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SHORT CORMMUNICATION

Development of microsatellite markers for the carnivorous plant *Genlisea aurea* (Lentibulariaceae) using genomics data of NGS

Yani C. Aranguren‑Díaz[1,2] · **Alessandro M. Varani**[3] · **Todd P. Michael**[4] · **Vitor F. O. Miranda**[1]

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Abstract

Genlisea aurea A.St.-Hil. is a carnivorous plant endemic species to Brazil in the Lentibulariaceae family. Very few studies have addressed the genetic structure and conservation status of *G. aurea* and the Lentibulariaceae. Microsatellites markers are advantageous tools that can be employed to predict the vulnerability of Lentibulariaceae species. Therefore, the development of molecular markers focusing the population analyses of *Genlisea* for future genetic studies and conservation actions are essential. Thus, we developed simple sequence repeats (SSRs) based on in silico analyses of *G. aurea* draft genome assembly. We characterized 40 individuals from several populations and identified 12 loci that were polymorphic, with heterozygosity between 0.123 and 0.650. We demonstrated that the *G. aurea* SSR markers work cross-species in *Genlisea filiformis, G. repens, G. tuberosa* and *G. violacea*. These markers will be important for future population, phylogeographic and conservation studies in *G. aurea* and other *Genlisea* species.

Keywords Conservation · Genetic diversity · Endemic species · Molecular markers · SSR

Introduction

The species of the genera *Genlisea, Utricularia* and *Pinguicula* (Lentibulariaceae) comprise the largest family of carnivorous plants with around 370 species known. There are 82 *Utricularia* and *Genlisea* species catalogued to Brazil, of which 26 are endemic, and *Genlisea* species are distributed in Central and South America and Africa. However, the greatest diversity of species is found in Brazil with 17 known species [1, 2].

✉ Vitor F. O. Miranda
vmiranda@fcav.unesp.br

1 Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, Departamento de Biologia Aplicada à Agropecuária, Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal, São Paulo 14884-900, Brazil

2 Universidad Simón Bolívar, Barranquilla, Colombia

3 Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, Departamento de Tecnologia, São Paulo, Brazil

4 J. Craig Venter Institute, 4120 Capricorn Ln., La Jolla, CA 92037, USA

Genlisea aurea A.St.Hil. is endemic to Brazil and distributed between the center and south of the country, in the Brazilian *Cerrado* and *Mata Atlântica* [2]. The Brazilian *Cerrado* and *Mata Atlântica* are some of the most biodiverse ecosystems in Brazil and the world with 31.5% and 49.5% of the endemic angiosperms respectively [3]. However, it is also one of the most fragile and threatened biomes due to reduction in size as a result of urbanization, agriculture and livestock [3, 4]. These biomes are among the 25 biodiversity hotspots [5], but have experienced a dramatic decrease in original vegetation [4–6]. Currently, 61 native species of Lentibulariaceae occur in the *Cerrado*, of which 20 are endemic; and in the *Mata Atlântica* 35 species of which 10 are endemic [2, 3].

Genlisea species lack roots and present small rosettes with dimorphic leaves: photosynthetic and achlorophyllous leaves. These latter leaves are specialized structures called *rhizophylls* that demonstrate positive geotropism [1]. The *rhizophylls* are subterranean, inverted Y-shaped tubular leaves, acting to fix the plants in the soil and also to trap small organism [7], usually microcrustaceans, acari and nematodes [8–10] or even algae, cyanobacteria and protozoans [11, 12], as a source of nutrients [11, 13].

Genlisea aurea has the second smallest genome known to date in plants, at 64 Mpb, and the genome size has been shown to vary among populations [14–16]. Despite a growing knowledge about the genome, currently there are no studies on the genetic structure of natural populations of *Genlisea* species, highlighting the need for genetic and population studies that could determine their conservation status. Moreover, the *Cerrado* and the *Mata Atlântica* are biodiversity hotspots [5] and the distribution of *G. aurea* is restricted to permanently wet to boggy habitats [1], making this species potentially vulnerable and is in risk.

Microsatellites are an excellent alternative for estimating genetic diversity. Microsatellite markers have been identified in *Utricularia reniformis* A.St.-Hil., and they have been shown to cross-amplify in some other *Utricularia* species [17]. However, we found that *Utricularia* microsatellite markers do not cross-amplify in *Genlisea* species. In this work, we presented 12 polymorphic microsatellite markers that we developed based *Genlisea aurea* Illumina sequencing data. These new *Genlisea* SSR markers will enable future studies of genetic diversity in populations of *G. aurea* and related species. *Genlisea* genetic diversity and population structure will inform conservation status and also enable conservation strategies.

