GENETIC DIVERSITY BETWEEN AND WITHIN POPULATIONS OF *Handroanthus heptaphyllus* (VELL.) MATTOS USING MICROSATELLITE MARKERS[1]

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ABSTRACT: *Handroanthus heptaphyllus* (Vell.) Mattos, popularly known as ipê-roxo, is a species of the family Bignoneaceae much appreciated for its beauty, excellent quality wood which is used for making medicinal products and also in reforestation programs of degraded areas, as well as landscaping and restoration. The aim of this study was to investigate the genetic diversity between and within populations of *H. heptaphyllus* using microsatellite markers. The 192 seedlings were produced from seeds collected on 30 trees into the two populations of natural forest fragments in Botucatu region, São Paulo, Brazil. Eight microsatellite loci were analyzed, with allelic polymorphism varying from six alleles for locus TAU22 to 14 alleles for loci TAU12, TAU30, and TAU31, with an expected mean number of alleles per locus ($\hat{A}_e$) of 4.9. The mean expected heterozygosity ($\hat{H}_e$) for the two populations was 0.785, the mean observed heterozygosity ($\hat{H}_o$) was 0.609, and the fixation index ($\hat{F}$) was low between populations, with a mean of 0.222. The gene differentiation between the two populations ($\hat{G}_{ST}$') was 0.100. We concluded that the higher genetic diversity is within populations; therefore, as far as germplasm collection programs in Botucatu region are concerned, it is recommended that a larger sampling of individuals should be considered within populations, thereby providing good genetic representativeness. The populations have enough genetic diversity to support genetic improvement and germplasm preservation programs.

Key words: Germplasm, molecular markers, genetic diversity.

DIVERSIDADE GENÉTICA ENTRE E DENTRO DE POPULAÇÕES DE *Handroanthus heptaphyllus* (VELL.) MATTOS POR MARCADORES MICROSSATÉLITES

RESUMO: *Handroanthus heptaphyllus (Vell.) Mattos, popularmente conhecida por ipê-roxo, é uma espécie pertencente à família Bignoneaceae, muito apreciada por sua beleza, madeira de excelente qualidade e utilizada em produtos medicinais e programas de reflorestamento de áreas degradadas, paisagismo e restauração. Neste trabalho, objetivou-se estudar a diversidade genética entre e dentro das populações de H. heptaphyllus por meio de marcadores microssatélites. Foram estudadas 192 plântulas, formadas a partir de sementes colhidas de duas populações, em um total de 30 árvores, de fragmentos florestais naturais na região de Botucatu - SP. Foram analisados oito locos microssatélites, com polimorfismo alélico, variando de seis alelos para o loco TAU22 a 14 alelos para os locos TAU12, TAU30 e TAU31, com número efetivo médio de alelos por loco* ($\hat{A}_e$) *igual a 4,9. As médias para a heterozigosidade esperada* ($\hat{H}_e$)*, para as duas populações foi de 0,785, a heterozigosidade observada* ($\hat{H}_o$) *foi de 0,609 e o índice de fixação* ($\hat{F}$) *variou pouco entre as populações, com média de 0,222. O valor médio da divergência genética entre as duas populações* ($\hat{G}_{ST}$'*) foi de 0,100. Conclui-se que a maior diversidade genética ocorre dentro das populações; portanto, em programa de coleta de germoplasma, para a região de Botucatu, é recomendado realizar uma maior amostragem de indivíduos dentro das populações, o que possibilitaria uma boa representatividade genética. As populações estudadas possuem diversidade genética para subsidiar programas de melhoramento genético e conservação de germoplasma.*

Palavras-chave: Germoplasma, marcadores moleculares, diversidade genética.

1 INTRODUCTION

The fragmentation of forests by explorers and the urbanization advent have both had a tremendous impact on biodiversity, upsetting the balance of ecosystems, isolating habitats and changing the behavioral pattern of both fauna and flora by disrupting gene flow between fragments (KAGEYAMA et al., 2003). As the result, the genetic diversity of forest tree species has been decreasing.

Typically, *Handroanthus heptaphyllus* (Velloso) Mattos occurs in areas of rain forests. Commonly known as ipê-roxo, the species has become increasingly popular

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for several environmental and economic issues. The bark is widely used for making medicinal syrups and the wood is hugely popular for its high economic value. It has also been used in recovery and restoration programs on degraded areas, given the decreasing occurrence of this species in natural habitats (ETTORI et al., 1996), being also used as an ornamental plant in urban public squares and for restoring riparian vegetation, since the species develops well on soils adjacent to watercourses (MOREIRA; SOUZA, 1987).

