Development and characterization of microsatellite loci for Cedrela fissilis Vell (Meliaceae), an endangered tropical tree species

Article *in* Silvae Genetica · December 2014

DOI: 10.1515/sg-2014-0031

CITATIONS
9

READS
101

9 authors, including:

Flávio B. Gandara
University of São Paulo
39 PUBLICATIONS **554** CITATIONS

SEE PROFILE

Evandro Vagner Tambarussi
Universidade Estadual do Centro-Oeste do Paraná (UNICENTRO)
121 PUBLICATIONS **513** CITATIONS

SEE PROFILE

Alexandre Magno Sebbenn
Instituto Florestal - Governo do Estado de São Paulo
400 PUBLICATIONS **4,187** CITATIONS

SEE PROFILE

Maria Andréia Moreno
University of São Paulo
19 PUBLICATIONS **136** CITATIONS

SEE PROFILE

Development and characterization of microsatellite loci for *Cedrela fissilis* Vell (Meliaceae), an endangered tropical tree species

By F. B. Gandara[1)], E. V. Tambarussi[2)], A. M. Sebbenn[3),*)], E. M. Ferraz[1)], M. A. Moreno[1)], A. Y. Ciampi[4)], R. P. Vianello[5)], D. Grattapaglia[4)] and P. Y. Kageyama[1)]

(Received 13th March 2015)

Abstract

The timber of the Neotropical tree *Cedrela fissilis* is used in construction, shipbuilding, carpentry and for medical purposes. In this studypolymorphic microsatellite (SSR) markers derived from an enriched genomic library were characterized using 120 adult trees from four different *C. fissilis* populations. No substantial genotypic linkage disequilibrium was detected among all possible pairs of SSR loci. The number of alleles per locus ranged from 2 to 20,the average allele number ranged from 8 to 9.7, depending on the population. The observed heterozygosity among the different SSR loci varied from 0.0 to 1.00 , the expected heterozygosity varied from 0.07 to 0.95 On the population level, the average observed and expected heterozygosities ranged from 0.50 to 0.63 and from 0.64 to 0.70, respectively. The average fixation index among populations ranged from 0.09 to 0.24. Thus, the SSR loci revealed high polymorphism rates and can be used to study the genetic diversity,structure, mating system, and gene flow in *C. fissilis*.

Key words: Cedar; Genetic diversity; Microsatellite markers; Neotropical tree species.

Introduction

Studies on population genetics can be helpful to design effective *in situ* management plans for tree species, in particular for those species which are subjected to forest fragmentation and logging. The Meliaceae family includes six species of the genus *Cedrela*, which are distributed from southern Mexico to northern Argentina (Styles, 1981). Three of the species, *Cedrela fissilis* Vell, *Cedrela odorata* L. and *Cedrela lilloi* C. DC., are native to Brazil. All of them arelisted as endangered species (IUCN, 2012).

C. fissilis is widely distributed in Brazil (ranging from latitudes 1° S (Pará State) to 33° S (Rio Grande do Sul State). It has a wide range of uses. Roundwood or sawnwood can be used for different construction purposes like e.g. ship- and aerospace-building, furniture making and the production of musical instruments. For the use as firewood, the wood is considered of good quality, but its high market value makes it inappropriate for this purpose (Carvalho, 1994). It is also used in medicine to combat fever, wounds and ulcers. Based on its wide range of utilization, populations of this species have decreased. Strategies for *in* and *ex situ* conservation of the remaining populations are necessary (Kageyama et al., 2003; Kageyama et al., 2014; Soldati et al., 2014).

Studies in populations of endangered tree species are necessary, since forest fragmentation can lead to species extinction or loss of tree populations. Microsatellite markers are well-suited for the use in in population genetic studies (Slatkin, 1995), due to high polymorphism rates. In this study, microsatellite markers were developed for *C. fissilis* and can be used for commercial and conservation interests.

Materials and Methods

Sampling of plant material

Sampling took place in four large areas, covering more than 750 ha of well-preserved mature forests with significant natural populations of *C. fissilis*. The four studied areas are located in the municipalities of Pidamonhangaba (VP) (22° 46' S, 45° 27' W, 1,200 ha), Sete Barras (SA) (24° 14' S, 48° 04' W, 41,700 ha) and Teodoro Sampaio (MD) (22° 35' S, 52° 12' W, 33,800 ha) in the state of São Paulo and in the municipality of Ilhota (MB) (26° 35' S, 48° 48' W, 750 ha) in the state of Santa Catarina. Sampling areas were chosen by their regional representation, preservation status, accessibility and available infrastructure. All areas consist out of forests that are situated in the Atlantic Forest Biome and represent the most threatened vegetation type in Brazil. 30 adjacent – and thus representing a continuous spatial sample – trees per population were chosen for sampling. Only adult individuals that occupied the canopy and/or showed signs of reproduction (presence of flowers or fruits) were sampled.

