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Electric eels galore: microsatellite markers for population studies

Lenice Souza-Shibatta[1], Dhiego G. Ferreira[2], Kátia F. Santos[2], Bruno A. Galindo[2], Oscar A. Shibatta[3], Silvia H. Sofia[4], Renata M. Giacomin[5], Douglas A. Bastos[6], Raimundo N. G. Mendes-Júnior[7] and Carlos David de Santana[8]

Correspondence:
Lenice Souza-Shibatta
lenicesouza@hotmail.com

Fourteen novel microsatellite loci are described and characterized in two species of electric eels, *Electrophorus varii* 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.

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1 Laboratório de Sistemática Molecular, Programa de Pós-Graduação em Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid, km 380, 86051-970 Londrina, PR, Brazil. (LSS) lenicesouza@hotmail.com (corresponding author).

2 Laboratório de Genética e Conservação (GECON), Universidade Estadual do Norte Paraná, Rua Portugal, 340 86300-000 Cornélio Procópio, PR, Brazil. (DGF) dhiegouenp@gmail.com; (KFS) kfabianadossantos@gmail.com; (BAG) bruno@uenp.edu.br.

3 Universidade Estadual de Londrina, Museu de Zoologia, Departamento de Biologia Animal e Vegetal, Centro de Ciências Biológicas, 86051-990 Londrina, PR, Brazil. (OAS) shibatta@uel.br.

4 Laboratório de Genética e Ecologia Animal (LAGEA), Departamento de Biologia Geral, Universidade Estadual de Londrina, Rod. Celso Garcia Cid, km 380, 86051-970 Londrina, PR, Brazil. (SHS) shsofiabelh@gmail.com.

5 Departamento de Biologia Geral, Universidade Estadual de Londrina, 86051-990 Londrina, PR, Brazil. (RMG) giacomin.rm@gmail.com.

6 Programa de Pós-Graduação em Ciências Biológicas (BADPI), Instituto Nacional de Pesquisas da Amazônia, 69060-001 Manaus, AM, Brazil. (DAB) avizdoug@gmail.com.

7 RESEX do Rio Cajari, Instituto Chico Mendes de Conservação da Biodiversidade, Rua Hamilton Silva, 1570, 68906-440 Macapá, AP, Brazil. (RNMJ) raimundo.mendes-junior@icmbio.gov.br.

8 Division of Fishes, Department of Vertebrate Zoology, MRC-159, National Museum of Natural History, P.O. Box 37012, Smithsonian Institution, 20013-7012 Washington, DC, WA, USA. (CDS) desantanac@si.edu.

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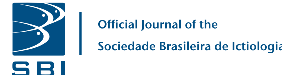

Catorze novos loci microsatélites são descritos e caracterizados em duas espécies de poraquês, *Electrophorus varii* e *E. voltai* de planícies alagadas e rios da floresta amazônica. Esses loci são polimórficos, altamente informativos e têm a capacidade de detectar níveis confiáveis de diversidade genética. 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. varii* 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*., 2001). 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* (DH5 – α 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 94ºC for 4 min, followed by 35 cycles at 94ºC for 40 s, 48ºC, 54ºC, or 60ºC (Tabs. 1–2) for 1 min, 72ºC for 1 min, and a final extension at 72ºC 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_o) and expected (H_e) 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_{IS}; 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^{-12} 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*, *Ele24* 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 varii*. Flanking primers, *Ta* = optimal annealing temperatures, k = number of alleles, H_o = observed heterozygosity, H_e = 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_o	H_e	PIC	(Q)	(I)	F_{IS}	Genebank Accession numbers
Elec 12	$(CA)_{14}$	F: CAGTTCAGTAGCAGGAGTATACAGG R: TTAGTGTGAGGTGGATTAACAATG	52°	7	203 – 241	0.769	0.692	0.661	0.483	0.125	-0.071	MN967054
Elec14	$(TG)_{28}$	F: GCTCTGTTGTGGTACGGC R: TGACTCGCAGGCTAACAGG	52°	9	191 – 260	1.000	0.795	0.774	0.663	0.049	-0.216	MN967055
Elec22	$(TG)_{15}$	F: GGAGCAGCAACCGGACTC R: GGCACTACAGTCTCCTCCAA	48°	4	171 – 177	0.231	0.388	0.363	0.214	0.399	0.437*	MN967056
Elec24	(GT)13$(GAAA)_4$	F: GATACTTCGAGCTCACGTCTTAG R: TCCTCATGTATCCCATTACCAAG	56°	2	214 – 216	0.000	0.355	0.292	0.146	0.479	1.000*	MN967057
Elec31	$(AG)_{18}$	F: TTGATCATTTAGCGTGGACTTAAC R: AGGCCACACTACTAATCAGAACG	45°	5	144 – 166	0.538	0.751	0.711	0.524	0.102	0.319*	MN967058
Elec39	$(GT)_{37}$	F: TCCAGGGACAGGACGTTG R: TCCAGCACACTCAGGTAGAGG	56°	15	166 – 228	0.846	0.902	0.895	0.805	0.017	0.102	MN967059
Elec43	$(TG)_{16}$	F: CCTGTTAGGCTGGTTAGATAATATG R: CAAGAAGCTAGACGCCATGC	60°	5	263 – 279	0.769	0.701	0.649	0.451	0.141	0.057	MN967060
Elec49	$(GT)_{17}$	F: ACTATCAGGTCTCAAAGGATTTTC R: GAGCACAGATCTGGTCATCTAGG	56°	4	178 – 202	0.231	0.334	0.317	0.184	0.460	0.345	MN96705461
Elec53	(GA)10(TG)8$(AG)_{19}$	F: GCAATATGATTCTGTTTGACTTCG R: GCACTGCCTGACAGATGG	52°	6	177 – 225	0.692	0.710	0.662	0.472	0.131	0.064	MN967062
Elec241	$(GT)_{14}$	F: CTGGTGGAGTTGATTACAGAGAG R: ACACTAACATATCCATCCACAAAG	56°	8	147 – 215	0.455	0.740	0.706	0.604	0.070	0.245*	MN967063
Elec244	$(TG)_{14}$	F: GAGGTGGATTAACAATGTAAACTGG R: CAGTTCAGTAGCAGGAGTATACAGG	56°	8	202 – 243	0.714	0.769	0.732	0.567	0.084	0.024	MN967064
Elec246	$(TG)_{24}$	F: CTCGGTCCTCCAGTCTTGC R: GTGACTCGCAGGCTAACAGG	52°	4	280 – 338	0.692	0.678	0.613	0.400	0.168	0.018	MN967065
Elec247	$(TG)_{13}$	F: TTAGTGTGAGGTGGATTAACAATG R: CATACATATGCACGTTCTCTTGC	56°	7	156 – 196	0.538	0.689	0.639	0.450	0.146	0.256	MN967066
Elec451	$(GT)_{14}$	F: GTAAGGAGAGCCGACAGCAC R: AAGGCAGTGTTGGAGTCACC	52°	1	169	–	–	–	–	–	–	MN967067
All loci				85		0.538	0.607	0.572	0.999	2.665^{-12}	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*, *Elec 24*, *Elec31* and *Elec247*) were moderately informative (PIC > 0.25 and < 0.5); and two loci