According to Jankowsky et al. (1990), the species of genus *Handroanthus* provide hard and heavy wood with low retractibility and density about 1,070 kg.m^{-3}; the wood is dark and has pale sapwood (PAULA; ALVES, 1997). Those characteristics have stirred the interest of timber companies and as a consequence the exploration has led to a large decrease in its natural occurrence. However, the species does not reproduce easily in natural environments and seed viability is very low under natural conditions (VIEIRA et al., 2010).

Despite the predatory exploration, the use of the species on a commercial scale is very limited and its seeds are still being collected from natural populations. So far, no genetic improvement programs have been created to offer improved seeds, whether for commercial purposes or even for creation of seed orchards.

In order to use a particular species for reforestation, conservation, or improvement programs, it is important to study its mating system and the genetic structure of populations through molecular markers, to know how genetic diversity is distributed within and between populations. It can help to acquire the necessary knowledge to ensure suitable population management, helping to stablish strategies to support genetic improvement and germplasm preservation programs (BROWN, 1978).

The seasonal semi deciduous forest is one of the forest subtypes that compose the Atlantic forest biome. Today, the total area of remnants of that type of forest in São Paulo State corresponds to less than 5% of the total area by the late nineteenth century (RAMOS et al., 2007) and *H. heptaphyllus* is a representative species of that vegetation. Therefore, the objective of the study was to investigate the genetic diversity between and within populations of *H. heptaphyllus* using microsatellite markers, looking to support genetic resource development and germplasm preservation programs for the species in region strongly affected by the impacts of forest fragmentation.

2 MATERIAL AND METHODS

2.1 Study site

The research was conducted in the municipality of Botucatu-SP, Brazil, in the region of Médio Tietê Basin. Due to heavy urbanization, the studied region presents many small forest fragments (Figure 1).

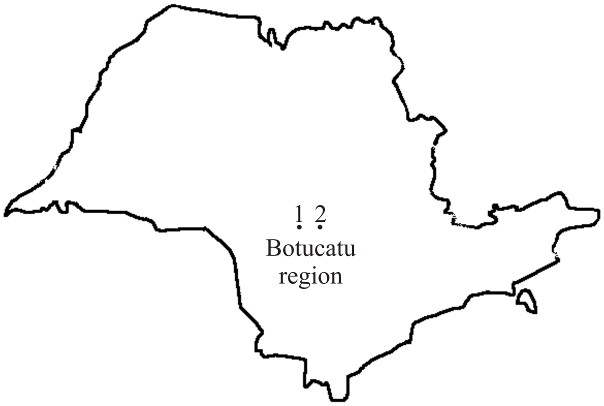

Figure 1 – Local of two studied *Handroanthus heptaphyllus* populations, in Botucatu region, São Paulo State, Brazil.

Figura 1 – *Localização das duas populações estudadas de Handroanthus heptaphyllus na região de Botucatu – SP.*

Two populations were demarcated in those forest fragments: population 1 (Lageado) consisted of 115 seedlings originated from 15 mother trees while population 2 (Indiana) was composed of 77 seedlings originated from 15 mother trees. The idea was to depict the representiveness occurrence of the species in the region, considering locally its low population density.

2.2 Microsatellite analysis

For each individual, three leaflets were collected from adult leaves and then stored at room temperature in a dry environment. For DNA extraction, 150 mg of plant material were used per sample. The protocol proposed by Ferreira and Grattapaglia (1995) was used. The primers were transferred from *Handroanthus aureus*, as developed by Braga et al. (2007), considering their respective annealing temperatures, through PCR procedures, for replication of DNA fragments (Table 1). The DNA fragments were separated by 6% bis/acrylamide gels. The gel was developed using nitrate of silver, according to the procedure described by Creste et al. (2001).

Table 1 – Primers developed by Braga et al. (2007) and transferred to *Handroanthus heptaphyllus*, with their respective annealing temperatures.

