Eletric eels galore: microsatellite markers for population studies

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Fourteen novel microsatellite loci are described and characterized in two species of electric eels, *Electrophorus variii* and *E. voltai* from floodplains and rivers of the Amazon rainforest. These loci are polymorphic, highly informative, and have the capacity to detect reliable levels of genetic diversity. Likewise, the high combined probability of paternity exclusion value and low combined probability of genetic identity value obtained demonstrate that the new set of loci displays suitability for paternity studies on electric eels. In addition, the cross-amplification of electric eel species implies that it may also be useful in the study of the closely related *E. electricus*, and to other Neotropical electric fishes (Gymnotiformes) species as tested herein.

Keywords: Amazon Rainforest, *Electrophorus*, Genetic diversity, Gymnotiformes, SSR.
Catorze novos loci microsatélites são descritos e caracterizados em duas espécies de poraquês, *Electrophorus variii* e *E. voltai* de planícies alagadas e rios da floresta amazônica. Esses loci são polimórficos, altamente informativos e têm a capacidade de detectar níveis confiáveis de diversidade genética. Da mesma forma, o alto valor de exclusão de paternidade combinado com a baixa probabilidade de identidade genética demonstra que o novo conjunto de loci exibe adequação para estudos de paternidade em poraquês. Além disso, a amplificação cruzada de espécies de peixes elétricos implica que também pode ser útil no estudo da espécie intimamente relacionada *E. electricus*, e de outras espécies de peixes elétricos neotropicais (Gymnotiformes).

Palavras-chave: Diversidade genética, *Electrophorus*, Floresta amazônica, Gymnotiformes, SSR.

INTRODUCTION

Electric eels (*Electrophorus* Gill, 1864) share with other species of Neotropical electric fishes (Gymnotiformes) a specialized electrogenic-electrosensory system used to navigate, and communication (Crampton, 2019). In addition to low-voltage electric organ discharges (EODs), electric eels generate high-voltage EODs for stunning prey and defense, as reported in the field by Humboldt in the 18th Century, and elegantly demonstrated in the laboratory by Catania (2019). For centuries, electric eels captivate minds, inspire scientific innovation, like the electric battery, which has been used as a model for understanding bioelectrogenesis (Finger, Piccolino, 2011; Gallant et al., 2014). Despite the broad public and scientific community interest, only recently species diversity on *Electrophorus* began to be explored in extent (de Santana et al., 2019). As a result, three electric eel species occurring in very distinct ecological environments were recognized: *E. electricus* (Linnaeus, 1766) and *E. voltai* de Santana, Wosiacki, Crampton, Sabaj, Dillman, Castro e Castro, Bastos & Vari, 2019 from Brazilian and Guyana shields in Highlands Amazon and *E. variii* de Santana, Wosiacki, Crampton, Sabaj, Dillman, Mendes-Júnior & Castro e Castro, 2019 from the Lowlands Amazon (de Santana et al., 2019).

The new finds offer an opportunity to study the genetics of populations of those distinct ecological and unique animals by characterizing their genetic variation, within and between populations, and the forces that affect their frequencies, such as migration, mutation, selection, and genetic drift. An excellent way to study the genetic composition of natural fish populations is by using molecular markers, which are powerful tools for quantifying genetic variation in individuals and populations, contributing to the management and conservation of species (Allendorf et al., 2010). According to Zane et al. (2002), the microsatellites (SSR – Simple Sequence Repeats), for instance, are considered useful for population studies because they are highly polymorphic markers. The population genetic analysis of species in the wild is of paramount importance for elucidating the factors and conditions that allow populations and species to be maintained and in the development of a strategy for its effective management (Moysés et al., 2005).
Published population genetic studies in Neotropical electric fishes are inexistent, and only a few attempts to develop microsatellite primers for Gymnotiformes were made (e.g., Moysés et al., 2005).

This study aims to develop candidate microsatellite loci to accurately access genetic diversity and help in future studies of population genetics of electric eels. Thus, this paper reports the development and characterization of novel microsatellite loci for *E. varii* and evaluates it in *E. voltai* to cross-amplification. Additionally, the primers were tested for cross-amplification in four species across Gymnotiformes.