Microsatellite development

Total genomic DNA was extracted from fresh leaves from a single *C. fissilis* tree collected on *Campus* of

1) Escola Superior de Agricultura "Luiz de Queiroz," Universidade de São Paulo, Av. Pádua Dias, 11, Caixa Postal 9, Piracicaba, SP, CEP: 13418-900, Brazil.

2) Universidade Federal de São Carlos, Departamento de Biologia, Rod. João Leme dos Santos, Km 110, Sorocaba, SP, CEP: 18052-780, Brazil.

3) Instituto Florestal de São Paulo, CP 1322, São Paulo, SP, CEP: 01059-970, Brazil.

4) Empresa Brasileira de Pesquisa Agropecuária, Embrapa Recursos Genéticos e Biotecnologia. Estação Parque Biológico - SAIN Final W5 norte, Brasilia, DF, 70770-900, Brazil.

5) Rosana Pereira Vianello, Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Arroz e Feijão. Rodovia Goiânia/Nova Veneza Km12. Laboratório de Biotecnologia. CP: 179, Goiania, GO, CEP: 74001-179, Brazil.

*) Corresponding author: Alexandre Magno Sebbenn. Instituto Florestal de São Paulo, CP 1322, São Paulo, SP, CEP: 01059-970, Brazil, Phone: +55 19 34351681. E-mail: alexandresebbenn@yahoo.com.br.

Table 1. – Microsatellite primers developed for *Cedrela fissilis*. The forward (F) and reverse (R) sequence, Repeat motif, size of the original fragment (bp) and annealing temperature (Ta°C) are shown for each primer pair.

Locus	Primer sequence	Repeat motif	Size	Ta(°C)
CF09	F: TTGTCCACGGTCTAAATTCCTT	$(ga)_9$	248 – 258	54
	R: TCCATAACCCGACCCATGA			
CF26	F: CCAAATTCCAGAGGAGAG	$(ga)_{19}$	146 – 194	56
	R: GTTCTGCTTCATCGAAGG			
CF32	F: GTACACTGCCTTAGTCCA	$(Ct)_{32}$	136 – 144	46
	R: ATTGAAAGACATCAGGC			
CF34	F: GTTGGCAGCATGATTACC	$(ga)_{20}$	128 – 168	56
	R: GAAGACTGTGTCTCTGAGTGG			
CF63	F: CCCACAAATTAACATCCCAA	$(ga)_{17}$	128 – 172	54
	R: CAGCCTAGAGCCGAATTCA			
CF66A*	F: CAGCAGTTCTGAAACAGTAA	$(ga)_{19}$	118 – 170	56
	R: ATTCAGCAACTTGAGAGC			
CF66B*	F: CAGCAGTTCTGAAACAGTAA	$(ga)_{19}$	202 – 254	56
	R: ATTCAGCAACTTGAGAGC			
CF78	F: GCCACAATATCTACTCTCAG	$(ct)_{17}$	122 – 168	56
	R: GTTTCCTCTCTTGGGTTT			
CF83	F: ACCATTCGAGCCCCACTACA	$(tc)_{20}$	312 – 328	54
	R: GCCAAGGCAACCGAATCA			

* Two different loci amplified by the same primer.

„Luiz de Queiroz“ College of Agriculture (University of São Paulo, Esalq/USP) using the protocol described by DOYLE and DOYLE (1987). The genomic DNA was digested using the restriction endonuclease *Sau*3AI (New England Biolabs, Ipswich, Massachusetts, USA) and separated on an agarose gel. DNA fragments between 280 and 600 bp were transferred to a cellulose membrane (NA-45). The library was enriched in microsatellite fragments using $(AG/TC)_{13}$ motifs linked to biotin. The enriched fragments were cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA), the ligation products were used to transform Epicurian Coli XL1-Blue *Escherichia coli*-competent cells (Stratagene, Agilent Technologies, Santa Clara, California, USA). Transformed cells were cultivated on agar plates containing 100 µg/mL ampicillin, 50 µg/mL X-galactosidase, and isopropyl β-D-1-thiogalactopyranoside (IPTG). Single white colonies were selected and stored at –80°C. A total of 884 recombinant colonies were obtained. Extraction of DNA from plasmid was carried out using the Wizard Miniprep system (Promega). All 884 recombinant clones were sequenced using the primers *Rsa* 21 (5'-TCTTGCTTACGCGTGGACTA-3') and *Rsa* 25 (5'-AGTCCACGCGTAAGCAAGAGCACA-3') and the the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems).

