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# Development and characterization of microsatellite markers for *Tibouchina hatschbachii* (Melastomataceae), an endemic and habitat-restricted shrub from Brazil

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**ABSTRACT.** *Tibouchina hatschbachii* Wurdack (Melastomataceae) is an autogamous shrub restricted to granite (GO) and sandstone (SO) rock outcrops from subtropical Brazil. We designed primers for the amplification of microsatellite regions for *T. hatschbachii,* and characterized these primers to estimate genetic diversity parameters and contemporary genetic structure patterns. Eight loci were successfully amplified and were characterized using 70 individuals from three natural populations. Polymorphic information content ranged from 0.200 to 0.772 per locus. All loci were polymorphic, with allele numbers ranging from two to eight. The low degree of polymorphism may be explained by the fact that *T. hatschbachii* has disjunct populations and a recent genetic bottleneck, and also that it is self-pollinated. The observed and expected heterozygosities ranged from 0.115 to 1.000 and from 0.112 to 0.800, respectively. We observed private alleles in all loci. These are important features that enable us to identify population differentiation and help to us understand gene flow patterns for *T. hatschbachii* in subtropical Brazil. Eight microsatellite loci from other species of *Tibouchina* amplified positively in *T. hatschbachii*.

**Keywords:** Melastomataceae, *Tibouchina*, granite, sandstone, rock outcrops, population genetics, simple sequence repeat (SSR).

## Desenvolvimento e caracterização de marcadores microssatélites para *Tibouchina hatschbachii* (Melastomataceae), um arbusto endêmico e com restrição de habitat da região subtropical do Brasil

**RESUMO.** *Tibouchina hatschbachii* Wurdack (Melastomataceae) é um arbusto autógamo, com ocorrência restrita em afloramentos rochosos graníticos (GO) e areníticos (SO) na região subtropical do Brasil. Neste trabalho, foram desenvolvidos marcadores para a amplificação de regiões microssatélites para *T. hatschbachii* e caracterizados esses *primers* para estimar parâmetros de diversidade genética. Oito loci foram amplificados com sucesso e caracterizados, utilizando 70 indivíduos de três populações naturais. O conteúdo de informação polimórfica variou de 0,200 a 0,772 por locus. Todos os loci foram polimórficos, com números de alelos que variam de dois a oito. O baixo grau de polimorfismo pode ser explicado pelo fato de que *T. hatschbachii* possui populações disjuntas e uma história recente de gargalo genético populacional, e também pelo fato de apresentar um sistema reprodutivo de autopolinização, tendendo a favorecer a baixa variação. As heterozigosidades observadas e esperadas variaram entre 0,115-1,000 e 0,112-0,800, respectivamente. Também foi observada a presença de alelos privados em todos os loci. Estas são características importantes que nos permitirão identificar a diferenciação entre populações e poderão ajudar na compreensão dos padrões de fluxo gênico atual de *T. hatschbachii* na região subtropical do Brasil. Oito loci microssatélites de outras espécies de *Tibouchina* amplificaram positivamente em *T. hatschbachii*..

**Palavras-chave:** Melastomataceae, *Tibouchina*, granito, arenito, afloramentos rochosos, genética populacional, *Simple Sequence Repeat* (SSR).

## Introduction

Plant population genetic studies have increased substantially in the last years (Collevatti, Castro, Lima, & Telles, 2012; Pinheiro et al., 2014; Reis, Ciampo-Gullardi, Bajay, Souza, & Santos, 2015), mainly with studies on microsatellite loci (Sunnucks, 2000), which are thought to be selectively neutral (Schlötterer, 1998) and display considerable levels of polymorphism and variation.

*Tibouchina hatschbachii* (Melastomataceae) is a shrub endemic to subtropical grassland areas in Brazil. It occurs in granite (GO) and sandstone (SO) rock outcrops, respectively in the Atlantic rain forest and in the southern portions of the Brazilian "Cerrado" (Wurdack, 1963; 1984; Meyer, Guimarães, & Goldenberg, 2009). These plants produce many flowers that offer only pollen for their visitors, playing a crucial role as a pollen source for bees in these habitats (Maia, Varassin, & Goldenberg, 2016). A previous study found high levels of autogamy in one population, as a result of a self-compatible reproductive system (Maia et al., 2016). Phylogeographic studies using cpDNA markers have showed genetic and geographical structure with genetically distinct lineages, and a recent reduction of effective population size. However, the contemporary genetic structure patterns that may still be limiting gene flow between lineages remain to be assessed. Moreover, it is still necessary to identify differences in the interpopulation gene flow patterns related to geographical and ecological barriers in the Brazilian subtropical region; and to propose conservation measures considering that *T. hatschbachii* could undergo drastic habitat reduction due to global climatic changes. The development of primers to evaluate microsatellite loci (SSR) will be useful to address these evolutionary and ecological questions involving contemporary gene flow (Ellegren, 2004).