**MATERIAL AND METHODS**

A partial enriched genomic library was constructed, and microsatellites were isolated and characterized following the protocol of Billotte et al. (1999). Tissue samples from *E. varii* and *E. voltai* were donated by the Instituto Nacional de Pesquisas da Amazônia (INPA), with invoice number: 009/96. Total genomic DNA was extracted from muscle tissue from a sample of *E. varii* (INPA 41112), according to Almeida (Almeida et al., 2010). Genomic DNA (5 μg) was digested, and the blunt-ended fragments were ligated to the adaptors (Edwards et al., 1996). Fragments were selected, amplified, and cloned into pGem-T Easy (Promega; www.promega.com) vectors using 5μL of the amplification product, 50 ng of vector, and 1 U of T4 DNA ligase in reaction buffer at 4°C (overnight). Cloning products were used to transform *Escherichia coli* (DH5α lineage) cells. The recombinant clones were selected and sequenced on an ABI 3500 XL automated sequencer. Sequences were analyzed, and primers were designed according to Hall (1999) and Rozen, Skaletsky (2000), respectively. The selected forward primers were marked with the M13 at the 5’ end (Schuelke, 2000). To test the potential presence of hairpin structures and problems with the primer-dimer, we follow the protocol of Vallone, Butler (2004). PCR amplifications were carried out on a panel consisting of 13 individuals of *E. varii* (INPA 41112 – 41122 and INPA 41124 – 41125) from three localities along the Curiaú River; and 14 individuals of *E. voltai* (LIA 4802 – 4806; INPA 41123; INPA 050453) – five from two localities of the Xingu River, one specimen collected in the Curiaú River and eight collected in the Iriri River. All specimens of electric eels were collected in the Amazon basin, Brazil. Cross-amplification tests were performed using four other Gymnotiformes species whose voucher specimens are deposited in the Museu de Zoologia da Universidade Estadual de Londrina (MZUEL) as follows: *Apteronotus cf. caudimaculosus* de Santana, 2003 (n=4; MZUEL 09538; Apteronotidae); *Eigenmannia trilineata* López & Castello, 1966 (n=5; MZUEL 09552; Sternopygidae); *Gymnotus sylvius* Albert & Fernandes-Matioli, 1999 (n=5; MZUEL 09546; Gymnotidae); and *Sternopygus macrurus* (Bloch & Schneider, 1801) (n=5; MZUEL 09454; Sternopygidae), all collected in the Laranjinha River, Paraná river basin. Reactions were performed according to Apolinário-Silva et al. (2018). Amplifications were made 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 (Tabs. 1–2) 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. GeneScan 600 Liz (Applied Biosystems) was used as the molecular weight standard.
Individuals were genotyped with GeneMarker 1.85 (SoftGenetics, State College, PA), followed by manually editing. Tests for Hardy-Weinberg Equilibrium (HWE) and the presence of linkage disequilibrium among the pairs of loci were calculated using GENEPOP 4.0.10; *P* values were subsequently adjusted applying the sequential Bonferroni correction (Rice, 1989). GenAlEx v.6.41 was used to estimate the observed (*H*<sub>o</sub>) and expected (*H*<sub>e</sub>) heterozygosities and the average number of alleles per locus. The paternity exclusion probability (*Q*) (Weir, 1996) and genetic identity probabilities (*I*) (Paetkau et al., 1995) were estimated using Identity 1.0. Estimates of the polymorphic information content (PIC) and potential null alleles were obtained through Cervus v.3.0 and Micro-Checker v.2.2.3, respectively. Default settings were used for all tests.

**RESULTS**

A set of 13 polymorphic and highly informative microsatellite loci for genetic studies of populations of *Electrophorus* were developed: a total of 45 out of 96 clones sequenced contained microsatellite regions, with 25 being suitable for primer design and PCR reactions. After testing different amplification conditions, 14 loci (almost all dinucleotide repeats) were successfully amplified. From those, one was monomorphic, and 13 were polymorphic for two electric eel species.