Sequencing was performed using an ABI 377 automated sequencer (Applied Biosystems, Foster City, California, USA) The program VecScreen was used to remove vector segments from each of the sequences (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Nine SSR loci, discovered within the sequenced clones, were selected for primer design. The selection criteria were high sequence quality and a minimum of 6 SSR motive repeats. Primer design was executed using the software Primer3 v0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0) according to the following criteria: annealing temperature ranging from 52–60°C and GC content between 40 and 60%. Each primer pair was designed to amplify a fragment of 150 to 300 bp (*Table 1*).

Marker analysis

For all adult trees, DNA was extracted from 100 mg of fresh leaves using the method of DOYLE and DOYLE (1987). All 8 microsatellite loci (*Table 1*) were amplified and scored in all 120 sampled *C. fissilis* individuals following the protocols of RITTER et al. (2011) and CRESTE et al. (2001). Allele scoring was carried out using the 10 bp DNA Ladder (Invitrogen) as size standard.

Data analysis

Genetic diversity was characterized as a mean across all loci using the following indices: total number of alleles (k), observed heterozygosity (H_o) and expected heterozygosity under Hardy-Weinberg equilibrium (H_e). To check for potential inbreeding (F), we used the within population fixation index. The statistical significance of F values was tested by permuting alleles among individuals, associated to a Bonferroni correction for multiple tests (95%, $\alpha = 0.05$). All analyses, including genotypic linkage disequilibrium, were performed using the FSTAT program (GOUDET, 1995).

Results

Eight hundred and eighty-four recombinant colonies of the *C. fissilis* genomic library were analyzed during this study. Microsatellites were found in 168 (19%) of the recombinant clones, demonstrating a low efficiency of the library enrichment. A significant genotypic linkage disequilibrium was detected for only two pairs of loci (CF26xCF66B and CF26xCF83) from the VP popu-

Table 2. – P value results of the estimates of genotypic disequilibrium between pairwise loci for four *Cedrela fissilis* populations. Adjusted P-value for 5% nominal level of Bonferroni correction is 0.000347.

Pairwise loci	Ilhota (MB)	Teodoro Sampaio (MD)	Sete Barras (SA)	Pindamonhangaba (VP)
CF09 X CF26	0.92847	0.16667	0.64583	0.15729
CF09 X CF32	0.30625	0.31944	0.3375	0.42049
CF09 X CF34	0.86701	0.30625	0.22847	0.62813
CF09 X CF63	0.86979	0.73368	0.12674	0.40833
CF09 X CF66A	0.76076	0.57153	0.30451	0.87708
CF09 X CF66B	0.88819	0.90868	0.21736	0.07222
CF09 X CF78	0.81632	0.62500	0.38194	0.21701
CF09 X CF83	0.73507	0.62188	0.02778	0.07431
CF26 X CF32	0.11806	0.05243	1.00000	0.15729
CF26 X CF34	0.12118	1.00000	0.22951	0.00417
CF26 X CF63	0.06458	0.45799	0.07813	0.17465
CF26 X CF66A	1.00000	1.00000	0.64514	0.06979
CF26 X CF66B	0.03715	0.03160	0.90694	0.00035*
CF26 X CF78	1.00000	1.00000	0.45660	0.43438
CF26 X CF83	0.78750	0.08229	0.53056	0.00035*
CF32 X CF34	0.27431	0.90625	0.33611	0.00208
CF32 X CF63	0.52431	0.15451	0.08958	0.00417
CF32 X CF66A	0.80938	0.62431	0.83924	0.04549
CF32 X CF66B	0.98646	0.89097	0.44896	0.06181
CF32 X CF78	0.72743	0.78889	0.65556	0.04931
CF32 X CF83	0.48403	0.92813	0.43576	0.33021
CF34 X CF63	1.00000	1.00000	0.08264	0.25347
CF34 X CF66A	0.12465	1.00000	1.00000	0.01528
CF34 X CF66B	0.29722	0.13854	0.01806	0.05035
CF34 X CF78	1.00000	0.44306	0.75799	0.53750
CF34 X CF83	0.29583	0.24132	0.08681	0.48993
CF63 X CF66A	1.00000	0.21424	0.18924	0.05417
CF63 X CF66B	0.51285	0.87153	0.11042	0.13021
CF63 X CF78	0.26771	0.5441	0.53368	0.11875
CF63 X CF83	0.72326	0.43611	0.78090	0.18681
CF66A X CF66B	1.00000	0.47882	0.53819	0.00764
CF66A X CF78	1.00000	1.00000	1.00000	0.25833
CF66A X CF83	0.16076	0.59063	0.42951	0.00278
CF66B X CF78	0.64722	0.27326	0.72153	0.27708
CF66B X CF83	0.24931	0.3066	0.75868	0.00104
CF78 X CF83	0.58854	0.02882	0.19965	0.07674

