CROP BREEDING AND APPLIED BIOTECHNOLOGY

NOTE

Crop Breeding and Applied Biotechnology
16: 355-360, 2016
Brazilian Society of Plant Breeding.
Printed in Brazil
http://dx.doi.org/10.1590/1984-70332016v16n4n52

Development and characterization of microsatellite loci for the Neotropical orchid *Trichocentrum pumilum*

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Abstract: *Studies of genetic diversity and structure are key elements in designing effective in situ and ex situ management plans, especially for species experiencing forest fragmentation. To investigate the level of genetic diversity in populations of* Trichocentrum pumilum*, eight polymorphic microsatellite loci were developed and used for genotyping 96 specimens from four disturbed populations. Low genetic diversity within populations was found (average number of alleles per locus ranging from 3.75 to 4.25, observed and expected heterozygosities from 0.238 to 0.333 and from 0.450 to 0.482, respectively). The fixation index (F_{IS}) ranged from 0.35 to 0.47, with significant values for all populations. No genotypic disequilibrium was detected. A mixed breeding system was found through an apparent outcrossing rate estimate. Our results suggest that these microsatellite loci are suitable for genetic studies of this species, showing low within population genetic diversity and moderate structure for T. pumilum populations.*

Key words: *Conservation genetics, genetic diversity, Orchidaceae, population genetics, tropical orchid species.*

INTRODUCTION

The orchid family is one of the largest groups among vascular plants, corresponding to 10% of all flowering plants (Otero and Flanagan 2006). *Trichocentrum pumilum* (Lindl.) M. W. Chase & N.H. Williams is an epiphytic orchid with common occurrence in Brazil (Barros et al. 2013). This species is often found in gallery forest between the vegetation of semi-deciduous forest. It is considered to be self-incompatible and pollinator-limited, being exclusively visited and pollinated by two bee species (*Tetrapedia diversipes* and *Lophopedia nigrispinis*) (Pansarin and Pansarin 2011). The flowers are yellowish or greenish yellow with brown spots. In January, during fruit development, each plant produces a new pseudobulb, which sometimes produces a new lateral inflorescence for the next flowering season. The fruits come into dehiscence from June/July (Pansarin and Pansarin 2011).

Microsatellite markers are highly variable and, therefore, highly advantageous when compared to other markers (Kalia et al. 2011). This molecular marker has been used in population genetic studies of orchid species, including tropical epiphytic species, such as *Dendrobium loddigesii* (Cai et al. 2012), *Cattleya* spp.

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Received: 13 May 2016
Accepted: 01 September 2016

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(Almeida et al. 2013, Novello et al. 2013, Tambarussi et al. 2016), among others. However, there are no previous reports of genetic studies for *T. pumilum* and no information on the level of genetic diversity of its populations. Thus, this study aimed at the development of microsatellite markers and genetic characterization of four disturbed *T. pumilum* populations in order to generate useful information aiming at conservation strategies.

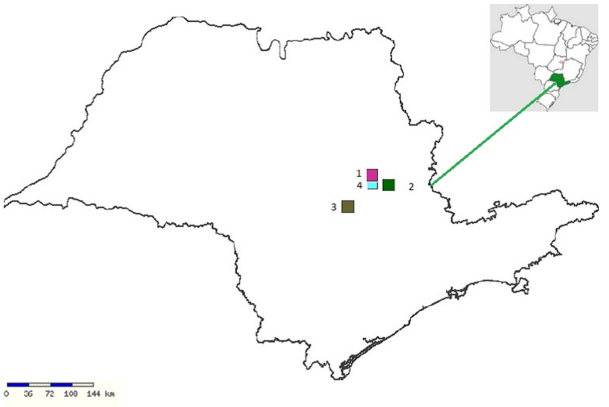

Figure 1. Collection sites of *Trichocentrum pumilum* in the State of São Paulo, Brazil. 1- Alto da Serra, São Pedro; 2- Iracemápolis; 3- Santa Maria da Serra; 4- São Pedro. Source: Atlas BIOTA FAPESP/ Center of Reference to Environmental Information (2015).

