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Note

Microsatellite markers for *Pinus pinaster* Ait.

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Abstract – Simple sequence repeats (SSRs) or microsatellites are valuable tools for genome mapping and population genetic studies for as they are codominant and highly polymorphic markers. Seventy-six SSR primer pairs from four *Pinus* species were tested to amplify microsatellites in *Pinus pinaster*. Twenty-six primer pairs were stemmed from a microsatellite library on *P. pinaster* and the other primer pairs were obtained in other species of the same genus (*P. radiata*, *P. strobus* and *P. halepensis*). Only three out of the 76 SSR primer pairs amplified at a single polymorphic locus in *P. pinaster*. The Mendelian inheritance of those three primer pairs was studied and their genetic map position was determined. The number of alleles and the level of heterozygosity were assessed in an analysis of a sample of 196 trees. The development of microsatellites in *Pinus* species has been reported to be a difficult task because of the size and complexity of their genome. The results of this study showed that cross-species amplification was quite unsuccessful.

***Pinus pinaster* / genetic variability / genetic mapping / microsatellite / cross-species amplification**

Résumé – Marqueurs microsatellites chez *Pinus pinaster* Ait. Les microsatellites (SSRs) sont des outils de choix pour la cartographie génétique et les études de génétique des populations parce qu'ils sont des marqueurs codominants et très polymorphes. Soixante-seize paires d'amorces de quatre espèces de pin ont été utilisées afin d'amplifier des microsatellites chez *Pinus pinaster*. Vingt-neuf paires d'amorces étaient issues d'une banque enrichie en microsatellites sur *P. pinaster* et les autres paires d'amorces avaient été obtenues sur d'autres espèces du même genre (*P. radiata*, *P. strobus* et *P. halepensis*). Sur un total de 76 paires d'amorces, seulement trois ont amplifié un seul locus microsatellite polymorphe chez *P. pinaster*. Leur ségrégation mendélienne a été étudiée et chaque locus a été localisé sur une carte génétique. Le nombre d'allèles et l'hétérozygotie ont été ensuite évalués en analysant un échantillon de 196 arbres. Le développement de microsatellites chez les espèces du genre *Pinus* s'est révélée difficile en raison de la taille et de la complexité de leur génome. Les résultats de cette étude ont montré que l'amplification inter-espèces n'a rencontré que peu de succès.

***Pinus pinaster* / variabilité génétique / cartographie génétique / microsatellite / amplification inter-spécifique**

Pinus pinaster Ait. is one of the most abundant conifer in South-Western Europe. It is an important species from an ecological (swamp draining and dunes protection) and economical (wood production, pulp and paper making industry) point of view. In France, it covers 1.4 M hectares which represents 10% of the forest surface. A breeding programme for *P. pinaster* was started in the sixties. It has now reached its third generation and has allowed the deployment of improved varieties. Genetic diversity studies were performed using terpenic,

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protein, allozymic and chloroplast microsatellite markers throughout the natural range of this species (Baradat et al., 1991 [1]; Barhman et al., 1992 [2]; Petit et al., 1995 [14]; Vendramin et al., 1998 [19]). Nuclear microsatellites are valuable codominant multiallelic DNA markers but not yet available in *P. pinaster* for testing the validity of controlled crosses, for fingerprinting clones and for studying the genetic diversity of the provenances used in the breeding programme. In this study, our aim was to test 76 primer pairs from four *Pinus* species to amplify microsatellite loci in *P. pinaster*.

We adopted two strategies to amplify microsatellites in *P. pinaster*. First, we tested 47 existing primer pairs, as described in the literature or by personal communication, from three other *Pinus* species (*table I*). Second, we constructed an enriched microsatellite library, from which we designed and tested 29 primer pairs.

