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Cross-amplification and multiplexing of cpSSRs and nSSRs in two closely related pine species (*Pinus sylvestris* L. and *P. mugo* Turra)

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Abstract: Background: Simple sequence repeats (SSRs) are widespread molecular markers commonly used in population genetic studies. Nowadays, next-generation sequencing (NGS) methods allow identifying thousands of SSRs in one sequencing run, which greatly facilitates isolation and development of new SSRs. However, their usefulness as molecular markers still must be tested empirically on a number of populations to select SSRs with best parameters for future population genetic research. An alternative approach, cheaper and faster than isolation and characterization of new SSRs, involves cross-amplification of SSRs in closely related species.
Aims: Our goal was to develop multiplex PCR protocols that will be useful in population genetic studies of Scots pine (*Pinus sylvestris* L.) and dwarf mountain pine (*P. mugo* Turra), and possibly other pine species.
Methods: We tested 14 chloroplast (cpSSRs) and 22 nuclear (nSSRs) microsatellite markers originally designed for Japanese black pine (*P. thunbergii* Parl.), *P. sylvestris* and loblolly pine (*P. taeda* L.) in four populations of *P. sylvestris* and *P. mugo* across different locations in Europe. We designed six multiplex PCRs, which were subsequently screened for their ability to provide repeatable and high quality amplification products using capillary electrophoresis.
Results: The transfer rate in our study was similar in both pine species, and it was very high for cpSSRs (93% and 86% for *P. sylvestris* and *P. mugo*, respectively) and moderate for nSSRs (59% for both species). We managed to design five well-performing multiplex reactions out of six initially tested. Most of the tested loci were polymorphic. Moreover, the allelic patterns detected at some cpSSRs were species-specific.
Conclusions: We provide a set of five multiplexes which can be used in genetic studies of both *P. sylvestris* and *P. mugo*. Chloroplast marker PCP30277 is a good candidate for a cheap species diagnostic marker suitable for tracking interspecific gene flow between hybridizing species of *P. sylvestris* and *P. mugo*.

Keywords: chloroplast microsatellites, dwarf mountain pine, hybridization, nuclear microsatellites, Scots pine

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Introduction

Microsatellites (=simple sequence repeats; SSRs or short tandem repeats; STRs) are the class of repetitive DNA sequences present in both eukaryotic and prokaryotic genomes. With respect to population genetics of forest tree species, microsatellites have proved to be useful neutral molecular markers in studies focusing on genetic diversity (e.g. Chybicki et al., 2011; Litkowiec et al., 2015; Wójkiewicz & Wachowiak, 2016), mating systems (e.g. Lian et al., 2001) and gene mapping (e.g. Echt et al., 2011) due to their high level of allelic variation and co-dominant mode of inheritance. The popularity of SSRs in genetic research of trees is also connected with the fact that they can be genotyped in one multiplex polymerase chain reaction (PCR). This technique allows amplification of two or more DNA fragments simultaneously. The possibility of multiplexing combined with capillary electrophoresis, which is based on a laser-induced fluorescence DNA technology, results in a cost-effective tool for genotyping large quantities of independent samples.

Till the next-generation sequencing (NGS) era, the development of novel microsatellite markers for forest tree species was difficult, costly and time-consuming. Currently, it is possible to identify thousands of microsatellite regions during one sequencing run of a genome or transcriptome. As a result, the isolation of new SSRs is no longer a real challenge, practically for any organism, including trees. Regardless of this, the usefulness of novel SSRs for population genetic studies still must be tested to verify which of them 1) provide repeatable, polymorphic and high quality amplification products, 2) are the most informative and 3) are transferable, which gives opportunity to perform genetic analyses at interspecific level.

The objects of our study were two very closely related pine species: Scots pine (*Pinus sylvestris* L.) and dwarf mountain pine (*P. mugo* Turra). At present these species have mostly allopatric distribution. *P. sylvestris* is the most widespread conifer in Europe and Asia, whereas *P. mugo* is typical to the mountain regions of Europe. We aimed at developing of efficient multiplex protocols for the amplification of chloroplast and nuclear SSRs (cpSSRs and nSSRs, respectively) in *P. sylvestris* and *P. mugo*, which we had pre-selected from a collection of 36 SSRs originally designed for *P. thunbergii* Parl., *P. sylvestris* and *P. taeda* L. (Table 1). The results of the cross-species amplification of cpSSRs and nSSRs are discussed in the light of their utility for future genetic research.