MATERIAL AND METHODS

Sampling procedures

Twenty-four individuals per population were sampled from four populations, at the municipalities of Iracemápolis (IRA), Santa Maria da Serra (SM), and São Pedro, in the State of São Paulo, Brazil (Figure 1). Populations sampled in São Pedro municipality were obtained in different areas of privately owned forest, one in São Pedro (SP) and the other in Alto da Serra (AS), at a higher altitude. All four populations were collected in forest fragments, next to agricultural landscape. The distance between populations varied from 1.6 (AS and SP) to 62.0 (IRA and SM) kilometers. Specimens of each population were deposited at the herbarium of the Escola Superior de Agricultura "Luiz de Queiroz", University of Sao Paulo, under the numbers ESA127076 and ESA127077.

DNA extraction, design, optimization of primers and sequencing/genotyping

DNA extraction from fresh leaves was based on Doyle and Doyle (1990), modified by Rodrigues et al. (2015). DNA quantification was performed on 1% agarose gels stained with GelRed (Biotium). Genomic library to obtain microsatellite markers was developed following Billote et al. (1999). DNA digestion, microsatellite enrichment, transformation of competent cells and sequencing of recombinant colonies were based on Tambarussi et al. (2013). We obtained 288 transformed clones, which were sequenced using universal T7 and SP6 primers with a BigDye v3.1 terminator kit on an ABI 3130*XL* Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, USA). The sequences obtained were transformed in a FASTA format by BioEdit Sequence Alignment Editor program (Hall 1999) and vectors and adapters sequences were excluded. The program Websat (Martins et al. 2009) allowed the identification of microsatellite sequences. The parameters used for the identification of microsatellites were: dinucleotide length ≥5nt; tri-, tetra-, penta-, hexanucleotide with a repeat length ≥3 nucleotides. Primers were designed using Primer3 (Rozen and Skaletsky 2000) considering the following criteria: annealing temperature ranging from 54 ° to 62 °C, guanine and cytosine content between 40 and 60% and range between 100 and 300 base pairs. Gene Runner v.3.1 software (Spruyt and Buquicchio 1994) was used to confirm the values for each of the parameters and to indicate the formation of secondary structures which are undesirable. Finally, Chromas2 software (McCarthy 1996) was used to assess the quality of the sequencing primers and the regions of the microsatellites. All forward primers were labeled with M13 sequence (5'- TGTAAAACGACGGCCAGT-3') following a labeling protocol (Schuelke 2000).

The optimization of the microsatellites loci, conducted using six individuals per population, allowed the establishment of the optimal protocol and the appropriate temperature for each amplification primer. Microsatellite fragments were amplified using a MyCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a total reaction volume of 10 µL, containing 20 ng of genomic DNA template, 1 U *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 1X polymerase chain reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.08% Nonidet P40), 0.25 mM each dNTP, 1.5mM $MgCl_2$, 2.5 pmols of forward and M13 label primers (FAM, HEX or NED dyes) and 5 pmols of reverse primers. Polymerase chain reaction was carried out according to Schuelke (2000), consisting of 94 °C (5 min), then 30 cycles at 94 °C (30 s)/T_a °C (45 s) (T_a= annealing temperature (Table 1)/72 °C (45 s), followed by 8 cycles at 94 °C (30 s)/53 °C (45 s)/72 °C (45 s), and a final extension at 72 °C for 10 min. Allele sizes were determined using the ABI 3130xl Genetic Analyzer System (Applied Biosystems) at the Research Centre on the Human Genome and stem Cells of University of São Paulo. SSR patterns

were scored based on the size standard ROX GSx500 (Life Technologies Inc.) using GENEMAPPER software v4.0 (Applied Biosystems). After selecting the polymorphic primers, 24 individuals from each population were genotyped using the same laboratory facilities and equipment cited above.

Data analysis

Fixation index (F_{IS}), genotypic disequilibrium, number of alleles per locus (k), observed (H_o) and expected heterozygosity (H_e) at Hardy-Weinberg equilibrium for each locus and as an average across all loci were estimated using FSTAT (Goudet 2002). To test whether $F_{IS}$and linkage disequilibrium between pairwise loci were significantly different from zero, we used Monte Carlo permutations of alleles between individuals and a Bonferroni correction (95%; α= 0.05). A global *F*-statistics analysis was obtained using GenAlex software (Peakall and Smouse 2012). The parameter apparent outcrossing rate ($\hat{t}_a$) was estimated considering the equation $\hat{t}_a$ = [(1–F)/(1+F)] (Vencovsky 1994) for each population.