Tabela 1 *– Primers, desenvolvidos por Braga et al. (2007), que foram transferidos para Handroanthus heptaphyllus, com suas respectivas temperaturas de anelamento.*

Primer	*Primer* sequence	Annealing temperature (°C)
Tau 12	F: CATCATCAACGTCAAGATCA R: CATTCTAGTCTTCCATAATAAGT	56
Tau 14	F: GGTAACGGATTGCTGGTTGT R: CATTGCGAATGGCCTATGGT	56
Tau 15	F: TTTGAGGGGTTGAAGCATTT R: CATTGTGGTCCCTCAACA	56
Tau 21	F: CTTTTGGGGGTCTTTGGAAT R: TGAAAGAGACAGAGACAAAGATACA	56
Tau 22	F: TATCTCTCCGCCGTACACCT R: CCAATCGAAGAGCCCATTTA	56
Tau 27	F: GGTAAATCATCTTCCGCTTCC R: ACTGCAGAATCGCCTTTTGT	56
Tau 30	F: TAGTTTAAGGGTGCCGTTGG R: CGAACATAAAGAGGCAACCCA	55
Tau 31	F: TCGTGCAGCTTTTGAGTCTG R: CTGCAAAACACAAAGCGAAA	57

2.3 Data analysis

Based on zymograms, the following parameters were estimated: allele frequencies ($\hat{p}_i$), mean number of alleles per locus ($\hat{A}$), derived from the arithmetic average of loci; expected number of alleles per locus ($\hat{A}_e = \frac{1}{1-\hat{H}_e}$) Nei (1978); observed heterozygosity ($\hat{H}_o = 1-\sum P_{ii}$); expected heterozygosity ($\hat{H}_e = 1-\sum p_i^2$) and fixation index ($\hat{F} = 1-\frac{\hat{H}_o}{\hat{H}_e}$), according to Weir (1996). The genetic structure between populations was derived from statistics proposed by Nei (1978), where: $\hat{H}_T$ is total gene diversity; $\hat{G}_{ST}$ is coefficient of gene diferentiation between populations and $\hat{H}_S$ is gene diversity within populations, using FSTAT software (GOUDET, 2002). The estimation of $\hat{G}_{ST}$, however, was standardized $\hat{G}'_{ST}$, according to Hedrick (2005), and presented as follow:

$$\hat{G}'_{ST} = \frac{\hat{G}_{ST}\left(1+\hat{H}_S\right)}{\left(1-\hat{H}_S\right)}.$$

3 RESULTS AND DISCUSSION

3.1 Genetic diversity and fixation index

Eight loci in two populations of *H. heptaphyllus* were found to be all polymorphic. A total of 63 alleles were found and the expected number of alleles per locus ($\hat{A}_e$) ranged from 3.4 (TAU 21) to 6.7 (TAU 14) in the population 1, and from 3.1 (TAU 30) to 8.0 (TAU 31) in the population 2, with mean value of 4.9 between populations (Table 2). Estimates of this parameter in other recent studies were found to range from 2.9 (MENDES, 2009) for *Cedrela fissilis* to 10.11 alleles (SILVA, 2010) for *Tabebuia aurea*.

The observed heterozygosity ($\hat{H}_o$) ranged from 0.461 (TAU 22, population 2) to 0.729 (TAU 31, population 2), while the expected heterozygosity ($\hat{H}_e$) ranged from a minimum of 0.673 (TAU 30, population 2) to a maximum of 0.875 (TAU 31, population 2), corresponding to a mean of 0.609 ($\hat{H}_o$) and 0.785 ($\hat{H}_e$) (Table 2). These mean estimates are higher than the results found by Mendes (2009) for *Cedrela fissilis* (0.566 and 0.646) and by Viégas (2009) for *Myracrodruon urundeuva*

Table 2 – Expected mean number of alleles per locus ($\hat{A}_e$), observed heterozygosity ($\hat{H}_o$), expected heterozygosity ($\hat{H}_e$) and fixation index ($\hat{F}$) for eight loci, in two populations of *Handroanthus heptaphyllus*.