* $P < 0.05$.

Table 3. – Population genetic parameters calculated for nine microsatellite loci developed for *C. fissilis*: sample size for each population (n), number of alleles (k), expected heterozygosity (H_e), observed heterozygosity (H_o) and fixation index (F).

Locus	Ilhota (MB) (n= 30)				Teodoro Sampaio (MD) (n= 30)				Sete Barras (SA) (n= 30)				Pindamonhangaba (n= 30) (VP)			
	k	H_o	H_e	F	k	H_o	H_e	F	k	H_o	H_e	F	k	H_o	H_e	F
CF09	2	0.08	0.07	-0.02	3	0.30	0.52	0.43	2	0.41	0.49	0.16	2	0.00	0.24	1.00*
CF26	16	0.63	0.93	0.32*	15	0.31	0.93	0.67*	9	0.80	0.89	0.10	10	0.52	0.89	0.42*
CF32	2	0.26	0.23	-0.13	2	0.30	0.35	0.13	2	0.07	0.07	-0.02*	2	0.07	0.38	0.82
CF34	10	0.68	0.86	0.21	13	0.93	0.89	-0.04	10	0.86	0.83	-0.04	9	0.83	0.87	0.05
CF63	13	0.72	0.89	0.19	17	0.57	0.90	0.37*	7	0.85	0.78	-0.09	12	0.77	0.86	0.11
CF66A	14	0.79	0.89	0.11	15	0.87	0.91	0.05	20	1.00	0.95	-0.06*	15	0.97	0.91	-0.07
CF66B	4	0.41	0.54	0.25	6	0.34	0.34	-0.01	7	0.52	0.74	0.30	8	0.77	0.79	0.02
CF78	11	0.78	0.77	-0.01	12	0.79	0.81	0.03	12	1.00	0.89	-0.13*	10	1.00	0.85	-0.18
CF83	4	0.14	0.58	0.75*	4	0.31	0.56	0.45*	4	0.20	0.67	0.70*	4	0.48	0.52	0.08
Mean	8.4	0.50	0.64	0.22*	9.7	0.52	0.69	0.24*	8.1	0.63	0.70	0.09	8.0	0.60	0.70	0.14*
Total	76	-	-	-	87	-	-	-	73	-	-	-	72	-	-	-

* $P < 0.05$.

lation (*Table 2*), showing that the eight loci do not seem to be genetically linked. The total number of alleles ranged from 72 to 87 depending on the population. The average number of alleles per loci ranged from 8.0 to 9.7 (*Table 3*). The observed heterozygosity strongly varied in the SA population (from 0.00 to 0.966) and the expected heterozygosity strongly varied (0.068 to 0.946) in VP population. The average observed heterozygosity ranged from 0.449 to 0.635 and the expected heterozygosity ranged from 0.642 to 0.700, depending on the population. The average fixation index ranged from 0.093 to 0.243, depending on the population. It was significantly higher than zero in the MB, MD and VP populations, suggesting inbreeding.

Discussion

The development of specific microsatellite markers is fundamental for new studies in the field of population genetics and the development of conservation strategies for endangered species (KONZEN, 2014). In this paper, we describe a set of microsatellite markers for *C. fissilis*. These markers will be important to study populations of *C. fissilis* and other species of the genus.

Few of the identified clones of the enriched genomic library were found to contain microsatellites, demonstrating a low efficiency of the library enrichment. However, a set of nine polymorphic SSR markers was obtained.