RESULTS AND DISCUSSION

Two hundred and eighty-eight positive clones from the library were sequenced. A set of 30 microsatellite primers were developed (Table S1). From these 30 loci, eight were found to be polymorphic (Table 1). A low to moderate genetic diversity was found within *T. pumilum* populations. The number of alleles per loci ranged from one to eight, with averages of 4.00, 4.12, 4.25 and 3.75 for AS, IRA, SM and SP populations, respectively (Table 2). For the total sample considering all populations, we found 43 different alleles, with four private alleles within populations (data not shown). H_o and H_e ranged from 0.238 to 0.333 and from 0.450 to 0.482, respectively; F_{IS} was significantly higher than zero for all populations, varying from 0.35 (SP) to 0.47 (SM), suggesting excess of homozygotes (Table 2). After Bonferroni correction, no genotypic disequilibrium was detected in the studied populations (data not shown). *F*-statistics (F_{IS} = 0.337; F_{ST} = 0.064; F_{IT} = 0.417) confirmed moderate levels of genetic structure, showing that most of the diversity is within populations.

T. pumilum apparently is not under threat or in danger of extinction (IUCN 2002). However, the four populations considered in our study, collected from fragmented forest areas in the State of São Paulo, showed low within genetic variability, similar to the findings of Pandey et al. (2015) with *Cypripedium kentuckiense,* a terrestrial orchid native to North America *(A* = 4.00; H_o = 0.436; H_e = 0.448), which might be indicative of the occurrence of evolutionary processes, such as population fragmentation, reduction in gene flow and genetic drift, leading to a decrease of genetic diversity. Higher levels of genetic diversity were found by Trapnell et al. (2015) evaluating five populations of the epiphytic *Dendrobium*

Table 1. Microsatellite loci developed for *Thichocentrum pumilum*, including the primer name, forward and reverse sequences, repeat motif, size range, annealing temperatures (Ta) and the GenBank registration number

Locus	Sequences 5´- 3´	Repeat Motif	Size Range (pb)	Ta (°C)	GenBank
TpuB06	F: CTAACCAGAGCTTCCGCTGT R: AGTCATCTGTCCGCTACCTG	$(TTC)_5$	302 -314	56	KT271869
TpuC04	F: TGCATTTCTCCATTTTCAGCC R: AGCTGACCCCACATAGTGC	$(AAC)_8$	277 -299	56	KT271870
TpuB7	F: GAAATCATCGTCGTCTTCTGC R: GTGGCGATTCTTCCATTGTTG	$(CT)_{23}$	125 -161	56	KT271875
TpuG7	F: GTTGTCCCCATGAAACCATTG R: TGCCACCACTTCACAGAACT	$(GT)_7$	165 -169	56	KT271881
TpuC3_2	F: GGGTTGGGGTAGTTCTCGTG R: CCGTTTGTTTCAGCTTTTCC	$(GTTT)_3$	299 -303	56	KT271888
TpuC8_2	F: CGCCTTACCTAGCAGTGACA R: GTATCACTCAAAAGCCACTCA	$(AC)_{13}$	286 -304	56	KT271889
TpuD12_2	F: CCTGAGCTAAAATGGGTTGC R: GCGTGGACTAGCAAGAAAAC	$(CA)_4(GA)_5$	147 - 141	56	KT271892
TpuH4_2	F: GTCCTTACGGTGACCTTTCT R: GCAGATAACCAACGAAAATGC	$(TTTC)_3$	218 - 220	58	KT271896

Table 2. Genetic diversity parameters in 24 individuals of *Trichocentrum pumilum* for each population using eight microsatellite loci, including the number of alleles (*k*), the observed (H_o) and expected heterozygosities (H_e), and the fixation index within populations (F_{IS})