***Tabela 2** – Número médio de alelos por loco ($\hat{A}_e$), heterozigosidade observada ($\hat{H}_o$), heterozigosidade esperada ($\hat{H}_e$) e índice de fixação ($\hat{F}$) para os oito locos e duas populações de Handroanthus heptaphyllus.*

Population	Locus	$\hat{A}_e$	$\hat{H}_o$	$\hat{H}_e$	$\hat{F}$
1	TAU 12	6.1	0.699	0.836	0.164
	TAU 14	6.7	0.666	0.851	0.217
	TAU 15	4.2	0.583	0.762	0.235
	TAU 21	3.4	0.567	0.702	0.192
	TAU 22	4.4	0.558	0.771	0.276
	TAU 27	4.9	0.667	0.797	0.163
	TAU 30	3.5	0.509	0.714	0.287
	TAU 31	5.7	0.632	0.823	0.232
	Mean	4.7	0.610	0.782	0.221
2	TAU 12	5.8	0.610	0.826	0.262
	TAU 14	5.3	0.667	0.811	0.178
	TAU 15	6.2	0.545	0.838	0.350
	TAU 21	3.3	0.616	0.694	0.112
	TAU 22	4.7	0.461	0.787	0.414
	TAU 27	5.0	0.603	0.798	0.244
	TAU 30	3.1	0.634	0.673	0.058
	TAU 31	8.0	0.729	0.875	0.167
	Mean	5.1	0.608	0.788	0.223
Total mean		4.9	0.609	0.785	0.222

(0.601 and 0.713), yet lower than results found by Silva (2010) for *Tabebuia aurea* (0.627 and 0,881), by Carvalho (2009) for *Copaifera langsdorffii* (0,757 and 0,893), and by Tarazi (2009) also for *Copaifera langsdorffii* (0,814 and 0,876), respectively for $\hat{H}_o$ and $\hat{H}_e$. In a study describing primers for *Tabebuia aurea*, Braga et al. (2007) obtained 0.913 for expected heterozygosity, which is above the mean value found in this work (0.785), and 0.578 for observed heterozygosity, which is below the mean value found at the present study (0.609).

The fixation index ($\hat{F}$) followed the same trend with a wider range of variation in population 2, with a minimum of 0.058 (TAU 30) and a maximum of 0.414 (TAU 22), and a mean of 0.222 when both populations were considered, suggesting deviations on the Hardy-Weinberg equilibrium due to homozygous excess, probably due to inbreeding. If compared with data of some tropical species literature, the mean estimate of $\hat{F}$ (0.222) is higher than the results found by Tarazi (2009) for *Copaifera langsdorffii* (0.071), by Carvalho (2009) also for *Copaifera langsdorffii* (0.152), and by Viegás (2009) for *Myracrouon urundeuva* (0.210), yet it is lower than results found by Mendes (2009) for *Cedrela fissilis* (0.250), by Silva (2010) for *Tabebuia aurea* (0.294), and by Braga et al. (2007) also for *Tabebuia aurea* (0.367).

Based on the estimated parameters ($\hat{A}_e$, $\hat{H}_o$ and $\hat{H}_e$) to help identify genetic diversity, and the fixation index ($\hat{F}$), it can be inferred that population 2 had slight superiority in the mean values of loci and a wider range of variation for those parameters. However, considering the size of those populations and the conditions of fragmentation there are an increase of inbreeding levels than expected over time as a function of likely relatedness of generations, potentially decreasing heterozygous and showing higher structuring within populations. Therefore, effective measures should be taken in order to prevent these populations from remaining isolated and to consequently avoid genetic diversity to decrease for generations. This issue was discussed extensively in studies by Bittencourt and Sebbenn (2007), Gaino et al. (2010), and Sebbenn et al. (2011), on *Araucaria angustifolia*, *Myracroduon urundeuva* and *Copaifera langsdorffii* respectively.

3.2 Genetic structure between and within populations

Results of total gene diversity ($\hat{H}_T$ = 0.794 or 100%) show higher diversity within populations ($\hat{H}_s$ = 0.785 or 98.8%) and lower gene diversity ($\hat{D}_{ST}$ = 0.09 or 1.2%) between populations. Considering only $\hat{G}_{ST}$, low variation was found between loci, the minimum gene diversity of 0.3% (TAU 27) and the maximum of 2.8% (TAU 14) were found. However, when the $\hat{G}'_{ST}$ proposed by Hedrick (2005) was used, the variation goes from 2.7% (TAU 27) to 30.3% (TAU 14), which is a considerable estimated value. Being more consistent and accurate parameter, $\hat{G}'_{ST}$ was the choice for discussion, indicating a mean variation of 10% between populations, which is something to be considered inasmuch as the presence or absence of alleles is concerned in either population (Table 3). This variation between the populations was becoming evident when studying the genetic diversity and fixation index parameters, in which a higher range of variation was found in population 2. It is interesting the use of $\hat{G}'_{ST}$ statistic for that dispersion measure.

