SPECIES SPECIFIC *cp*DNA MARKERS USEFUL FOR STUDIES ON THE HYBRIDISATION BETWEEN *PINUS MUGO* AND *P. SYLVESTRIS*

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ABSTRACT

PCR-RFLP technique has been used to detect species-specific mutations of organelles DNA for closely related dwarf mountain pine (*Pinus mugo*) and Scots pine (*P. sylvestris*). Restriction fragment patterns have been compared of amplification products for *trn*L-*trn*F *cp*DNA and for *cox*I and *orf*25 genes of *mt*DNA. The difference has been found in the DraI and HinfI restriction patterns of the amplification products for *trn*L-*trn*F region of *cp*DNA with two haplotypes detected. The haplotype M is characteristic for *P. mugo* and the haplotype S for *P. sylvestris*. These markers may be useful for the analysis of the natural hybridisation and introgression between these species postulated for some sympatric populations on the basis of morphological analysis. No differences have been disclosed in the studied *mt*DNA regions.

KEY WORDS: PCR-RFLP, *cp*DNA, *mt*DNA, *Pinus sylvestris, Pinus mugo,* hybridisation.

INTRODUCTION

Some sympatric populations of *Pinus mugo* (Turra) and *Pinus sylvestris* (L) contain plants which, according to morphometric studies, may represent hybrids or introgressants between these species. Among others, the formation of hybrids or introgressants is postulated for some populations from the Alps (Marcet 1967; Christensen 1987), the Sudeten and the Carpathians (Staszkiewicz and Tyszkiewicz 1969, 1972; Bobowicz 1990; Staszkiewicz 1994) and Rila Mts. (Dobrinov 1965; Yurukov and Tashev 1992). The estimated frequency of hybrids ranges from rare presence (Christensen and Dar 1997) to the formation of hybrid swarms (Bobowicz 1990; Viewegh and Cambalova 1993).

The hybridisation hypothesis has been tested with genetic markers including protein studied with serological techniques (Prus-Głowacki et al. 1978, 1980, 1981), *cp*DNA restriction patterns (Fillipula et al. 1992) and isoenzyme polymorphism (Prus-Głowacki and Szweykowski 1983; Fillipula et al. 1992; Neet-Sarqueda 1994; Siedlewska and Prus-Głowacki 1994). However, the use of the latter markers makes difficulties due to the absence of diagnostic alleles in putative parental species (Strauss et al. 1992). Some simply inherited and diagnostic markers are necessary for studies of this kind.

Our studies have been aimed at finding a species specific plastid and mitochondrial markers that would be suitable for the estimation of the intensity and direction of the hybridisation between *Pinus mugo* and *P. sylvestris* in natural populations.

MATERIAL AND METHODS

The studies have been performed on 14 individuals of Scots pine and 9 of dwarf mountain pine (Table 1). Most individuals of Scots pine have originated from Scots pine 1982, provenance trial in the Institute of Dendrology, Polish Academy of Science in Zwierzyniec near Kórnik, coordinated by the International Union of Forest Research Organisation (IUFRO). The individuals originate from 11 European countries and they represent the European range of *P. sylvestris*. Three of the analysed plants come from populations in the vicinity of Poznań. The plant from "Bór na Czerwonem" peatbog near Nowy Targ has a phenotype typical for the high peatbog ecotype of Scots pine. Dwarf mountain pine individuals mostly come from a collection grown in the Botanical Garden of the Polish Academy of Sciences in Warsaw-Powsin and they originate from natural stands in the Tatra Mts. (Marczewski 1993). The remaining individuals originate from the "Bór na Czerwonem" peatbog and they manifest morphological traits typical for *P. mugo*.

DNA was isolated from winter buds with the use of the CTAB technique (Murray and Thompson 1980). After the isolation, the DNA was suspended in the TE buffer (10 mM

TABLE 1. List of studied plants, their origin and haplotype of *cp*DNA.