	Alto da Serra				Iracemápolis			
Locus	*k*	H_o	H_e	F_{IS}	*k*	H_o	H_e	F_{IS}
TpuB06	2	0.167	0.160	-0.07	2	0.208	0.190	-0.09
TpuC04	7	0.772	0.760	-0.02	8	0.834	0.792	-0.05
TpuB7	8	0.318	0.880	0.64*	8	0.042	0.839	0.95*
TpuG7	3	0.150	0.620	0.76*	3	0.143	0.606	0.76*
TpuC3_2	3	0.374	0.630	0.41	3	0.524	0.629	0.16
TpuC8_2	6	0.416	0.460	0.08	5	0.381	0.376	-0.01
TpuD12_2	1	NE**	0.000	NE	2	1.000	0.130	-0.05
TpuH4_2	2	0.095	0.260	0.63*	2	0.091	0.245	0.62*
Mean	4.00	0.333	0.460	0.39*	4.12	0.318	0.476	0.38*
SD***	2.62	0.231	0.308	0.345	2.59	0.284	0.277	0.43
Total	32	-	-	-	33		-	-
	Santa Maria				**São Pedro**			
Locus	*k*	H_o	H_e	F_{IS}	*k*	H_o	H_e	F_{IS}
TpuB06	3	0.174	0.170	-0.05	2	0.170	0.082	-0.02
TpuC04	6	0.400	0.590	0.32	6	0.539	0.732	0.09
TpuB7	8	0.273	0.840	0.68*	8	0.292	0.841	0.65*
TpuG7	3	0.227	0.680	0.66*	3	0.266	0.635	0.60*
TpuC3_2	3	0.250	0.230	-0.10*	3	0.195	0.632	0.14
TpuC8_2	7	0.458	0.780	0.41	4	0.574	0.732	0.26
TpuD12_2	2	0.000	0.080	1.00*	2	0.085	0.082	-0.02
TpuH4_2	2	0.125	0.260	0.51*	2	0.088	0.121	0.66*
Mean	4.25	0.238	0.450	0.47*	3.75	0.276	0.482	0.35*
SD	2.38	0.146	0.300	0.40	2.19	0.188	0.327	0.30
Total	34	-	-	-	30	-	-	-

**P<0.05* after a Bonferroni correction for multiple tests
**NE: not estimated
***SD is the standard deviation

calamiforme from Australia (*A* = 3.6; H_o = 0.489; H_e = 0.591), Mallet et al. (2014) with 10 populations of the epiphytic *Jumellea rossii* (*A* = 9.2; H_o = 0.463; H_e = 0.750), Kartzinel et al. (2013) studying 12 populations of *Epidendrum firmum* (*A* = 12.4; H_o = 0.785; H_e = 0.834), as well as Pinheiro et al. (2013) studying populations of *E. denticulatum*, including a population from a neighboring town in our study (Itirapina/SP), with H_o ranging from 0.331 to 0.516, and H_e from 0.395 to 0.529, all with nuclear SSR markers.

The apparent outcrossing rate ($\hat{t}_a$) showed values below 0.60 for each *T. pumilum* population, averaging 0.508, which is an indication that this species presents a mixed mating system, which may explain the higher levels of fixation index and lower levels of genetic diversity. Similar values of apparent outcrossing rate ($\hat{t}_a$ =0.43) was found for *Cattleya walkeriana* (Tambarussi et al. 2015). Besides the mixed breeding system found for *T. pumilum*, population fragmentation may also be a factor leading to lower genetic diversity values, since small fragmented populations may suffer severe loss of genetic diversity due to reduction in gene flow and genetic drift, and may experience extinction of the population in the long-term (Hamrick and Godt 1989). These are still speculative ideas, but future studies must also explore the putative role of fragmented and non-fragmented forest areas related to the loss of genetic diversity that could be tested with an increased number of populations.

In conclusion, the eight polymorphic loci reported in this study have proven to be useful for population genetics studies in *T. pumilum*. This is the first genetic study on this species, showing important data related to the genetic diversity of four populations and a preliminary indication of its mixed reproductive system, which should be of use in conservation programs and for further studies with other *T. pumilum* populations.

ACKNOWLEDGEMENTS

The authors wish to thank FAPESP for scholarships, especially the one attributed to the first author, which was also used to finance this study (Grant #2013/13918-6). EAV was supported by a CNPq research fellowship. The authors wish to thank Patricia Dias Santos for the contribution in the primers development.

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