Methods

Four populations of *P. sylvestris* (128 individuals) and four populations of *P. mugo* (105 individuals) across different locations in Europe were analysed in this study (Table 2). The collected samples were stored in -20°C until DNA extraction. Genomic DNA was extracted from 50-100 mg of needle tissue, following the CTAB protocol as described by Dumolin et al. (1995). RNase A was added to the final incubation step. The DNA concentration was measured with BioPhotometer (Eppendorf AG, Germany) and adjusted to 15 ng/μl.

We selected 14 chloroplast and 22 nuclear microsatellite markers available in the published literature (Table 1). CpSSRs were initially developed for *P. thunbergii*, whereas nSSRs for *P. sylvestris* and *P. taeda*. The markers were combined into six multiplex PCRs and screened for their ability to provide repeatable and high quality polymorphic amplification products of expected size. The loci were finally amplified in five multiplex PCRs in Applied Biosystems Veriti and 2720 thermal cyclers (Life Technologies, USA). The PCRs were carried out in a total volume of 10 μl, using the Qiagen Multiplex PCR kit (Qiagen, Germany). Each reaction contained about 45 ng of template DNA, 1x Qiagen Multiplex PCR Master Mix, 0.5x Q-Solution and 0.05-0.1 μM each of forward and reverse primers. All primers were tested individually prior to the performance of multiplex reactions. We used equimolar concentration of primers in the initial amplification procedures, which were subsequently adjusted to obtain an even intensity of the fluorescence signal. Amplification conditions were optimised across all multiplexes for both pine species. Details of final PCR parameters are described in Table 1. The fluorescently labelled PCR products were separated on a capillary sequencer, the Applied Biosystems 3130 Genetic Analyzer (Life Technologies, USA). The GeneScan 500 LIZ Size Standard (Life Technologies, USA) was used as an internal size standard. The raw data were scored with the GeneMapper Software ver 4.0 (Life Technologies, USA), checked manually and converted into discrete allele sizes with the use of the AlleloBin software (Prasanth et al., 2006).

Two parameters were calculated for each species for cpSSRs: the number of alleles (A_N) and unbiased diversity (A_{uh}) using GenAlEx ver 6.5 (Peakall & Smouse, 2006). A_{uh} was computed as mean across all populations for each species. With regard to nSSRs, we used the multiple sample score test (U test for heterozygote deficit, Raymond and Rousset 1995), implemented in GENEPOP ver 4.3 (Rousset, 2008), to assess the significance of departures from Hardy-Weinberg equilibrium (HWE) for each locus, separately for each species. The frequency of null alleles (NAF) was estimated using FreeNA (Chapuis & Estoup, 2007) separately for each population and each species. A_N, effective number of alleles (A_E), observed and expected heterozygosity (H_O and H_E,

Table 1. A list of multiplexes and thermocycling conditions for *P. sylvestris* and *P. mugo*. Multiplex 4 (nSSR) is omitted as the loci (psyl17 (Sebastiani et al., 2012), ptTX3116 (Elsik & Williams, 2001), SPAC11.6, SPAC 11.8, SPAC 12.5 (Soranzo et al., 1998) failed to amplify in both *P. sylvestris* and *P. mugo*. Each reaction consisted of the following steps: I – initial denaturation, II – denaturation, III – annealing, IV – elongation, V – final elongation