Species	Plant No.	Location	Country	Haplotype
P. sylvestris	S 1	Wielkopolski National Park	Poland	S
	S 2	Poznań	Poland	S
	S 3	Mosina near Poznań	Poland	S
	S 4	"Bór na Czerwonem" peatbog	Poland	S
	S 5*	Serebryanskoe	Russia	S
	S 6*	Silene	Latvia	S
	S 7*	Miłomłyn	Poland	S
	S 8*	Spała	Poland	S
	S 9*	Rychtal	Poland	S
	S 10*	Sumpberget	Sweden	S
	S 11*	Zahorie	Slovakia	S
	S 12*	Maocnica	Yugoslavia	S
	S 13*	Prusacka Rijeka	Bosnia	S
	S 14*	Catacik	Turkey	S
P. mugo	M 1	"Bór na Czerwonem" peatbog	Poland	M
	M 2	"Bór na Czerwonem" peatbog	Poland	M
	M 3	"Bór na Czerwonem" peatbog	Poland	M
	M 4**	Tatra Mts	Poland	M
	M 5**	Tatra Mts	Poland	M
	M 6**	Tatra Mts	Poland	M
	M 7**	Tatra Mts	Poland	M
	M 8**	Tatra Mts	Poland	M
	M 9**	Tatra Mts	Poland	M

*Plants from the provenance trial in the area of the Experimental Forest, the Institute of Dendrology, Polish Academy of Science in Zwierzyniec near Kórnik.
**Plants from the collection growing in the Botanical Garden of the Polish Academy of Sciences in Warsaw–Powsin and A. Mickiewicz University, Poznań (M4).

Tris, 1 mM EDTA, pH 8.0). Three fragments of organellar DNA were amplified, including: the non-coding region between *trn*F and *trn*L genes (primer 1: 5'- CGA AAT CGG TAG ACG CTA CG-3'; primer 2: 5'-ATT TGA ACT GGT GAC ACG AG-3') of *cp*DNA (Taberlet et al. 1991), *orf*25 gene (primer 1: 5'-ATG CTA TTT GCT GCT ATT CC-3', primer 2: 5'-AGG ACT ATC AAG CCT TCT CG-3') and *cox*I gene (primer 1: 5'-TTA TTA TCA CTT CCG GTA CT-3', primer 2: 5'-AGC ATC TGG ATA ATC TGG-3') the region of *mt*DNA (Brandt et al. 1992). The PCR reactive mixture contained 100 ng of total DNA, 0.2 mM of each nucleotide, 0.5 µM of each of the primers, 1x reaction buffer, 0.5 U Taq polymerase (Biometra®) in the total volume of 20 µl. Preliminary denaturation of DNA for 5 min at 96°C was followed by 35 cycles of denaturation at 94°C for 0.5 min, primer annealing at 53°C for 1 min, DNA synthesis at 72°C for 1.5 min. The reaction was terminated with 10 min incubation at 72°C. Amplification efficiency was verified by the electrophoretic separation of 5 µl of PCR products in parallel with 3 µl of a marker (pKO3/HinfI) in 1.6% agarose gel in 1x TAE buffer (40 mM Tris-HCl, 1mM EDTA, pH 8.0) and staining with ethidium bromide.

The PCR products (10 µl) were subjected to restriction analysis at 37°C for 2 h (at 60°C for 3h using TaqI restriction enzyme). The region of *trn*F – *trn*L was subjected to restriction analysis using AluI, AvaII, BstnI, DraI, HinfI, SauIIIA and TaqI enzymes. The product of *cox*I gene was digested with AluI, HinGI, HinfI, SauIIIA and TaqI and the product of *orf25* gene was additionally digested with AvaII, BstnI, HindII and HpaII. Following the digestion, the samples were separated in 5-8% polyacrylamide gel at 5V/cm, in 1xTBE buffer (0.1M Tris-HCl, 1 mM EDTA, pH 8.3) (Sambrook et al. 1989) and pUC19/HaeIII, TaqI was used as a molecular weight marker. Following staining with ethidium bromide, the gels were analysed by UV light.