The microsatellite library, enriched with CA and GA repeats, was constructed from *P. pinaster* genomic DNA, as described by Edwards et al. (1996) [8]. The protocol was modified for hybridisation and washing as followed. Nylon membranes were prehybridised in 6X SSC 5X Denhardt's 1% SDS at 65 °C for 48 h renewing the solution after 24 h. Hybridisation was performed in the same conditions for 20 h. Washing was performed three times in 2X SSC, 1% SDS 65 °C for 15 min, then 1X SSC, 1% SDS for 10 min at 65 °C. A first PCR was performed on DNA that was bound and then eluted from the membrane. PCR products were used for a second round of enrichment. After this second step of enrichment, PCR products were cloned according to the protocol outlined in the Topo TA Cloning kit (Invitrogen, The Netherlands). They were sequenced using LI-COR automatic sequencers 4000 and 4000L (LI-COR Inc., Nebraska, USA). A total of 65 clones containing a microsatellite were detected from 80 clones randomly chosen from the library. Primers were designed for 29 SSRs using the primer software (version 5.0, Whitehead Institute for Biomedical Research, 1991).

The extraction of DNA and the amplification of microsatellites were performed as followed. Genomic DNA was extracted from needles as described by Doyle and Doyle (1991) [5]. The PCR was carried out in a Thermal Cycler Perkin Elmer GeneAmp PCR system 9600, using 0.4 units of Gibco BRL *Taq* Polymerase (Life Technologies, Inc. Gaithersburg MD, USA), and approximately 6 ng of genomic DNA in a total volume of 10 µl containing 200 µM of each nucleotide and 0.2 µM of each primer. Optimized $MgCl_2$ concentrations are indicated in table I. Each forward primer was labelled with the infra-red fluorescent dye IR800 (purchased at MWG Biotech). After a preliminary denaturation step at 94 °C for 4 min, PCR amplifications were performed for 35 cycles under the following conditions: 30 s at 94 °C, 30 s at the annealing temperature (see table I), and 45 s at 72 °C, with a final extension step of 10 minutes at 72 °C. After the amplification, 2 µl of PCR product were mixed with 7 µl of loading buffer (78% formamide, 10 mM EDTA pH 7.6, 0.1% bromophenol blue and 0.1% xylene cyanol), heated for 5 min at 75 °C and quickly cooled on ice. Afterwards 1 µl of denatured SSR fragments was loaded into a 25 cm long denaturating gel containing 8% acrylamide/bisacrylamide (19:1), 6 M urea and 0.4X TBE (134 mM TRIS, 45 mM boric acid, 2.5 mM EDTA). Electrophoresis was performed in the LI-COR automated sequencers using a 1X TBE running buffer at 1500 V, 40 mA and 45 °C of plate temperature. The RFLPscan version 3.0 (Scanalytics) software was used to score the SSR fragments.

Only three (one from *P. halepensis* and two from *P. pinaster*) out of the 76 primer pairs screened amplified at a single highly polymorphic locus in *P. pinaster*

Table I. Amplification of *Pinus* microsatellites in *Pinus pinaster.*

Species (number of primer pairs tested)	Sub-section	Amplification[d]		Banding pattern[e]		
		+	–	SML	SPL	C
Pinus radiata (n = 11)[a]	Attenuatae	73%	27%	12%	0%	88%
Pinus strobus (n = 11)[b]	Strobi	100%	0%	73%	0%	27%
Pinus halepensis (n = 25)[c]	Halepenses	68%	32%	23%	6%	71%
Pinus pinaster (n = 29)	Australes	79%	21%	34%	9%	57%

[a] 7 pairs from G.F. Moran (unpublished results), 2 pairs from [17] Smith and Devey (1994), 2 pairs from [10] Fisher et al. (1998).
[b] 4 pairs from [6] Echt et al. (1996) and 7 pairs available at URL http:/www.resgen.com.
[c] 25 pairs from G.G. Vendramin (unpublished).
[d] + : amplification; - : no amplification.
[e] SML: single monomorphic locus; SPL: single polymorphic locus; C: complex banding pattern.