The genetic diversity of the different *C. fissilis* populations was moderate but similar to the values obtained in studies of microsatellite markers in other species of the Meliaceae family, such as *Cedrela balansae* ($H_e = 0.643$), *Swietenia humilis* ($H_e = 0.548$) and *Swietenia macrophylla* ($H_e = 0.657$), (WHITE et al., 1999; NOVICK et al., 2003; SOLDATI et al., 2013). However, for *C. fissilis*, other studies have reported higher values of genetic diversity ($H_e = 0.820$) (KAGEYAMA et al., 2003).

Genotypic linkage disequilibrium was detected for two pairs of SSR loci within the VP population and may have occurred due to the effects of forest fragmentation, producing genetic drift. Genotypic linkage disequilibrium between pairwise loci in plants is caused by selfing, mating among relatives and bottlenecks (HARTL and CLARK, 1989). A possible recent bottleneck could be a cause of genotypic disequilibrium in the studied populations.

The presented new and informative microsatellite markers can be used to investigate populations of *C. fissilis* for breeding, conservation and environmental reforestation plans. The markers can be used for inferring genetic diversity, mating system and gene flow in *C. fissilis*.

Accession numbers

The sequences were deposited in the GenBank database with the following accession numbers: KP238561 [CF09]; KP238562 [CF26]; KP238563 [CF32]; KP238564 [CF34]; KP238565 [CF63]; KP238566 [CF66A and B]; KP238567 [CF78]; KP238568 [CF83].

Acknowledgements

We thank the Cenargen (Embrapa Recursos Genéticos e Biotecnologia) for help with the lab work and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 1998/2448-7) for funding this research. ALEXANDRE MAGNO SEBBENN and DARIO GRATTAPAGLIA are recipient of research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

CARVALHO, P. E. R. (1994): Espécies Florestais Brasileiras: Recomendações silviculturais, potencialidades e uso da madeira. EMBRAPA-CNPF, Brasilia, Brazil, 640p.

CRESTE, S., A. T. NETO and A. FIGUEIRA (2001): Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. Plant Molecular Biology Reporter **19**: 299–306.

DOYLE, J. J. and J. L. DOYLE (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry **19**: 11–15.

GOUDET, J. (2002): Fstat (Version 2.9.3.2.): a computer program to calculate F-statistics. Journal of Heredity **86**: 485–486.

IUCN (2012): IUCN Red List of Threatened Species. Version 2012.2. http://www.iucnredlist.org (March 11, 2015).

KAGEYAMA, P. Y., D. CARON, F. GANDARA and J. D. SANTOS (2004): Conservation of Mata Atlântica forest fragments in the State of São Paulo, Brazil. *In:* VINCETI, B., AMARAL, W., MEILLEUR, B. (Eds.), Proceedings of the Symposium on Challenges in Managing Forest Genetic Resources for Livelihoods: Examples from Argentina and Brazil. International Plant Genetic Resources Institute, Rome, Italy, pp. 167–185.

KAGEYAMA, P. Y., A. M. SEBBENN, L. A. RIBAS, F. B. GANDARA, M. CASTELLEN, M. B. PERECIM and R. VENCOVSKY (2003): Diversidade genética em espécies arbóreas tropicais de diferentes estágios sucessionais por marcadores genéticos. Scientia Forestalis **64**: 93–107.

KONZEN, E. R. (2014): Towards conservation strategies for forest tree endangered species: the meaning of population genetic statistics. Advances in Forestry Science **1**: 45–51.

RITTER, L. M. O., M. M. BAJAY, M. MONTEIRO, R. G. V. C. SOUZA, M. A. MORENO and P. Y. KAGEYAMA (2011): Development of microsatellite markers for *Qualea grandiflora* (Vochysiaceae), a typical species of the Brazilian cerrado. American Journal of Botany 99: e97–e98 (doi: 10.3732/ajb.1100405).

SLATKIN, M. (1995): A measure of population subdivision based on microsatellite allele frequencies. Genetics **139**: 457–462.

SOLDATI, M. C., M. V. INZA, L. FORNES and N. ZELENER (2014): Cross transferability of SSR markers to endangered *Cedrela* species that grow in Argentinean subtropical forests, as a valuable tool for population genetic studies. Biochemical Systematics and Ecology **53**: 8–16.

STYLES, B. T. (1981): Swietenioideae. In: Pennington, T.D., Styles, B.T., Taylor, D.A.H. (Eds.), Meliaceae. Flora Neotropica Monograph. Botanical Garden, New York, USA, pp. 359–418.