In *E. varii*, a total of 85 different alleles were detected, varied from 2 (*Elec24*) to 15 (*Elec39*), with an average of 6.4 alleles per locus. The observed and expected heterozygosity ranged from 0.000 (*Elec24*) to 1.000 (*Elec14*) and from 0.334 (*Elec49*) to 0.902 (*Elec39*), respectively. After sequential Bonferroni correction for multiple comparisons (*α* = 0.05, *k* = 91), no evidence of linkage disequilibrium between any pair of loci examined was observed. In the HWE tests, two loci, *Elec24* and *Elec241*, presented significant deviation after correction for multiple tests (sequential Bonferroni correction *α* = 0.05 and *k* = 14). These loci were also the only ones showing possible null alleles, inferred from excess homozygous genotypes, explaining the observed deviation from HWE. It was observed that the same loci that had a significant deviation in the HWE, plus loci *Elec22* and *Elec31*, also had significant values of the endogamic coefficient (*F*<sub>IS</sub>; Tab. 1). The mean PIC for the 13 polymorphic loci was 0.572 following a scale proposed by Botstein et al. (1980), 10 loci (*Elec12*, *Elec14*, *Elec 21*, *Elec31*, *Elec39*, *Elec43*, *Elec53*, *Elec241*, *Elec246* and *Elec247*) were highly informative and three loci (*Elec22*, *Elec24* and *Elec49*) were moderately informative. The probabilities of identity and paternity exclusion were equal to 2.665<sup>-12</sup> and 0.999, in that order (Tab. 1).

All 14 microsatellite primers developed for *E. varii* were successfully cross-amplified in *E. voltai*. Thirteen are polymorphic loci and produced a total of 74 different alleles, with allele number ranging from 2 (*Elec31* and *Elec451*) to 12 (*Elec49*), with an average of 5.2 alleles per locus (Tab. 2). The observed and expected heterozygosity varied from 0.071 (*Elec12*, *Elec24* and *Elec451*) to 1.000 (*Elec14*) and from 0.069 (*Elec451*) to 0.908 (*Elec49*), correspondingly. After Bonferroni sequential correction for multiple comparisons (*α* = 0.05, *k* = 91), no evidence of linkage disequilibrium between any pair of loci examined was detected.
TABLE 1 | Description and characterization of 14 microsatellite loci isolated from Electrophorus variii. Flanking primers, Ta = optimal annealing temperatures, k = number of alleles, H_e = observed heterozygosity, H_o = expected heterozygosity estimated from 13 individuals, Q = paternity exclusion probability, I = probability of genetic identity, F_is = endogamy coefficient, PIC = polymorphic information content, GenBank accession numbers. * Significant value for the endogamy coefficient (F_is).