At present, 24 microsatellite loci are available for the genus *Tibouchina* (12 loci for *T. pulchra*, Brito, Vigna, & Souza, 2010; 12 loci for *T. papyrus*, Telles et al. 2011). Nevertheless, polymorphisms found for one species do not necessarily corresponds to a polymorphism found in loci from other species, even among phylogenetically related species (Ellegren, Primer, & Sheldon, 1995; Oliveira, Pádua, Zucchi, Vencovsky, & Vieira, 2006). This shows how important is the development of specific microsatellite loci for species focused in a study.

In this study, for the first time we isolated and characterized eight pairs of specific primers that amplify polymorphic microsatellite loci of *Tibouchina hatschbachii*, in order to study population genetic structure and gene exchange among populations. Furthermore, we tested cross-amplification (transferability) of 24 microssatelites loci developed for other *Tibouchina* species in *T. hatschbachii*.

## Material and methods

Leaves from 70 individuals from three natural populations of *T. hatschbachii* were sampled in Paraná and São Paulo States, in Brazil (Figure 1): 25 individuals from Buraco do Padre - BP (voucher *Maia, F.R. 102*, Municipality of Ponta Grossa, Paraná, 25°09'07.60"S, 49°54'21.68"W), 25 individuals from Piraí do Sul - PS (voucher *Maia, F.R. 85,* municipality of Piraí do Sul, Paraná, 24°46'70.28"S, 50°02'12.22"W) and 20 individuals from *Estação Ecológica da Barreira* - EEB (voucher *Maia, F.R. 94,* municipality of Itararé, São Paulo, 24°11'77.80"S, 49°36'31.94"W). The genomic DNA was extracted from macerated leaves following the standard 2% CTAB protocol (Doyle & Doyle, 1990).

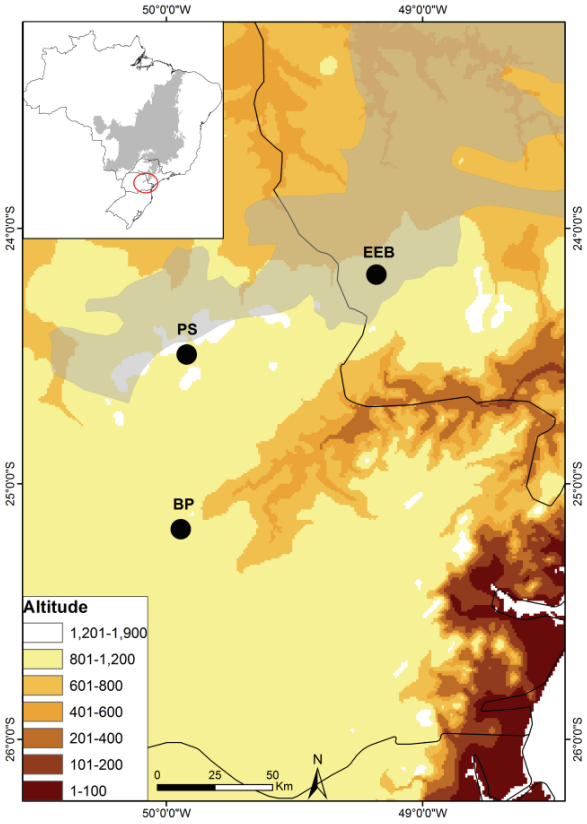


**Figure 1.** Map with the geographic location of *Tibouchina hatschbachii* populations sampled in the Brazilian subtropical region. BP = Buraco do Padre; PS = Piraí do Sul; EEB = Estação Ecológica da Barreira.