TABLE 2 | Cross-amplification of 14 microsatellite *loci* and genetic diversity per *locus* in *Electrophorus voltai*. Flanking primers, k = number of alleles, H_o = observed heterozygosity, H_e = expected heterozygosity estimated from 14 individuals, Q = paternity exclusion probability, I = probability of genetic identity, F_{IS} = endogamy coefficient, PIC = polymorphic information content. * Significant value for the endogamy coefficient (F_{IS}).

Locus name	k	Allele size range (bp)	H_o	H_e	PIC	(Q)	(I)	F_{IS}
Elec12	3	203 – 211	0.071	0.135	0.131	0.068	0.752	0.500*
Elec14	6	209 – 260	1.000	0.719	0.679	0.492	0.119	-0.358
Elec22	4	173 – 179	0.143	0.403	0.364	0.209	0.395	0.666*
Elec24	3	212 – 216	0.071	0.564	0.466	0.266	0.287	0.881*
Elec31	2	148 – 166	0.500	0.375	0.305	0.152	0.460	-0.300
Elec39	6	150 – 172	0.857	0.760	0.724	0.547	0.093	-0.090
Elec43	7	251 – 315	0.760	0.791	0.724	0.594	0.074	0.228
Elec 49	12	220 – 242	0.786	0.908	0.901	0.812	0.015	0.171
Elec53	8	199 – 225	0.643	0.832	0.811	0.668	0.048	0.261*
Elec241	10	161 – 191	0.538	0.861	0.846	0.749	0.027	0.408*
Elec244	1	212	0.000	0.000	–	–	–	–
Elec246	5	280 – 362	0.500	0.548	0.516	0.338	0.236	0.125
Elec247	5	154 – 162	0.214	0.508	0.478	0.306	0.272	0.602*
Elec451	2	169 – 177	0.071	0.069	0.067	0.033	0.869	0.001
All loci	74	–	0.429	0.532	0.500	0.999	2.981^{-11}	0.228*

(*Elec12* and *Elec451*) had low informative potential (PIC < 0.2). The loci set showed a low value of genetic identity combined probability (2.9×10^{-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*, *Elec53*, *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.

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AUTHOR CONTRIBUTION

Lenice Souza-Shibatta: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing-original draft, Writing-review and editing.
Dhiego G. Ferreira: Formal analysis, Investigation, Methodology, Writing-original draft, Writing-review and editing.
Kátia F. Santos: Formal analysis, Investigation, Methodology.
Bruno A. Galindo: Formal analysis, Investigation, Writing-original draft.
Oscar A. Shibatta: Investigation, Writing-original draft, Writing-review and editing.
Silvia H. Sofia: Investigation, Methodology, Writing-review and editing.
Renata M. Giacomin: Formal analysis, Investigation, Methodology.
Douglas A. Bastos: Data curation, Investigation, Resources, Writing-original draft, Writing-review and editing.
Raimundo N. G. Mendes-Júnior: Data curation, Investigation, Methodology, Resources, Writing-review and editing.
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.

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