RESULTS

The amplification products obtained for the *trn*F-*trn*L region of *cp*DNA have a length of about 950 bp and those for the *orf*25 and *cox*I regions of *mt*DNA are around 500 bp and 700 bp respectively. All restriction products of amplified *orf*25 and *cox*I genes of *mt*DNA have shown no difference between Scots and dwarf mountain pine. The restriction patterns of *trn*F-*trn*L region are also identical to all restriction enzymes used with the exception of DraI and HinfI. One restriction site to DraI has been detected for *P. mugo* and none for *P. sylvestris,* which resulted in two bands of the digested DNA in *P. mugo* contrasting to one band of undigested product in *P. sylvestris* (Fig. 1). Additionally, following the digestion of *trn*F-*trn*L product with HinfI, the difference has been detected in the fragments of approximately 50 bp in length (Fig. 2). These two differences are correlated and form two distinct haplotypes – haplotype S, characteristic for all studied samples of *P. sylvestris* and haplotype M, restricted to *P. mugo* (Table 1).

DISCUSSION

Polymorphism of plastid and mitochondrial DNA has been explored recently in many studies on hybridisation and introgression in gymnosperms, including pines (Wagner 1992). Both genomes are inherited in the paternal (*cp*DNA) or maternal (*mt*DNA) lines and do not undergo sexual recombi-

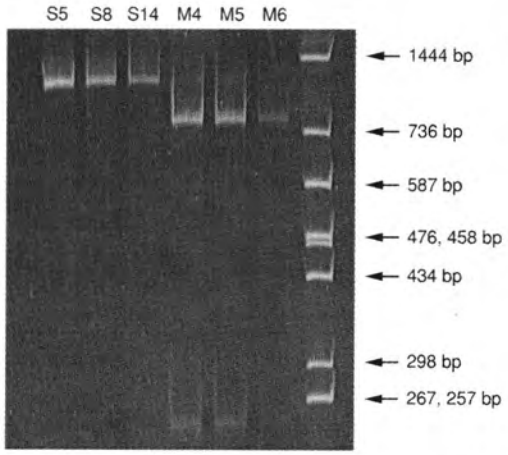

Fig. 1. Restriction analysis of PCR amplified region between *trn*F and *trn*L genes of plastid DNA of Scots pine (S) and dwarf mountain pine (M) using DraI restriction enzyme.

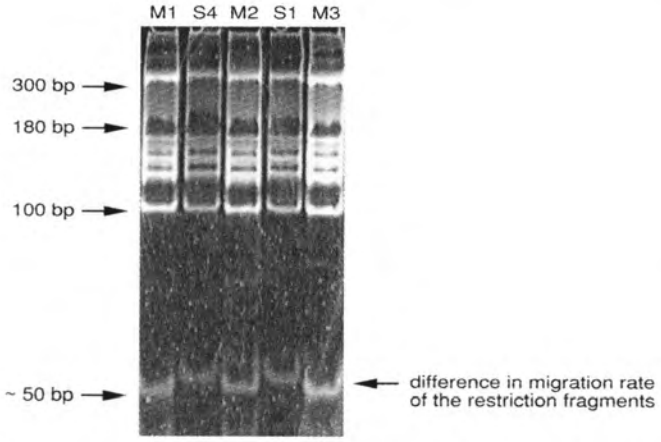

Fig. 2. Restriction analysis of PCR amplified region between *trn*F and *trn*L genes of plastid DNA of Scots pine (S) and dwarf mountain pine (M) using HinfI restriction enzyme.

nation (Neale and Sederoff 1989, Hipkins et al. 1994). These unique genetic systems offer a better chance to evaluate the direction and the intensity of hybridisation and gene flow than those provided by the recombining nuclear markers. Having a pair of species specific markers, one from *cp*DNA and one from *mt*DNA, it is possible to follow the gene flow between parents and the presence of markers in hybrids in natural populations. Among others, this approach has been used in the analysis of the hybridisation and introgression between *Picea glauca*, *P. engelmannii* and *P. sitchensis* (Sutton et al. 1991) and between *Pinus pumila* and *P. pentaphylla* (Watano et al. 1995, 1996).