A microsatellite-enriched genomic library was constructed following specific protocol (Billotte, Lagoda, Risterucci, & Baurens, 1999), with a few modifications, using DNA from one individual from PS population. Genomic DNA was digested with Rsa I enzyme (Invitrogen, Carlsbad, California, USA) in incubation at 37°C for 3 hours, and the digested fragments were enriched in microsatellite fragments using $(CT)_8$ and $(GT)_8$ motifs. Digested fragments were ligated to the double-stranded AfaI adapters Afa21 (5′-CTCTTGCTTACGCGTGGACTA-3′) and Afa25

(5′-TAGTCCACGCGTA AGCAAGAGCACA-3′) and incubated for 2 hours at 20°C. Hybridized DNA was captured by streptavidin-coated magnetic probe beads (MagneSphere Magnetic Separation Products, Promega Corporation, Madison, Wisconsin, USA). The enriched fragments were amplified by polymerase chain reaction (PCR), the product was cloned into pGEM -T Easy Vector (Promega Corporation), and ligation products were used to transform Epicurian Coli XL1-Blue *Escherichia coli* – competent cells (Stratagene, Agilent Technologies, Santa Clara, California, USA).

A total of 95 positive clones were sequenced using the universal T7 primer combination and a BigDye v3.1 terminator kit on an ABI3730 DNA Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA). The selection of sequences containing microsatellites was performed using WebSat (Martins, Lucas, Neves, & Bertioli, 2009). Primers were designed using PRIMER3 (Untergasser et al., 2007) according to the following criteria: size of primers preferably between 18 and 22 bp, melting temperature (Tm) between 45°C and 60°C, amplified product length between 100 and 300 bp, and GC content between 40% and 60%.

The amplification was performed with a forward primer synthesized with a 19 bp M13 tail (5'-CACGACGTTGTAAAACGAC-3'; Schuelke, 2000), a reverse locus-specific primer, and a universal M13 primer labeled with the fluorescent dyes FAM or HEX (Applied Biosystems). All PCR amplifications were performed in 25 µL volumes containing 20-50 ng DNA template, PCR buffer1x (10 mM Tris-HCl, pH 8.3; 50 mM KCl), primer forward (0.8 µM), primer reverse (0.8 µM), universal M13 (0.8 µM), $MgCl_2$ (1.5 mM), dNTP (0.3 mM) and 1 U of *Taq*DNA polymerase (Invitrogen Plat). The PCR program for all loci consisted of 1 min of initial denaturation at 96°C followed by 30 cycles of denaturation at 95°C for 1 min, a primer-specific annealing temperature (50 to 56°C) for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Amplification products were verified with electrophoresis on 3% agarose gels with 0.1 mg/ml of ethidium bromide in 1 × TBE buffer (89 mM Tris–borate, 2 mM EDTA, pH 8.0) and sent to Macrogen sequencing service (Korea). Samples were automatically genotyped with the software GeneMarker2.4 (SoftGenetics).

The CERVUS program was used to calculate the polymorphic information content (PIC) (Kalinowski, Taper & Marshall, 2007). The number of alleles per locus ($N_A$), fixation index ($F$), observed ($H_O$) and expected heterozygosities ($H_E$), and the respective confidence intervals were estimated with diveRsity package (diveRsity package; Keenan, McGinnity, Cross, Crozier, & Prodöhl, 2013) in R (R Core Team, 2015). Confidence intervals were calculated using 1000 bootstraps, with resampling loci. Exact tests for departure from Hardy-Weinberg Equilibrium (HWE) using the Markov-chain test, and tests for linkage disequilibrium (LD) using a likelihood-ratio test were performed using the Arlequin version 3.5 software (Excoffier & Lischer, 2010), and corrected for multiple comparisons using a sequential Bonferroni correction (95%, $\alpha = 0.05$). Private alleles were estimated using the GeneAlex 6.3 (Peakall & Smouse, 2012).

Twelve microsatellite loci developed for *T. pulchra* (Brito et al., 2010) and other twelve for *T. papyrus* (Telles et al., 2011) were tested in *T. hatschbachii*. The PCR conditions were performed as previously described, but with an annealing temperature of 60°C for all tested primers. The amplifications were visualized in 3% agarose gels. The loci were considered successfully amplified when at least one band of the expected size was observed. A 100bp DNA ladder (Promega) was used as molecular size marker.