Materials and methods

In silico search

We made an in silico search of simple sequence repeats, present the draft assembly of the *Genlisea aurea* genome based on Illumina paired-end sequencing data (http://www.genlisea.org—unpublished data). This search was performed using the algorithm *SSR_pipeline* [18] employing the search parameter repeated sequences di, tri and tetra nucleotide with eight to sixteen repetitions and 50 bp flanking sequences. After that we designed specific primers with Primer3 [19] to amplify these regions, and selected twenty-one primer pairs that were estimated to generate products longer to 100 bp. The primers-pairs were selected to be between 20 and 22 bp, less than a 5 °C T_m (melting temperature) difference between the forward and reverse, a GC % between 45–55%, and not having primer dimers or secondary structures.

Sampling and DNA extraction

We collected tissue from six populations and a total of 40 individuals from three geographic regions (Northeast, Center-West, and Southeast of Brazil) covering most distribution area of *Genlisea aurea* (Table 1). The samples were dried in silica gel and DNA extractions were done using the cetyltrimethylammonium bromide (CTAB) protocol [20]. The DNA was quantified on a spectrophotometer (NanoDrop 800, Thermo Scientific Corp, San Jose, CA, USA). Vouchers representing each population were deposited in Herbarium JABU (University of Sao Paulo State, UNESP/FCAV, Jaboticabal, Brazil).

Polymerase chain reaction and genotyping

After PCR testing, we selected SSR primers that amplified bands with the expected sizes. The amplicons were visualized on a 3% agarose gel. From these effective primers, new forward primers were designed with a M13 tail (CACGAC GTTGTAAAACGAC) on the 5′ end (Schuelke, 2000). In addition, three primers were made with M13 sequence tag 6-FAM-, HEX- and NED- (Applied Biosystems). The 12 microsatellites regions were amplified in individual reactions in 10 µL, using 1X PCR buffer (10 mM Tris pH 8.4, 50 mM KCl, 3 mM $MgCl_2$), 0.2 µM M13-forward primer, 0.8 µM the reverse primer, 0.8 µM primer fluorescently labeled M13, 0.15 mM of dNTPs, 0.5 U polymerase and 30 ng of DNA. The amplification profiles required an initial denaturation at 94 °C for 4 min; followed by 35 cycles of 30 s at 94 °C, 45 s at 45–50 °C, and 45 s at 72 °C, with a final extension at 72 °C for 5 min. After, 96-well plates were prepared for capillary electrophoresis by mixing the three PCR product labeled with different fluorescence dyes (0.5 µL each), 0.5 µL GeneScan™ 500 ROX size standard ladder (Applied Biosystems) and 8 µL of formamide. The separation and identification of alleles by capillary electrophoresis were performed on the Genetic Analyzer ABI

Table 1 *Genlisea aurea* individuals and populations studied

Population	Sample (individual)	Localization
1	BA011, BA021, BA031, BA041, BA051	Municipality Lençois, Bahia State
2	BA012, BA022, BA072, BA082, BA132, BA212	Municipality Rio de Contas, Bahia State
3	BA033, BA053, BA083, BA093, BA133	Municipality Rio de Contas, Bahia State
4	SA04, SA07, SA08, SA09, SA10, SA11, SA12, SA13, SA14, SA15, SA16, SA23, SA30	Municipality Campos do Jordão, São Paulo State
5	GA031, GA051, GA071, GA101, GA111	Municipality Alto Paraíso, Goiás State
6	GA012, GA042, GA062, GA122, GA132, GA162	Municipality Alto Paraíso, Goiás State

3730 XL DNA Analyzer (Applied Biosystems, Foster City, California, CA), and genotyping was performed using the GeneMapper® program.

Statistical analyses

We estimated the size of the alleles including the M13 tail, the number of alleles per locus (k), and observed and expected heterozygosity (H_O and H_E), using GenAlEx 6.5 [21]. The inbreeding coefficient (F_{IS}) and the tests for deviation from Hardy–Weinberg equilibrium (HWE) were calculated with GENEPOP 4.2.2 program [22] and determined the null alleles with Micro-Checker [23]. Furthermore, it was made a quantitative matrix with allelic sizes and PCA multivariate analysis. The PCA was conducted with randomization test of 999 runs and using the method based on covariances for the 40 samples from the three regions of Brazil (Northeast, Center-West, and Southeast).