In the first studies of this type, the restriction fragment length polymorphism (RFLP) of the entire *cp*DNA has been used to analyse hybridisation between *Pinus contorta* and *P. banksiana* (Wagner et al. 1987) and between *Pinus tabulaeformis* and *P. yunanensis* (Wang and Szmidt 1990). Hybridisation between *P. mugo* and *P. sylvestris* in some putative hybrid populations from Bohemia and Slovakia has also been tested by using RFLP analysis of *cp*DNA (Fillipula et al. 1992). This method is, however, relatively expensive and time-consuming as it involves DNA hybridisation steps. The simplest approach involves comparison of the restriction pattern of amplification products of specific DNA regions (PCR-RFLP) to detect species-specific differences that are based on gains or losses of restriction sites (Ziegenhagen, Fladung 1998). PCR-RFLP markers of *cp*DNA have successfully been used to find molecular differences between *P. halepensis* and *P. brutia* (Boscherini et al. 1994) and in the studies of hybridization between *P. taeda* and *P. echinata* (Edwards-Burke et al. 1997).

In this study a marker (gain/loss of restriction site for DraI within *trn*F-*trn*L region of *cp*DNA) has been discovered and the marker differentiates between closely related dwarf mountain and Scots pines. Another difference (HinfI restriction pattern of the same product) probably represents insertion-deletion of a small fragment of DNA. However, the use of the second marker may encounter some technical difficulties to bring it out in analyses because this distinction is related to the slight difference in the migration of small fragments of DNA.

Since the plants used in our study include individuals from various geographic regions, the detected differences in haplotypes seem to be species specific and suitable for detection of natural hybrids between *P. mugo* and *P. sylvestris*. In order to confirm the result, we plan to enlarge the number of samples especially that of *P. mugo* as well as to study F1 hybrids from controlled crosses (Prus-Głowacki, Stephan 1998) to verify the predicted uniparental inheritance of these mutations.

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LITERATURE CITED

BOBOWICZ M.A. 1990. Hybrids of *Pinus mugo* Turra × *Pinus sylvestris* L. from the Bór na Czerwonem peatbog in Nowy Targ Valley (in Polish). Wyd. Nauk. UAM, Poznań.

BOSCHERINI G., MORGANTE M., ROSSI P., VENDRAMIN G.G. 1994. Allozyme and chloroplast DNA variation in italian and greek population of *Pinus leucodermis*. Heredity 73: 284-290.

BRANDT P., SUNKEL S., UNSELD M., BRENNICKE A., KNOOP V. 1992. The nad4L gene is encoded between exon c of nad5 and orf25 in the *Arabidopsis* mitochondrial genome. Molecular and General Genetics 236: 33-38.

CHRISTENSEN K., DAR G.H. 1997. A morphometric analysis of spontaneous and artificial hybrids of *Pinus mugo* x *P. sylvestris* (Pinaceae). Nordic Journal of Botany 17: 77-86.

CHRISTENSEN K. 1987. A morphometric study of the *Pinus mugo* Turra complex and its natural hybridization with *P. sylvestris* L. (Pinaceae). Fesses Repertorium 98 11-12: 623-635.

DOBRINOV I. 1965. Prinos kam izučavanie na estestvenite chibridi meždu belija bor (*Pinus sylvestris* L.) i kleka (*Pinus montana* v. mughus Willk.) v Balgaria. Nauč. Tr. 13: 39-48.

EDWARDS-BURKE M. A., HAMRICK J.L., PRICE R.A. 1997. Frequency and direction of hybridization in sympatric populations of *Pinus taeda* and *P. echinata* (Pinaceae). Am. J. Bot. 84, 7: 879-886.

FILLIPULA S., SZMIDT A.E., SAVOLAINEN O. 1992. Genetic comparison between *Pinus sylvestris* and *P. mugo* using isozymes and chloroplast DNA. Nord. J. Bot. 12: 381-386.

