci* examined was observed. Only the *locus* Mcot09 showed significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$). This *locus* showed the presence of null alleles, inferred from excess homozygous genotypes which could explain the observed deviation from HWE. Additionally, the *locus* Mcot08 revealed an excess of homozygotes and the possible presence of null alleles, but did not show any significant deviation from the HWE. It was also observed that the *loci* Mcot08 and Mcot12 were the only ones showing significant values of endogamic coefficient ($F_{IS}$) (Table 1).

## Discussion

A high combined probability of paternity exclusion value (0.999) and a low combined probability of genetic identity value ($2.16 \times 10^{-6}$) were obtained, showing that the set of *loci* described herein, exhibits good applicability for studies of parentage and population differentiation of *M. cottoides*.

The average PIC was 0.528, ranging from 0.115 to 0.927. According to Botstein *et al.* (1980), PIC values above 0.5 indicate highly informative markers; from 0.25 to 0.50, moderately informative; and below 0.25, slightly informative. Of the 13 *loci* obtained, eight proved to be highly informative (Mcot02, Mcot03, Mcot04, Mcot07, Mcot08, Mcot09, Mcot10 and Mcott12) with PIC values equal to or higher than 0.589; two were moderately informative (Mcot05 and Mcot11) presenting PIC values equal to or higher than 0.337. Only one *locus* (Mcot13) was slightly informative, exhibiting a PIC value equal to 0.115.

All 13 microsatellite primers developed for *M. cottoides* were successfully cross-amplified in two from four Pseudopimelodidae species (Table 2). Only two *loci* (Mcot04 and Mcot09) failed to amplify in *P. pulcher* and *M. garavelloi*, respectively. From the set of *loci* tested, *Microglanis garavelloi* and *M. parahybae* exhibited 10 polymorphic *loci*, while *M. cibelae* and *P. pulcher* showed, respectively, 11 and 12 polymorphic *loci* each.

**Table 1.** Description of 13 microsatellite *loci* (11 polymorphic and two monomorphic) isolated from the Neotropical fish *Microglanis cottoides*. Flanking primers, $T_a$ = optimal annealing temperatures, $k$ = number of alleles, allele size range (bp), $H_o$ = observed heterozigosity, $H_e$ = expected heterozygosity estimated from 30 individuals, $Q$ = paternity exclusion probability, $I$ = probability of genetic identity, $F_{IS}$ = endogamy coefficient, PIC = polymorphic information content, GenBank accession numbers. * Significant deviations from Hardy-Weinberg equilibrium ($P < 0.05$). ** Significant value for the endogamy coefficient ($F_{IS}$).

| *Locus* | Sequence | Primers (5'- 3') | $T_a$ (°C) | $k$ | Allele size range (pb) | *Ho* | *He* | (*Q*) | (*I*) | PIC | $F_{IS}$ | GenBank |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mcot01 | $(GT)_7$ | F: TCATGATGGCGACTGAACTC<br>R: TTATTGCGTGTGCTTTCTGG | 48 | 1 | 244 | 0.000 | 0.000 | 0.000 | 1.000 | 0.000 | - | KF134914 |
| Mcot02 | $(AC)_9$ | F: AACTGCCTGCAAAACAACC<br>R: TTACCCTGACAACCAGCTCAC | 54 | 8 | 110 - 126 | 0.792 | 0.786 | 0.593 | 0.074 | 0.758 | 0.014 | KF134915 |
| Mcot03 | $(TG)_{16}$ | F: AGCCCAATCTGTCTGAGAGC<br>R: ATCATCACCACGTGGAACAC | 54 | 17 | 183 - 221 | 0.917 | 0.915 | 0.828 | 0.013 | 0.909 | 0.019 | KF134916 |
| Mcot04 | $(TG)_{18}$ | F: AGCGGCTGTAATAGGCTGTG<br>R: CGGCTGTTCTTCCTTTACAG | 48 | 20 | 159 - 211 | 0.917 | 0.931 | 0.861 | 0.008 | 0.927 | 0.037 | KF134917 |
| Mcot05 | $(GTAAAA)_3$ | F: CGTTGAAGAGTAACACAGACGTG<br>R: AAGCGGAGGTTCAATTTCG | 54 | 2 | 126 - 132 | 0.458 | 0.430 | 0.168 | 0.417 | 0.337 | -0.045 | KF134918 |
| Mcot06 | $(GT)_7$ | F: TCATGATGGCGACTGAACTC<br>R: TTATTGCGTGTGCTTTCTGG | 48 | 1 | 245 | 0.000 | 0.000 | 0.000 | 1.000 | 0.000 | - | KF134919 |
| Mcot07 | $(GT)_{10}$ | F: TTCACTCAAGAGGCAGCTGTAG<br>R: ACCGTGTTCGTCGCTAATG | 54 | 15 | 127 - 183 | 0.917 | 0.875 | 0.759 | 0.025 | 0.865 | -0.026 | KF134920 |
| Mcot08 | $(AC)_{19}$ | F: CGCAGCACACCAGACTTATG<br>R: TCATGGGTAACGACACCAAG | 54 | 17 | 156 - 204 | *0.792 | 0.908 | 0.815 | 0.015 | 0.901 | **0.148 | KF134921 |
| Mcot09 | $(TGAA)_4$ | F: CAGGTGGGTTCCGGATTAAC<br>R: CAGTAGAGACTCTCATAACAATGCAC | 54 | 7 | 97 - 125 | 0.958 | 0.681 | 0.428 | 0.156 | 0.627 | -0.388 | KF134922 |
| Mcot10 | $(ATGA)_6$ | F: GTGGGATCCGGATTAACCTC<br>R: AAATAACAGGAAAGTTTTTATCCACTC | 48 | 7 | 118 - 138 | 0.667 | 0.666 | 0.409 | 0.170 | 0.607 | 0.019 | KF134923 |
| Mcot11 | $(GT)_5$ | F: TCATTTGGAACCAGGACTCTG<br>R: GGAATCGAACCCATGAACTC | 54 | 4 | 165 - 188 | 0.417 | 0.386 | 0.215 | 0.400 | 0.363 | -0.057 | KF134924 |
| Mcot12 | $(ATGA)_6$ | F: GTGGGATCCGGATTAACCTC<br>R: CCGTCAGTATGTCAGTCAGTCAG | 60 | 5 | 166 - 182 | 0.375 | 0.641 | 0.393 | 0.180 | 0.589 | **0.432 | KF134925 |
| Mcot13 | $(TGT)_8$ | F: GAAGGTGTGGATTGAGAGCAG<br>R: CAGATTACCCATCCGTCCAT | 60 | 3 | 125- 131 | 0.125 | 0.119 | 0.0598 | 0.780 | 0.115 | -0.029 | KF134926 |
| All *loci* | | | | 108 | 8.3 | 0.580 | 0.579 | 0.999 | $2.16 \times 10^{-6}$ | 0.538 | 0.021 | |