Results and discussion

A total of 173,777 sequences with di- (63.3%), tri- (32.2%) and tetra- (4.5%) nucleotide tandem repeats were found in 44,307 contigs of the *G. aurea* draft assembly. 461 sequences with 8–16 repeats were selected for further evaluation. From the 461 sequences, 21 were selected that were estimated to generate products longer to 100 bp. After PCR essays in 40 *G. aurea* specimens from six different populations (Table 1), twelve loci tested were polymorphic (Table 2). The number of alleles ranged from five to eleven alleles. Observed heterozygosity ranged from 0.123 to 0.650, and expected heterozygosity from 0.366 to 0.843. Significant deviation from HWE ($P < 0.001$) detected for 9 loci, and these 9 loci showed potential for null alleles ($P < 0.01$). This sample is possibly in Hardy–Weinberg equilibrium with loci GAR6, GAR8, GAR12, GAR14, GAR15, GAR16, GAR17, GAR18, GAR19 showing signs of a null allele.

In the PCA (Fig. 1), there is a grouping of individuals isolated from each region (Northeast, Center-West, and Southeast of Brazil) and the dispersion of samples reflects the high polymorphism observed in the population statistics and suggests sharing of haplotypes between population from different geographic regions. These results indicate that these 12 markers can be potentially useful for various genetic studies in populations of *Genlisea aurea*, allowing distinguish relationships among individuals and populations.

We also tested the cross-species amplification on three individuals of four related species: *Genlisea filiformis* A.St.-Hil., *G. repens* Benj., *G. tuberosa* Rivadavia, Gonella &

Table 2 Details for 12 polymorphic microsatellites loci for the forty samples of *Genlisea aurea*

Locus	Primer sequence 5′-3′	Repeat motif	Size (bp)	K	H_O	H_E	F_{IS}	F null
Gar06	F:TAGTGGCAGTGTACTTGCGG R:TATCCGTTGTGGACTTCGGC	TA	113–165	11	0.427	0.843	0.505	0.227
Gar08	F:TTTGCACTCCGCGTCTATCT R:GGAACCTCGTCCACGAAGAA	TG	132–170	15	0.600	0.832	0.290	0.127
Gar11	F:AGGAGACACTTCATTCCTGC R:GTTGAGACCAGTGCATCAGC	CCT	102–153	8	0.650	0.671	0.044	–
Gar12	F:CAACCCCAAACAACATCATCCA R:TGCTGCTTAGTGCAACAACAA	ATT	98–163	7	0.325	0.568	0.438	0.155
Gar14	F:AAGAAGGCATAGGCACCACG R:CCGCTGGATTTGCTCCTTCT	CAT	89–144	9	0.275	0.827	0.674	0.302
Gar15	F:CCAATCGCATTACACCACCC R:CCAGAACTGGATTTCGCCCT	ATG	112–169	11	0.500	0.824	0.404	0.176
Gar16	F:TCCATCAGACAGTCAACCAGG R:CCGGTAAGTAAGGGTCATGT	CAT	142–169	8	0.350	0.675	0.491	0.194
Gar17	F:TCCGCTTCCTTCGACCTTAG R:GCGGACTTTTAGACACAGAAGG	CTT	66–117	7	0.123	0.744	0.836	0.355
Gar18	F:GGCACCGGTTTTGAGTTTCG R:GCGTACCTGGTTAGGTTGCT	TACA	101–133	7	0.475	0.791	0.410	0.176
Gar19	F:TGAAGGAGGTCGTCGGGG R:ACATGGGAATGGGAACGACA	ATGA	102–146	6	0.250	0.622	0.606	0.229
Gar20	F:TTCGAATCAGTGAGCCC R:AACTATTGCAGGCAGTCATC	CTTT	95–107	6	0.525	0.475	−0.092	–
Gar21	F:CTGGCCATTGACATCGGGTA R:TGTCTGAAAGACGACAACCA	ATTC	97–109	5	0.350	0.366	0.057	–

Primers are either forward (F) or reverse (R). The amplified fragment sizes show base pairs (bp). The number of alleles k, heterozygosity observed (H_O), inbreeding coefficient (F_{IS}), heterozygosity expected (H_E). F null estimated frequency of null alleles ($P < 0.01$)