## Results and discussion

From the 95 recombinant colonies sequenced, 16 clones contained simple sequence repeats, of which eight presented proper flanking regions for primer design (Table 1). Polymorphic information content ranged from 0.200 to 0.772 per locus (average 0.492) and all markers were informative (Table 1). The highest PIC value was found in the That02 and That04 loci, which contained the most alleles (Table 1). All eight designed primer pairs were polymorphic at the analyzed populations (Table 2). Within the populations of *Tibouchina hatschbachii*, the $N_A$ per locus ranged from two to eight, the mean $N_A$ per locus was 3.812 and $H_O$ and $H_E$ varied, respectively, from 0.115 to 1.000 and from 0.112 to 0.800 (Table 2). We also suspect that the low heterozygosity levels may be related to high levels of autogamy found in populations of these species (Frankham, Ballou, & Briscoe, 2008; Silva-Arias, Mäder, Bonato, & Freitas, 2015; Maia et al., 2016). In addition, endemic species with an aggregated distribution pattern tend to have low heterozygosities (Gitzendanner & Soltis, 2000), since they suffer greater influence of genetic drift and inbreeding. In fact, the low diversity values were similar to those found for *T. papyrus* ($N_A$ per locus ranged from one to six), a congeneric and endemic species of "rock fields" ("campos rupestres") in the Brazilian Cerrado (Telles et al. 2011).

**Table 1** Characterization of eight microsatellite loci for *Tibouchina hatschbachii*[1].

| Locus | Repeat motif | Primer sequences (5′ – 3′) | Size (bp)[2] | Ta (°C)[3] | $N_A$ | PIC | GenBank |
|---|---|---|---|---|---|---|---|
| That01 | $(GAAAA)_3$ | F: TTTAGTTGCCACCTCATGACC | 194 | 60 | 5 | 0.465 | KU060776 |
| | | R: GCTCAGAGCCTTGGTAGCTT | | | | | |
| That02 | $(GAT)_4$ | F: GACATTCGCGAACTCCATTT | 180 | 60 | 8 | 0.772 | KU060777 |
| | | R: TCATAAGTTTGTCGAGTCTCC | | | | | |
| That03 | $(TTA)_4$ | F: TTCTCCAAACATCAATGCACA | 221 | 58 | 5 | 0.583 | KU060778 |
| | | R: AAATCGTTTGTTTGGCTTCG | | | | | |
| That04 | $(AAC)_7$ | F: ATCAAAATGGGTCAGGTCCA | 199 | 58 | 9 | 0.712 | KU060779 |
| | | R: AAGTGTGCCGTGCGTGAG | | | | | |
| That05 | $(TCG)_4$ | F: CGTGCTGGTCATTGTCATCT | 199 | 66 | 6 | 0.401 | KU060780 |
| | | R: AGGATGAAAGCGAAGGTGAA | | | | | |
| That06 | $(TGAA)_3$ | F: TCTTTCGGGAATGAAATAATCG | 181 | 56 | 3 | 0.200 | KU060781 |
| | | R: TGAAATGGTGGAAAATTTGGA | | | | | |
| That07 | $(TAAA)_4$ | F: TGGGATTTGGAAACCTTGTC | 187 | 58 | 6 | 0.409 | KU060782 |
| | | R: GTCAAGGCCGACAAATATGAA | | | | | |
| That08 | $(TA)_5$ | F: GACATTGGACTGATCCGACA | 199 | 56 | 2 | 0.396 | KU060783 |
| | | R: CGCAATGATTTTGGATGACA | | | | | |

[1] All values are based on 70 samples from three populations of *Tibouchina hatschbachii*; [2] Fragment sizes does not include the M13 tail (5′-CACGACGTTGTAAAACGAC-3′) attached to the forward primer; [3] Ta = annealing temperature; $N_A$ = number of alleles per locus; PIC = polymorphic information content;

**Table 2.** Results of initial polymorphic microsatellite marker screening in three populations (70 individuals) of *Tibouchina hatschbachii*.