Multiplex	Loci	Step	*P. sylvestris*	*P. mugo*
1 (cpSSR)	Pt15169, Pt26081, Pt30204, Pt36480, Pt45002, Pt71936 (Vendramin et al., 1996)	I	95°C, 15 min.	95°C, 15 min.
		II	94°C, 15 sec.	94°C, 30 sec.
		III	58°C, 90 sec.	58°C, 45 sec.
		IV	72°C, 90 sec.; go to II × 27	72°C, 90 sec.; go to II × 30
		V	72°C, 10 min.	72°C, 10 min.
2 (cpSSR)	PCP1289, PCP26106, PCP30277, PCP36567, PCP41131, PCP45071, PCP87314, PCP102652 (Provan et al., 1998)	I	95°C, 15 min.	95°C, 15 min.
		II	94°C, 15 sec.	94°C, 30 sec.
		III	60°C, 90 sec.	60°C, 45 sec.
		IV	72°C, 90 sec.; go to II × 27	72°C, 90 sec.; go to II × 30
		V	72°C, 10 min.	72°C, 10 min.
3 (nSSR)	psyl2, psyl16, psyl18, psyl19, psyl25, psyl36, psyl42, psyl44, psyl57 (Sebastiani et al., 2012)	I	95°C, 15 min.	95°C, 15 min.
		II	94°C, 30 sec.	94°C, 30 sec.
		III	57°C, 90 sec.	55°C, 90 sec.
		IV	72°C, 90 sec.; go to II × 37	72°C, 90 sec.; go to II × 37
		V	72°C, 10 min.	72°C, 15 min.
5 (nSSR)	ptTX2146 (Elsik et al., 2000), ptTX3107 (Elsik & Williams, 2001), SPAG 7.14 (Soranzo et al., 1998)	I	95°C, 15 min.	95°C, 15 min.
		II	94°C, 30 sec.	94°C, 30 sec.
		III	55°C, 90 sec.	56°C, 90 sec.
		IV	72°C, 90 sec.; go to II × 29	72°C, 90 sec.; go to II × 34
		V	72°C, 10 min.	72°C, 15 min.
6 (nSSR)	ptTX3025, ptTX3032 (Elsik et al., 2000), ptTX4001, ptTX4011 (Zhou et al., 2002), SPAC 11.4 (Soranzo et al., 1998)	I	95°C, 15 min.	95°C, 15 min.
		II-1	94°C, 30 sec.	94°C, 30 sec.
		III-1	60°C Δ↓1°C/cycle, 40 sec.	65°C Δ↓1°C/cycle, 40 sec.
		IV-1	72°C, 90 sec.; go to II-1 × 9	72°C, 60 sec.; go to II-1 × 9
		II-2	94°C, 30 sec.	94°C, 30 sec.
		III-2	50°C, 40 sec.	55°C, 60 sec.
		IV-2	72°C, 90 sec.; go to II-2 × 35	72°C, 60 sec.; go to II-2 × 31
		V	72°C, 10 min.	72°C, 7 min.

respectively) were calculated in GenAlEx ver 6.5 across all populations separately for each species.

Results & Discussion

The transfer rates were very similar in both *P. sylvestris* and *P. mugo*. We managed to transfer 13 (93%) and 12 (86%) out of 14 initially tested chloroplast microsatellites to *P. sylvestris* and *P. mugo*, respectively. Locus Pt36480 was successfully transferred only to *P. sylvestris*. Similar high values of transfer rates for cpSSRs were noted previously by Dzialuk and Burczyk (2004), who proposed a multiplex PCR that consisted of six loci for population studies in *P. sylvestris*. With regard to nuclear microsatellites, the transfer rates were moderate (59%) for both pines. Similarly to our results, moderately low (26%) transfer rates were demonstrated by Celiński et al. (2013), who tested the transferability of 19 nSSRs from *P. sylvestris* and *P. taeda* to *P. mugo*. In our study, 13 out of 22 nSSRs were amplified successfully in both species, but some loci that failed to amplify or gave poor results in *P. sylvestris* turned out to be useful for *P. mugo* and vice-versa (ptTX3107 and SPAC 11.4 only for *P. sylvestris*, whereas psyl16 and ptTX4001 only for *P. mugo*). Our results clearly show that the amplification of cpSSRs was more successful than nSSRs, which is most likely associated with the fact that the mutation rate of chloroplast DNA is lower than of nuclear DNA (Willyard et al., 2007). As a result, the high sequence conservation among chloroplast genomes of conifers allows successful amplification of cpSSRs designed for *P. thunbergii* in closely (as in our study) or more distantly related conifer species.

Allelic variation of the analysed loci was high with mean 7.12 and 6.32 alleles per locus for *P. sylvestris* and *P. mugo*, respectively. Nearly all successfully amplified

Table 2. Descriptive statistics of the studied cpSSR and nSSR markers in *P. sylvestris* (S) and *P. mugo* (M)*. A_N – number of alleles; A_{uh} – unbiased diversity (mean for all populations); A_E – effective number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; NAF – null allele frequency (range for all populations). Test for heterozygote deficit: ns – not significant; * – $p < 0.05$; ** – $p < 0.01$; *** – $p < 0.001$