| Locus   | Sequence repeat | Primer sequences (5’-3’) | T_a (°C) | k         | Allele size range (bp) | H_e | H_o | PIC (Q) | F_is | Genebank Accession numbers |
|---------|----------------|--------------------------|---------|-----------|------------------------|-----|-----|---------|------|---------------------------|
| Elec12  | (CA)_n         | F: CAGTTCAGTAGACGAGATACAGG  | 52°     | 7         | 203–241                | 0.769| 0.692| 0.661   | 0.483| 0.125                     | MN967054 |
|         |                | R: TTAGTTGAGTGGATACAGTG    |         |           |                        |     |      |         |      |                           |          |
| Elec14  | (TG)_n         | F: GCTCTGTTGTGTACGGC       | 52°     | 9         | 191–260                | 1.000| 0.795| 0.774   | 0.663| 0.049                     | MN967055 |
|         |                | R: TGACTCGAGGAAGTACGG      |         |           |                        |     |      |         |      |                           |          |
| Elec22  | (TG)_n         | F: GGAGCAGCAAGCAAGCTC      | 48°     | 4         | 171–177                | 0.231| 0.388| 0.363   | 0.214| 0.399                     | MN967056 |
|         |                | R: GGCACAGTCTCTTCCATTCA    |         |           |                        |     |      |         |      |                           |          |
| Elec24  | (GT)13(GAAA)_n| F: GATCGTTGAGCTCTGTTGTA     | 56°     | 2         | 214–216                | 0.000| 0.335| 0.292   | 0.146| 0.479                     | MN967057 |
|         |                | R: TCCCTAGTGAATCCATTCAAG   |         |           |                        |     |      |         |      |                           |          |
| Elec31  | (AG)_n         | F: TGGATCTTGAGGACTTACAA     | 45°     | 5         | 144–166                | 0.538| 0.731| 0.711   | 0.524| 0.102                     | MN967058 |
|         |                | R: AGGCCACACTAATACGGAAG    |         |           |                        |     |      |         |      |                           |          |
| Elec39  | (GT)_10        | F: TCCAGGCAAGGAGCTG        | 56°     | 12        | 166–228                | 0.846| 0.902| 0.895   | 0.805| 0.017                     | MN967059 |
|         |                | R: TCCAGCACATCCAGGAGAG    |         |           |                        |     |      |         |      |                           |          |
| Elec43  | (TG)_n         | F: CCGTTGAGCAGGTAGATATAG   | 60°     | 5         | 263–279                | 0.769| 0.701| 0.649   | 0.451| 0.141                     | MN967060 |
|         |                | R: CAAGAGCTAGACGCCATGC     |         |           |                        |     |      |         |      |                           |          |
| Elec49  | (GT)_4         | F: ACTATGAGGTTCATAGAAGT    | 56°     | 4         | 178–202                | 0.231| 0.334| 0.317   | 0.184| 0.460                     | MN96705461|
|         |                | R: GAGCAGAAGCCTGTTGATTAG  |         |           |                        |     |      |         |      |                           |          |
| Elec53  | (GA)10(TG)8(AG)_n| F: GCATATGATCTTTGACTCTCG   | 52°     | 6         | 177–225                | 0.692| 0.710| 0.662   | 0.472| 0.131                     | MN967062 |
|         |                | R: GCATCGTCCTACGATAGG      |         |           |                        |     |      |         |      |                           |          |
| Elec241 | (GT)_8         | F: CTGTTGGAGGTAGATACAGAG   | 56°     | 8         | 147–215                | 0.455| 0.740| 0.706   | 0.604| 0.070                     | MN967063 |
|         |                | R: ACACTAAACTATCATCAACCA   |         |           |                        |     |      |         |      |                           |          |
| Elec244 | (TG)_4         | F: GAGTAGAGATACAGAGATTCG   | 56°     | 8         | 202–243                | 0.714| 0.769| 0.732   | 0.567| 0.084                     | MN967064 |
|         |                | R: CAGTTCAGTGACGATTACAGG  |         |           |                        |     |      |         |      |                           |          |
| Elec246 | (TG)_4         | F: CTGTTGCTCTGACAGTCTGC   | 52°     | 4         | 280–338                | 0.692| 0.678| 0.613   | 0.400| 0.168                     | MN967065 |
|         |                | R: GTGACTGCGGATCTACAGG    |         |           |                        |     |      |         |      |                           |          |
| Elec247 | (TG)_4         | F: TATGTTGAGGTGGTAGATACAGT| 56°     | 7         | 156–196                | 0.538| 0.689| 0.639   | 0.450| 0.146                     | MN967066 |
|         |                | R: CATACATGCGACTCTCTTCGGC |         |           |                        |     |      |         |      |                           |          |
| Elec451 | (GT)_4         | F: GTAAGGAGGGGAGGAGGACAC  | 52°     | 1         | 169                | 0.000| 0.000| 0.000   | 0.000| 0.000                     | MN967067 |
|         |                | R: AAGGCCAGTGGGAGGACAC    |         |           |                        |     |      |         |      |                           |          |

All loci                                                 85     | 0.538| 0.607| 0.572 | 0.999 | 2.665 | 0.153*|

Hardy-Weinberg Equilibrium deviations were significant for four loci (Elec22, Elec24, Elec241 and Elec247) after correction for multiple tests (sequential Bonferroni correction, α = 0.05 and k = 14). At the same time, these loci were the only ones showing null alleles (inferred from excess homozygous genotypes), which could explain the observed deviation from HWE. In addition, these same loci, plus Elec12 and Elec53, also showed significant values of the inbreeding coefficient (F_is; Tab. 2). The mean Polymorphic Information Content (PIC) for the 13 loci was 0.500, indicating that the loci set is highly informative (Tab. 2). Seven loci (Elec14, Elec39, Elec43, Elec49, Elec53, Elec241 and Elec246) were highly informative (PIC > 0.5); four loci (Elec22, Elec24, Elec31 and Elec427) were moderately informative (PIC > 0.25 and < 0.5); and two loci
(Elec12 and Elec451) had low informative potential (PIC < 0.2). The loci set showed a low value of genetic identity combined probability (2.9x10^{-11}) and high-shared probabilities of paternity exclusion (0.999), which suggest a high discriminatory power for population genetic studies (Tab. 2).