| | BP (n=25) | | | | PS (n=25) | | | | EEB (n=20) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | $N_A$ | $H_O$ | $H_E$ | $F$ | $N_A$ | $H_O$ | $H_E$ | $F$ | $N_A$ | $H_O$ | $H_E$ | $F$ |
| That01 | 4 | 0.560 | 0.633 | 0.115★ | 3 | 0.310 | 0.272 | -0.133 | 3 | 0.381 | 0.430 | 0.300 |
| That02 | 6 | 0.280 | 0.672 | 0.583★ | 7 | 0.423 | 0.785 | 0.461★ | 6 | 0.200 | 0.750 | 0.734★ |
| That03 | 3 | 0.880 | 0.586 | -0.500 | 2 | 1.000 | 0.500 | -1.000★ | 4 | 0.810 | 0.650 | -0.233 |
| That04 | 4 | 0.760 | 0.650 | -0.168 | 6 | 0.269 | 0.369 | 0.269 | 8 | 0.600 | 0.800 | 0.247★ |
| That05 | 2 | 0.320 | 0.480 | 0.333 | 3 | 0.577 | 0.567 | -0.016 | 5 | 0.250 | 0.380 | 0.350★ |
| That06 | 2 | 0.240 | 0.211 | -0.136 | 2 | 0.308 | 0.261 | -0.181 | 2 | 0.450 | 0.350 | -0.290 |
| That07 | 3 | 0.440 | 0.362 | -0.214 | 4 | 0.115 | 0.112 | -0.040 | 2 | 0.350 | 0.400 | 0.122 |
| That08 | 2 | 0.800 | 0.493 | -0.623 | 2 | 0.923 | 0.497 | -0.857★ | 2 | 0.900 | 0.490 | -0.818★ |
| Mean | 3.250 | 0.535 | 0.520 | -0.161★ | 3.625 | 0.490 | 0.420 | -0.187★ | 4 | 0.493 | 0.530 | 0.095★ |

*Note*: $N_A$ = number of alleles per locus in each population; $F$ = fixation index; $H_E$ = expected heterozygosity; $H_O$ = observed heterozygosity; $N$=sample size for each population; BP: Buraco do Padre; PS: Piraí do Sul; EEB: Estação Ecológica da Barreira; ★ Deviations from HWE were not statistically significant (ns) or were statistically significant at $p \leq 0.001$. .

The disjunct distribution can also be another factor acting on the genetic diversity (Telles et al., 2011).

On the other hand, the allelic richness found in microsatellite loci of *T. hatschbachii* (ranging from two to eight alleles) was smaller than the one found for *T. pulchra* (ranging from 4 to 31 alleles; Brito et al., 2010). We believe that this may result from different distribution patterns found for these species. While *T. hatschbachii* has a restrict and disjunt distribution that may reduce genetic diversity (as shown above), *T. pulchra* has a broad and continuous distribution along the Atlantic Forest (Meyer, Guimarães, & Goldenberg, 2010), which reduces the effects of the genetic drive and endogamy on these populations.

Except for That06 and That07, all loci deviated from HWE in one or all populations, especially in EEB population, in which four out of eight loci showed this deviation, most likely due to deficiency in heterozygotes found here. No LD was detected between pairs of loci.

Eleven private alleles were found in all the loci (four in the PS population and seven in the EEB population) showing that the two populations are differentiating. These results may explain the divergent lineages found for *Tibouchina hatschbachii*, but this needs to be tested on a larger sample. Anyway, these are important features that enable us to identify population differentiation (Collevatti et al., 2012; Reis et al., 2015) and can help to us understand contemporary gene flow patterns between genetic lineages identified for *T. hatschbachii*.

Cross-species amplification tests revealed six microsatellite loci (50%) from *T. pulchra* (TP01; TP03; TP05; TP25; TP27 and TP33) and two loci (16,66%) from *T. papyrus* (TPAP 16 and TPAP17) that amplified satisfactorily in *T. hatschbachii*, indicating that these loci can also be useful for studies on population genetics for other *Tibouchina* species. However, we did not test polymorphisms for these markers, which means that many of them can be monomorphic and consequently with no use in these studies. The transferability success can also be a consequence of low divergence in DNA sequences among these *Tibouchina* species, especially with species *T. pulchra* (50% positive amplification).

This suggests that some markers may be readily transferable between species of the same genus or family (Ellegren et al., 1995; Oliveira et al., 2006; Barbará et al., 2007).

## Conclusion

The eight polymorphic loci isolated for *T. hatschbachii* proved to be useful for studying genetic diversity of this species and will be used to test hypotheses regarding the maintenance of barriers to gene exchange among genetic lineages of *T. hatschbachii*, that were shown in previous phylogeographic studies using cpDNA markers. Such markers will provide not only knowledge on the genetic diversity and population genetic structure for this species, but also genetic conservation of these stocks in the Brazilian subtropical region.

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