Locus	Size range [bp]	A_N	A_{uh}	A_E	H_O	H_E	NAF
	S/M	S/M	S/M	S/M	S/M	S/M	S/M
Pt15169	124–130/121–126	7/5	0.75/0.56	–/–	–/–	–/–	–/–
Pt26081	110–112/109–112	3/4	0.26/0.48	–/–	–/–	–/–	–/–
Pt30204	140–148/143–149	9/7	0.79/0.79	–/–	–/–	–/–	–/–
Pt36480	143–145/–	3/–	0.18/–	–/–	–/–	–/–	–/–
Pt71936	148–154/145–149	7/5	0.64/0.62	–/–	–/–	–/–	–/–
PCP1289	108–111/107–108	4/2	0.35/0.17	–/–	–/–	–/–	–/–
PCP26106	146–148/145–148	3/4	0.28/0.50	–/–	–/–	–/–	–/–
PCP30277	134–140/115–120	7/6	0.77/0.75	–/–	–/–	–/–	–/–
PCP36567	110–112/110–112	3/3	0.12/0.47	–/–	–/–	–/–	–/–
PCP41131	139–143/140–159	5/10	0.16/0.69	–/–	–/–	–/–	–/–
PCP45071	153–156/146–151	4/6	0.45/0.55	–/–	–/–	–/–	–/–
PCP87314	112–114/112–116	3/5	0.32/0.68	–/–	–/–	–/–	–/–
PCP102652	114–116/114	3/1	0.03/0.00	–/–	–/–	–/–	–/–
psyl2	207–213/198–210	3/5	–/–	1.29/1.54	0.21/0.32	0.22/0.34	$0.00–0.13^{ns}$/ $0.00–0.13^{ns}$
psyl16	–/201–213	–/6	–/–	–/3.03	–/0.64	–/0.67	$–/0.00–0.06^{ns}$
psyl18	292–307/292–304	6/5	–/–	1.28/1.18	0.16/0.12	0.21/0.15	$0.00–0.08^{*}$/ $0.00–0.12^{**}$
psyl25	216–219/213–219	2/3	–/–	1.02/1.57	0.02/0.38	0.02/0.36	0.00^{ns}/ $0.00–0.03^{ns}$
psyl36	250–262/250–262	5/5	–/–	1.27/1.12	0.22/0.07	0.21/0.10	0.00^{ns}/ $0.00–0.13^{**}$
psyl42	167–179/169–177	7/4	–/–	3.25/2.10	0.69/0.51	0.69/0.50	$0.00–0.03^{ns}$/ $0.00–0.05^{ns}$
psyl44	169–178/169–175	4/2	–/–	1.19/1.29	0.15/0.26	0.16/0.22	$0.00–0.06^{ns}$/ 0.00^{ns}
psyl57	190–208/190–205	7/6	–/–	2.35/2.63	0.62/0.62	0.57/0.61	$0.00–0.02^{ns}$/ $0.00–0.09^{ns}$
ptTX2146	180–252/153–264	17/17	–/–	3.86/3.24	0.74/0.64	0.74/0.63	$0.00–0.04^{ns}$/ $0.00–0.01^{ns}$
ptTX3107	153–183/–	8/–	–/–	4.39/–	0.44/–	0.77/–	$0.15–0.26^{***}$/–
SPAG 7.14	177–257/185–265	30/28	–/–	14.29/11.56	0.77/0.80	0.93/0.91	$0.00–0.14^{***}$/ $0.00–0.15^{***}$
ptTX3025	266–299/266–275	7/4	–/–	1.90/1.27	0.43/0.19	0.47/0.21	$0.00–0.12^{*}$/ $0.00–0.11^{*}$
ptTX4001	–/205–221	–/6	–/–	–/2.66	–/0.53	–/0.58	$–/0.00–0.05^{ns}$
ptTX4011	256–280/262–284	10/9	–/–	3.10/3.27	0.62/0.60	0.68/0.67	$0.00–0.15^{**}$/ $0.00–0.18^{**}$
SPAC 11.4	130–166/–	18/–	–/–	7.10/–	0.88/–	0.85/–	$0.00–0.02^{ns}$/–
Mean		**7.12/6.32**	**0.39/0.52**	**3.56/2.80**	**0.46/0.44**	**0.50/0.46**	**0.04/0.03**

*Populations analysed in the study (long./lat.):
S: Joutsa, Finland (25°45'0"/64°41'24"); Tatras, Poland (20°21'36"/49°25'12"); Divčibare Mts, Serbia (44°6'0"/19°59'24"); St. Miguel d'Engolasters, Andorra (42°40'12"/0°46'12").
M: Sudetes, Poland (15°47'50"/50°44'40"); Carnic Alps, Italy (13°15'35"/46°32'45"); Carpathians, Romania (24°32'19"/45°36'30"); Dinaric Alps, Bosnia and Herzegovina (18°13'08"/43°45'00").