**Table 2.** Cross-amplification of 13 *loci* in four species of Pseudopimelodidae. Amplifications and polymorphisms were tested in five individuals of each species. - indicates no amplification. *k* = the number of alleles per *locus*.

| | Primers | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mcot01 | Mcot02 | Mcot03 | Mcot04 | Mcot05 | Mcot06 | Mcot07 | Mcot08 | Mcot09 | Mcot10 | Mcot11 | Mcot12 | Mcot13 |
| Species | Allele size range (bp) and the number of alleles per *locus* observed (*k*) | | | | | | | | | | | | |
| *Microglanis cibelae* | 244-246 | 114-122 | 104-218 | 180-196 | 114-132 | 148-168 | 244-246 | 168-214 | 113-125 | 122-132 | 165 | 166-174 | 128 |
| | *k*= 2 | *k*= 4 | *k*= 2 | *k*= 5 | *k*= 3 | *k*= 5 | *k*= 2 | *k*= 5 | *k*= 3 | *k*= 4 | *k*= 1 | *k*= 3 | *k*= 1 |
| *Microglanis garavelloi* | 232 | 114-124 | 195-197 | 181-191 | 112-132 | 155-161 | 232-256 | 152-172 | - | 116-130 | 184 | 168-172 | 126-129 |
| | *k*= 1 | *k*= 3 | *k*= 1 | *k*= 3 | *k*= 3 | *k*= 3 | *k*= 2 | *k*= 3 | - | *k*= 3 | *k*= 1 | *k*= 3 | *k*= 2 |
| *Microglanis parahybae* | 251 | 116-126 | 202-208 | 207-221 | 114-132 | 145-155 | 244-252 | 162-176 | 114-124 | 122-126 | 184 | 170-178 | 122 |
| | *k*= 1 | *k*= 5 | *k*= 3 | *k*= 4 | *k*= 2 | *k*= 4 | *k*= 1 | *k*= 5 | *k*= 4 | *k*= 3 | *k*= 1 | *k*= 3 | *k*= 1 |
| *Pseudopimelodus pulcher* | 238-258 | 100-134 | 182-214 | - | 110-156 | 145-153 | 245-247 | 158-170 | 115-197 | 122-126 | 186-192 | 170-174 | 101-128 |
| | *k*= 4 | *k*= 5 | *k*= 6 | - | *k*= 2 | *k*= 4 | *k*= 2 | *k*= 5 | *k*= 5 | *k*= 2 | *k*= 2 | *k*= 2 | *k*= 5 |

The successful cross-species amplification of microsatellite *loci* described herein can be attributed to the high conservatism of the flanking microsatellite regions, which is expected among close related species, as reported elsewhere (Barbará *et al*., 2007). Thus, the set of primers presented in the current study appear as promising tools for future population genetic studies involving these five species of Neotropical fish.

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