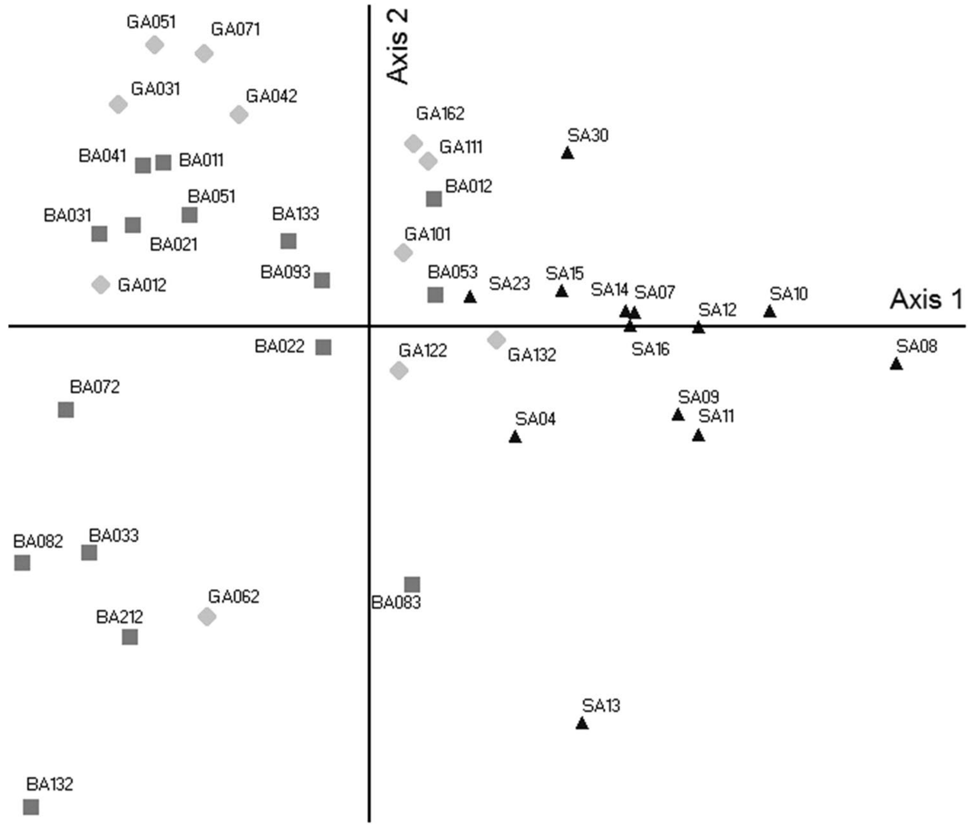

Fig. 1 Principal component analysis of samples of forty *Genlisea aurea* of six populations representing three geographic areas using allele's size for the development loci. (■) Samples from Northest (Bahia State); (◆) samples from Center-West (Goias State); (▲) samples from Southeast (São Paulo State)

Table 3 Cross-species amplification in *Genlisea filiformis, G. repens, G. tuberosa* and *G. violacea* using microsatellite developed for *G. aurea*

Locus	*G. filiformis*	*G. repens*	*G. tuberosa*	*G. violacea*
Gar06	175	190–210	210	100
Gar08	120	–	120	120
Gar11	–	100	100	150
Gar12	350	260	250	260
Gar14	110	110	110	110
Gar15	130	130	130	130
Gar16	180–200	150	–	150
Gar17	160	180	160	160
Gar18	–	–	–	120
Gar19	100	100	100	100
Gar20	100	100	100	150
Gar21	80	80	80	80

Numbers denote the amplicons size

"–" Denotes no amplification

A. Fleischm., and *G. violacea* A.St.-Hil. (Table 3). All loci showed transferability, and only 4 were partially positive (locus Gar08 was negative for *G. repens*, Gar11 for *G. filiformis*, Gar16 for *G. tuberosa*), with Gar18 positive for *G. repens* only (Table 3). However, further analyses will be done (in preparation) to determine their usefulness for estimating genetic diversity in other species.

Our analyses resulted in twelve microsatellites markers for *Genlisea aurea*, which are effective and informative for futures studies on phylogeography and conservation. Furthermore, our study demonstrated that the microsatellites cross amplify in additional species as *G. filiformis, G. repens, G. tuberosa* and *G. violacea*. Thus, these markers are potentially useful in diversity analyses of these species and possibly other related Lentibulariaceae taxa.

Conclusions

From genomic sequencing data from next generation, we searched and characterized SSR in individuals of *Genlisea aurea* from different populations of Brazil, covering most distribution area for the species. This resulted in twelve polymorphic microsatellite markers for *G. aurea*, which could be used for future studies in population genetics and conservation, and phylogeography. In addition, our study demonstrates that these microsatellites are transferable in other species as *G. filiformis, G. repens, G. tuberosa*, and *G. violacea*. Therefore, these twelve microsatellite markers are potentially useful for population analysis of *Genlisea* and also possibly for other species of Lentibulariaceae.

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Compliance with ethical standards

Conflict of interest The author declares there is no conflict of interests.

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