Cross-amplification testing of all 14 Electrophorus loci in four other Gymnotiformes was conducted. Six microsatellite loci successfully amplified in Apteronotus albifrons (Linnaeus, 1766) (Elec12, Elec39, Elec49, Elec247 and Elec451) and E. trilineata (Elec12, Elec14, Elec39, Elec49, Elec247, Elec451). Three effectively worked in G. sylvius (Elec12, Elec53, Elec247), and two in S. macrurus (Elec12, Elec49). The locus Elec12 was polymorphic for all species tested, ranging from three (G. sylvius and S. macrurus) to five (A. albifrons and E. trilineata) alleles per locus. Elec47 presented four alleles in G. sylvius, three in A. albifrons, and two in E. trilineata. On the other hand, the microsatellite loci Elec14, Elec39, and Elec451 were monomorphic for tested species, and loci Elec22, Elec24, Elec31, Elec43, Elec241, and Elec244, did not amplify for any of the four tested species.

**DISCUSSION**

Deviations of the HWE and significant F_{IS} values for some loci, mainly in E. voltai, are likely to be caused by the mixture of individuals originating from different populations. Freeland (2005) suggested that the inclusion of elements of multiple genetic units in a single panel could cause the Wahlund effect, i.e., excess homozygosity and significant estimations of F_{IS}. Similar results were observed by Apolinário-Silva et al. (2018), which
used a panel consisting of 34 individuals derived from genetically distinct units for microsatellite validation.

Microsatellite primers are generally highly species-specific (Zane et al., 2002). However, we have verified that all 14 primers pairs, developed for *Electrophorus varii*, satisfactorily amplify for *E. voltai*. The cross-species amplification implies that it may also be useful in *E. electricus* (which is more closely related to *E. voltai* – see de Santana et al., 2019) as well as in other Gymnotiformes species not tested herein. Heterologous primers can be successfully used in different species of fishes, and the quality of amplification depends on the degree of genetic conservation of positions bordering microsatellite regions (Abdul-Muneer, 2014). Consequently, the low amplification rate primers in the four species of Gymnotiformes can be explained by the lack of conservation of microsatellite sites. Equally, the successful amplification described in *E. voltai* can be attributed to the elevate conservation of the microsatellite flanking regions, which according to Barbará et al. (2007), is expected among closely related species. Accordingly, the lowest cross-amplification found for *Gymnotus*, currently hypothesized as the putative sister taxon to *Electrophorus* (Alda et al., 2018), was unexpected (see discussion on *Electrophorus* interrelationships in de Santana et al., 2019), indicating that *Electrophorus* current hypothesis of interrelationships deserves further attention.

**ACKNOWLEDGMENTS**

We are grateful to JAR Capiberibe for his financial support to this research (Parliamentary Amendment n° 20470007), IBAMA/SISBIO IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis), ICMBio (Instituto Chico Mendes-MMA), to INPA for donating tissue samples, and to C. Ruas (UEL), for helping build the library. OAS was granted by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 303685/2018–2). This paper was supported by the Project Diversity and Evolution of Gymnotiformes from the São Paulo Science Foundation (FAPESP)/ Smithsonian Institution (# 2016/19075–9) and Global Genome Initiative (GGI–Peer–2017–149) to CDS.

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AUTHOR CONTRIBUTION

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Carlos David de Santana: Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing-original draft, Writing-review and editing.

ETHICAL STATEMENT
This study was carried out in strict accordance with the recommendations provided in the Guide for the Care and Use of Laboratory Animals. The collection was authorized by the System of Authorization and Information on Biodiversity – SISBIO (n°. 40522-6). The sampling protocol was approved by the Ethics Committee on the Use of Animals – CEUA of the Instituto Nacional de Pesquisas da Amazônia (n°. 044/2016).

COMPETING INTERESTS
The authors declare no competing interests.

HOW TO CITE THIS ARTICLE
• Souza-Shibatta L, Ferreira DG, Santos KF, Galindo BA, Shibatta OA, Sofia SH, Giacomin RM, Bastos DA, Mendes-Júnior RNG, de Santana CD. Electric eels galore: microsatellite markers for population studies. Neotrop Ichthyol. 2020; 18(4):e200081. https://doi.org/10.1590/1982-0224-2020-0081