HIPKINS V.D., KRUTOVSKII K.V., STRAUSS S.H. 1994. Organelle genoms in conifers: structure, evolution and diversity. Forest Genetics 1, 4: 179-189.

MARCET E. 1967. Über den Nachweis spontaner Hybriden von *Pinus mugo* Turra und *Pinus sylvestris* auf Grund von Nedelmerkmalen. Ber.Schweiz. Bot. Ges. 77: 314-361.

MARCZEWSKI A. 1993. Dwarf mountain pine population from Tatra National Park in the Botanical Garden of the Polish Academy of

Sciences in Warsaw–Powsin (in Polish), pp. 47-52. In: Cichocki W. (Ed.): Protection of Tatra mountains in view of the threats (in Polish). Wydawnictwo Muzeum Tatrzańskiego, Zakopane.

MURRAY M.G., THOMPSON W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8: 4321-4325.

NEALE D.B., SEDEROFF R.R. 1989. Paternal inheritance of chloroplast and maternal inheritance of mitochondrial DNA in loblolly pine. Theor. Appl. Genet. 77: 212-216.

NEET-SARQUEDA C. 1994. Genetic differentiation of *Pinus sylvestris* L. and *Pinus mugo* aggr. populations in Switzerland. Silvae. Genet. 43, 4: 207- 215.

PRUS-GŁOWACKI W., STEPHAN B.R. 1998. Immunochemical and isoenzymatic characterization of hybrids from controlled crosses between *Pinus montana* var. *rostrata* and *Pinus sylvestris*. Forest Genetics 5, 3: 155-163.

PRUS-GŁOWACKI W., SZWEYKOWSKI J. 1983. Studies on isoenzyme variability in populations of *Pinus sylvestris* L., *Pinus mugo* Turra, *Pinus uliginosa* Neumann and individuals from a hybrid swarm population. Bull. Sci. Amis. Poznań D, 22: 107-122.

PRUS-GŁOWACKI W., SADOWSKI J., SZWEYKOWSKI J., WIATROSZAK I. 1981. Quantitative and qualititative analysis of needle antigens *Pinus sylvestris*, *Pinus mugo*, *Pinus uliginosa* and *Pinus nigra* and some individuals from a hybrid swarm population. Genet. Polon. 22, 4: 447-454.

PRUS-GŁOWACKI W., SZWEYKOWSKI J. 1980. Serological characteristics of some putative hybrid individuals from a *Pinus sylvestris* × *P. mugo* swarm population. Acta Soc. Bot. Pol. 49, 1-2: 127-142.

PRUS-GŁOWACKI W., SZWEYKOWSKI J., SADOWSKI J. 1978. Studies on serological similarity of *Pinus sylvestris* L., *P. mugo* Turra and individuals from a hybrid swarm population. Genet. Polon. 19, 3: 321-338.

SAMBROOK J., FRITSCH E.F., MANIATIS T. 1989. Molecular cloning. Á laboratory manual. Second edition. Cold Spring Harbor Laboratory Press.

SIEDLEWSKA A., PRUS-GŁOWACKI W. 1994. Allozyme variability in *Pinus mugo* Turra population from Topieliska peat-bog near Zieleniec. Genet. Polon. 35, 4: 285-301.

STASZKIEWICZ J. 1994. Variability of *Pinus mugo* × *P. sylvestris* (Pinaceae) hybrid swarm in the Tisovnica nature reserve (Slovakia). Polish Bot. Stud. 5: 33-41.

STASZKIEWICZ J., TYSZKIEWICZ M. 1972. Variability of natural hybrids of *Pinus sylvestris* L. × *Pinus mugo* Turra (=*P. rotundata* Link) in South-West Poland and in the selected stands in Bohemia and Moravia (in Polish). Fragm. Flor. Geobot. 18, 2: 173-191.

STASZKIEWICZ J., TYSZKIEWICZ M. 1969. Natural hybrids of *Pinus mugo* Turra × *Pinus sylvestris* L. in Nowy Targ Valley (in Polish). Fragm. Flor. Geobot. 15, 2: 187-212.