Table 3 – Gene diversities: total ($\hat{H}_T$), within ($\hat{H}_s$) and between populations ($\hat{D}_{ST}$), and the statistics $\hat{G}_{ST}$ of Hedrick (2005) and Nei (1978) between populations of *Handroanthus heptaphyllus*, for eight microsatellite loci, in Botucatu region, SP, Brazil.

Tabela 3 – *Diversidades gênicas: total ($\hat{H}_T$), dentro ($\hat{H}_s$) e entre populações ($\hat{D}_{ST}$), como também as estatísticas $\hat{G}_{ST}$ de Hedrick (2005) e Nei (1978) entre as populações de Handroanthus heptaphyllus, para os oito locos microssatélites, na região de Botucatu, SP.*

Locus	$\hat{H}_s$	$\hat{H}_T$	$\hat{D}_{ST}$	$\hat{G}_{ST}$	
				Nei	Hedrick
TAU 12	0.831	0.838	0.007	0.008	0.087
TAU 14	0.831	0.855	0.024	0.028	0.303
TAU 15	0.800	0.811	0.011	0.013	0.117
TAU 21	0.698	0.701	0.003	0.004	0.022
TAU 22	0.779	0.789	0.010	0.012	0.097
TAU 27	0.797	0.800	0.003	0.003	0.027
TAU 30	0.694	0.703	0.009	0.013	0.072
TAU 31	0.849	0.858	0.009	0.011	0.135
All	0.785	0.794	0.009	0.012	0.100

Another factor that supports $\hat{G}'_{ST}$ statistic is the study of Hamrick (1983) with wind-dispersed seeds of 38 tree species. Although the author worked with isoenzymes, the estimated gene differentiation between populations ($\hat{G}_{ST}$) was 0.056 or 5.6%, which is higher than the present result for *H. heptaphyllus* populations (1.2%). However, when estimating $\hat{G}'_{ST}$ statistic, according to Hamrick (1983), the value was 0.093 or 9.3%, close to the estimate for *H. heptaphyllus* (0.100 or 10%) at the present study.

Populations of *Tabebuia aurea* were studied by Silva (2010) in Assis-SP, Pedregulho-SP, Selvíria-MS and Três Lagoas-MS, Brazil, who found a $\hat{G}_{ST}$ mean of 0.025 or 2.5% and a mean $\hat{G}'_{ST}$ of 0.444 or 44.4%. Estimates above 10% for $\hat{G}_{ST}$ were also found by Viegás (2009) between two populations of *Myracrodruon urundeuva* (Aramina-SP and Selvíria-MS, Brazil): 15.9% (pollen) and 23.5% (ovule). This high gene differentiation between populations was expected because of the geographic distance between them, obstructing the gene flow. Isolation and differing historical events in each population, therefore, result in higher gene differentiation, as opposed to geographic proximity which facilitates gene exchange (VIÉGAS, 2009), as is the statement of two populations of *H. heptaphyllus* in Botucatu region.

3.3 Seed collection for genetic resource development

One of the objectives of this study was to create conditions to support germplasm preservation programs for *H. heptaphyllus*, in Botucatu region, São Paulo, Brazil, area that is strongly affected by the impacts of forest fragmentation. According to Sebbenn (2002, 2003, 2006), an estimated number of trees for seed collection intended for forest tree improvement would be 29 to 76, with an average of 45. Therefore, even if collecting from both populations, only 30 trees are found in the study region, which is below the average considered optimal and just one tree above the minimum considered reasonable. It gives an idea on deforestation level of the studied region, which makes seed collection virtually impossible for establishment of seed orchards, since that would require 44 to 114 unrelated trees, with an average of 67 parent trees. To stablish seed orchard it is necessary a gene enrichment from the other preferably neighbor regions.

4 CONCLUSIONS

The populations of *H. heptaphyllus* have shown allelic polymorphism in all eight microsatellite loci.

The species genetic diversity is compatible with results from other tropical tree species. The existing genetic diversity is nonetheless sufficient to support genetic improvement and germplasm conservation programs for the species. However, there are signs of inbreeding as a result of forest fragmentation in Botucatu region, Brazil.

High genetic variation was found within populations, suggesting that a larger sampling of individuals is required, however the divergence between populations cannot be ignored.

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