Table II. Characteristics of three primer pairs for amplifying maritime pine microsatellite loci, with expected (H_E) and observed (H_0) levels of heterozygosity based on 196 individuals.

Locus	Primers (5'→3')	Micro-satellite sequence	Length of PCR product	T_a (°C)[a]	$MgCl_2$ (mM)	Number of alleles	H_E	H_O	Map location[b]	EMBL Accession number
FRPP91	F:GTACTCCCACATAAAATGAGACTT R:CCGAAATACATTGCAGGTTA	$(CT)_{20}$	168	61	2.25	25	0.862	0.684	9	AJ012085
FRPP94	F:GGCAAACCTCTTTTAGAGTGC R:TTTGTCGATTTTTCTTGAAATCTAA	$(CT)_{22}$	162	60	2.5	17	0.726	0.571	5	AJ012086
ITPH4516	F:TGATGCAAACAAGTTCCATG R:AGCACTCGCTAAACTATGAAGG	$(CT)_{27}$	159	61	2.25	20	0.894	0.684	3	AJ012087

[a] T_a, annealing temperature; [b] Linkage group according to the genetic map of Costa et al. (2000).
See also URL http://www.pierroton.inra.fr/genetics/pinus/map2.html

genomic DNA (*table I*). Their Mendelian pattern of inheritance was tested and their allelic variations were examined for 196 individuals, that belonged to eight different populations from southwest France. The characteristics of these three SSRs are summarized in *table II.* Each microsatellite locus revealed a high amount of polymorphism (mean number of alleles = 20.7). The average observed heterozygosity was 0.65. We used a haploid (megagametophyte) mapping pedigree to show that they exhibit a Mendelian pattern of inheritance and we could position them in a previously constructed linkage map (Costa et al., 2000 [4]). About a third of the primer pairs analyzed in this study resulted in single locus-specific amplification. Among these loci, 87.5% were monomorphic and 12.5% were polymorphic. The majority of the remaining primer pairs gave either no amplification (22.4%) or produced multiband patterns (46%) (*table I*). The difficulty of developing informative (single polymorphic locus) microsatellites has already been reported in other conifer species (Echt et al., 1996 [6]; Pfeiffer et al., 1997 [15]) and can be attributed to their large genome size and complexity (Wakamiya et al., 1993 [20]; Kinlaw and Neale, 1997 [12]).

In this study, we showed that only one primer pair from other *Pinus* species (*P. halepensis*) could be transferred to *P. pinaster*. According to Farjon (1984) [9], *P. radiata* belongs to the same section as *P. pinaster* whereas *P. strobus* belongs to the section *Strobus* and *P. halepensis* to the section *Pinea*. However, a natural hybrid between *P. halepensis* and *P. pinaster* was mentioned by Schütt (1959) [16], which may explain our result. As reported by Echt and May-Marquardt (1997) [7], we also found that SSR information do not transfer across *Pinus* species. However, ten polymorphic SSRs markers developed in *P. halepensis* produced single variable bands segregating in a Mendelian manner in the species *P. brutia* (G.G. Vendramin, personal communication). In this case, cross-species amplification seemed to be easier because these *Pinus* species show a low degree of divergence (Bucci et al., 1998 [3]). Similarly, some studies have shown that SSRs isolated from several species amplify the corresponding and polymorphic PCR products in closely related species. Kijas et al. (1995) [11] tested two primer sets in 10 different *Citrus* species and two related genera and found conservation of the sequences. Using 17 sets of primers developed from sessile oak, *Quercus petraea*, Steinkellner et al. (1997) [18] found that two of the loci were polymorphic in all the *Quercus* species tested. In general, the success of the amplification diminishes with increasing species divergence (Steinkellner et al., 1997 [18]; Whitton et al., 1997 [21]; Lefort et al., 1999 [13]). Further development of *P. pinaster* microsatellites will be focused on an enriched cDNA library.

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