SUTTON B.C.S., FLANAGAN D.J., GAWLEY J.R., NEWTON C.H., LESTER D.T, EL KASSABY Y.A. 1991. Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. Theor. Appl. Genet. 82: 242-248.

STRAUSS S.H., BOUSQUETT A., HIPKINS V.D., HONG Y.P. 1992. Biochemical and molecular genetic markers in biosystematic studies of forest trees. New Forests 6:125-158.

TABERLET P., GIELLY L., PAUTON G., BOUVET J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17: 1105-1109.

WAGNER D.B. 1992. Nuclear, chloroplast, and mitochondrial DNA polymorphism as biochemical markers in population genetic analyses of forest trees. New Forests 6: 373-390.

WAGNER D.B., FURNIER G.R., SAGHAI-MAROOF M.A., WILIAMS S.M., DANCIK P.B., ALLARD R.W. 1987. Chloroplast DNA polymorphism in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci. USA 84: 2097-2100.

WANG X.R., SZMIDT A. 1990. Evolutionary analysis of *Pinues densata* (Masters), a putative Tertiary hybrid. Theor. Appl. Genet. 80: 641-647.

WATANO Y., IMAZU M., SHIMIZU T. 1996. Spatial distribution of *cp*DNA and *mt*DNA haplotypes in a hybrid zone between *Pinus pumila* and *P. parviflora* var. *pentaphylla* (Pinaceae) J. Plant Res. 109: 403-408.

WATANO Y., IMAZU M., SHIMIZU T. 1995. Chloroplast DNA typing by PCR-SSCP in the *Pinus pumila-P. parviflora* var. *pentaphylla* complex (Pinaceae). J. Plant Res. 108: 493-499.

VIEWEGH J., CAMBALOVA H. 1993. Variabilita hybridnéeho roja Pinus x celakovkiorum A. et. Gr. posudzovaná niekolkými metódami zhlukovej analyzý. Biológia (Bratislava) 48: 39-44.

YURUKOV S., TASCHEW A. 1992. Studies of natural hybrids between Scots pine (*Pinus sylvestris* L.) and Mountain pine (*Pinus mugo* Turra) in the South-east Rila Mts. Nauka za Gorata 29: 39-43.

ZIEGENHAGEN B., FLADUNG M. 1998. PCR-RFLP analysis of chloroplast DNA of various gymnosperms: A rapid tool for assessing genetic variation at the above-species level. pp. 403-406. In: Karp A., Isaac P.G, Ingram D.S.(eds) Molecular Tools for Screening Biodiversity.

SPECYFICZNE GATUNKOWO MARKERY *cp*DNA PRZYDATNE W BADANIACH NAD HYBRYDYZACJĄ POMIĘDZY *P. MUGO* I *P. SYLVESTRIS*

STRESZCZENIE

Celem znalezienia specyficznych gatunkowo mutacji organellowego DNA dla kosodrzewiny (*Pinus mugo*) i sosny zwyczajnej (*P. sylvestris*) zastosowano analizę PCR-RFLP. Porównano obrazy restrykcyjne produktów amplifikacji rejonu *trn*L-*trn*F *cp*DNA oraz genów *cox*I i *orf*25 *mt*DNA.

Wykryto różnicę w obrazie restrykcyjnym produku amplifikacji rejonu *trn*L-*trn*F *cp*DNA dla enzymów DraI i HinfI i występowanie dwóch haplotypów. Haplotyp M jest charakterystyczny dla kosodrzewiny, a haplotyp S dla wszystkich zbadanych osobników sosny. Markery te mogą być przydatne do analizy hybrydyzacji i introgresji pomiędzy tymi gatunkami postulowanej na podstawie analizy morfologicznej. Nie znaleziono różnic międzygatunkowych dla badanych rejonów mitochondrialnego DNA.

SŁOWA KLUCZOWE: PCR-RFLP, *cp*DNA, *mt*DNA, *Pinus sylvestris, Pinus mugo,* hybrydyzacja.