cpSSRs were polymorphic, exhibiting between two to ten alleles. Only PCP102652 was monomorphic in *P. mugo* (114 bp), whereas almost all individuals of *P. sylvestris* (99%) carried the 115 bp variant. In the case of nSSRs, A_N was lower for markers developed by Sebastiani et al. (2012) (the 'psyl' series; A_N between two and seven) than for other nSSRs (from four for ptTX3025 in *P. mugo* up to 30 for SPAG 7.14 in *P. sylvestris*). The mean value of unbiased diversity (mean A_{uh}) parameter, calculated for cpSSRs, did not differ statistically between the studied pines (mean $A_{uh} = 0.39$ and mean $A_{uh} = 0.52$ for *P. sylvestris* and *P. mugo*, respectively; Student's t-test: $p = 0.20$). As for nSSRs, the difference between the mean effective number of alleles (A_E) was also not significant (3.56 for *P. sylvestris* vs. 2.80 for *P. mugo*; U Mann-Whitney

test: p = 0.63). Significant heterozygote deficit was observed for six loci (psyl18, psyl36, ptTX3107, SPAG 7.14, ptTX3025, and ptTX4011). The frequency of null alleles (NAF) differed across loci and, to a lesser extent, between species (NAF = 0.00-0.26). The mean observed and expected heterozygosity (H_O and H_E, respectively) were similar in both species (mean H_O = 0.46, mean H_E = 0.50 and mean H_O = 0.44, mean H_E = 0.46 for *P. sylvestris* and *P. mugo*, respectively; Student's t-test: p = 0.84 for H_O and p = 0.69 for H_E). For most loci H_O was only slightly lower than H_E. Some loci, however, displayed H_O greater than H_E. Microsatellites with higher number of repeats generally displayed higher heterozygosity values (Table 2). Based on our results, we recommend to omit some nSSR loci with the frequency of null alleles exceeding 5%, including psyl18, ptTX3107, SPAG 7.14, and ptTX4011. Alternatively, a proper correction methods should be applied as, according to the simulation study by Chapuis and Estoup (2007), the levels of classical parameters used to describe population differentiation are overestimated in the presence of null alleles.

Loci that exhibit species-specific allelic patterns are ideal for studies of interspecific gene flow and identification of hybrid zones. In the present work, the most pronounced differences were apparent for 2 cpSSRs: PCP45071 and PCP30277. Alleles scored for these loci did not overlap when the two species were taken into account. Only 2 bp difference was observed for PCP45071 and it does not seem to be a species-specific polymorphism as compared to other studies (Wójkiewicz & Wachowiak, 2016). The difference for PCP30277 was at least 14 bp (Table 2), and this locus can be useful as a diagnostic marker to track interspecific gene flow in the species' contact zones. Regarding interspecific differences for nSSRs, we observed opposing tendencies for psyl2 and SPAG 7.14. Higher variants in *P. sylvestris* as compared to *P. mugo* were identified for psyl2, whereas lower sizes were typical for SPAG 7.14. Variants scored for *P. mugo* represented a subset of those identified in *P. sylvestris* for four loci: psyl42, psyl44, psyl57, and ptTX3025. For these markers, longer alleles, preferred in *P. sylvestris*, were absent in *P. mugo*. The same A_N was observed for ptTX2146 for both *P. sylvestris* and *P. mugo*, but some individuals of *P. mugo* had alleles shorter and others longer than *P. sylvestris*. As oppose to cpSSRs, there was no locus which had non-overlapping alleles when compared in both pine species (Table 2).

Conclusions

We provide five well-performing multiplexes consisting of sets of chloroplast and nuclear microsatellites that can be applied in population and conservation genetic studies of both *P. sylvestris* and *P. mugo*, and possibly of other pine species, e.g. from the *P. mugo* complex. The markers seem particularly useful for the assessment of the background neutral genetic variation that is necessary to further look for genetic signatures of natural selection in candidate genomic regions. Due to their high genetic variability, they could also be applied in the identification and tracking of plant material. Furthermore, the marker that exhibits species-specific allelic patterns (PCP30277) seems ideal for studies of interspecific gene flow in the species' contact zones. Such studies accompanied by analyses of sequence variation at candidate genomic regions will help to address questions related to the role of hybridization in evolution of *P. sylvestris* and *P. mugo* (Wachowiak et al., 2015, 2016). Our study clearly confirms that cross-amplification seems to be a good first choice alternative to the *de novo* development of microsatellite markers, especially for species with poor genomic resources. The possibility of genotyping using multiplex PCRs makes their application additionally time and cost-effective.

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