DENDROBIOLOGY
2004, vol. 51, 67–72

Witold Wachowiak, Alina Bączkiewicz,
Konrad Celiński, Wiesław Prus-Głowacki

Species-specific chloroplast DNA polymorphism in the *trn*V-*rbc*L region in *Pinus sylvestris* and *P. mugo*

Received: 20 February 2004, Accepted: 26 March 2004

Abstract: Four *cp*DNA regions were analyzed with the use of PCR-RFLP technique and nucleotide sequences of two *mt*DNA regions were characterized in order to find *P. sylvestris* and *P. mugo* species specific markers useful for studies of the species hybridization. The difference in the restriction fragment patterns of *trn*V-*rbc*L region after digestion with *Mva*I endonuclease was detected. The analyses of the species representatives from various geographic regions revealed that the observed polymorphism is species specific. No differences have been disclosed in the analyzed *trn*S-*trn*T, *trn*K1-*trn*K2, *trn*C-*trn*D *cp*DNA regions. The *P. sylvestris* and *P.mugo* *mt*DNA sequences of *orf*25 and *cox*I regions proved to be identical.

Additional key words: Scots pine, dwarf pine, hybridisation, DNA markers, *mt*DNA,

Addresses: W. Wachowiak, Polish Academy of Science, Institute of Dendrology, ul. Parkowa 5, 62-035 Kórnik, Poland wachwit@amu.edu.pl
A.Bączkiewicz, Department of Genetics, Institute of Experimental Biology, Adam Mickiewicz University, Międzychodzka 5, 60-371 Poznań, Poland
K.Celiński, Department of Genetics, Institute of Experimental Biology, Adam Mickiewicz University, Międzychodzka 5, 60-371 Poznań, Poland
W. Prus-Głowacki, Department of Genetics, Institute of Experimental Biology, Adam Mickiewicz University, Międzychodzka 5, 60-371 Poznań, Poland

Introduction

The mechanism of uniparental inheritance of organelle genomes may be applied in the studies on hybridisation and introgression between closely related forest tree species. In conifers, the plastid genome is transmitted paternally whereas the mitochondrial genome in the maternal line (Neale et al. 1986; Neale and Sederoff 1988). The use of species specific chloroplast (*cp*DNA) and mitochondrial (*mt*DNA) markers allows for identification of hybrids displaying the genomes from different species. These studies also enable to define the direction and intensity of hybridisation and its influence on the genetic structure of the species sympatric populations. The markers of organelle genomes have been applied in the analyses of hybridisation processes among the species of *Abies* (Isoda et. al. 2000), *Picea* (Bobola et. al.1996; Perron and Bousquet 1997; Germano and Klein 1999) or *Pinus* (Wagner et. al. 1987; Wang and Szmidt 1990).

Biometric and biochemical (isozymes) analyses of the individuals from Scots pine (*Pinus sylvestris*) and dwarf pine (*P. mugo*) sympatric stands have not resulted in formulating one coherent hypothesis about

the postulated processes of the species hybridisation (Prus-Głowacki and Szweykowski 1983; Staszkiewicz 1993; Siedlewska and Prus-Głowacki 1994). The estimation of the hybridisation intensity vary from rare formation of hybrids (Neet-Sarqueda et al. 1988; Christensen and Dar 1997) to the formation of hybrid swarms, consisting of F1 hybrids, hybrids of subsequent generations and introgressants (Staszkiewicz and Tyszkiewicz 1969; Bobowicz 1990; Vievegh and Cambalowa 1993). These different opinions result from the absence of diagnostic features for particular species, which would allow for certain hybrids identification. Considering the absence of biometrical and biochemical diagnostic traits the application of *cp*DNA and *mt*DNA markers specific to the analyzed species seems to be a good approach aimed at verifying the hypotheses about the frequency and the direction of the species hybridization.

So far there have been described species specific for *P. sylvestris* and *P. mugo cp*DNA markers in the *trn*F-*trn*L region (Wachowiak et al. 2000). The present study is the continuation of the analyses aiming at *mt*DNA markers development and detecting additional differences in the *cp*DNA regions which would be useful to prove the gene exchange among the species.

Methods

The *P. sylvestris* and *P. mugo* representatives used in this study have been derived from different geographical regions (Table 1). *P. sylvestris* S1-S20 individuals (apart from S9) come from the Scots pine 1982 provenience trail in the Institute of Dendrology, Polish Academy of Science in Zwierzyniec near Kórnik, coordinated by IUFRO (International Union of Forest Research Organization). The trial consists of 20 populations representing the European range of *P. sylvestris* (Boratyński 1991). The S9 *P. sylvestris* individual was collected in Uścikuwiec. The M1-M14 *P. mugo* was derived from the populations in the Botanical Garden of the Polish Academy of Science, in Warsaw – Powsin and they originate from the natural stands in the Tatra Mts. (Marczewski 1993). The plant material from the remaining *P. mugo* individuals were collected in their natural populations (Table 1).

The needles from trees (ca. 100 mg of fresh material) undergone DNA extraction using the method described by Dumolin et al. (1995). Four *cp*DNA and two *mt*DNA regions were amplified with the use of the primers described in Table 2. PCR was run in a Personal Cycler (PTC-200, MJ Research, USA). PCR-amplification was carried out in a total volume of 25 μl containing about 20 ng of template DNA, 2.5 mM $MgCl_2$, 100 μM of each dNTP, 0.2μM of each primer and 0.25 U of *Taq* polymerase with the respective 1× PCR buffer (*Taq* polymerase and 10× PCR buffer were purchased from Fermentas, Lithuania). Preliminary denaturation of DNA for 5 min at 95°C was followed by 30 cycles of denaturation at 93°C for 30 sec, primer annealing for 45 sec at 57.5° (for *trn*V-*rbc*L, *trn*C-*trn*D regions) or 53°C (for *trn*S-*trn*T, *trn*K1-*trn*K2, *orf*25 and *cox*I regions), DNA synthesis at 72°C for 2 min for *cp*DNA and 1 min for *mt*DNA primers. The reaction was terminated with 10 min incubation at 72°C.

PCR-RFLP analyses of the amplified *cp*DNA regions were performed with the use of restriction enzymes described in Table 3. The enzymes applied for digestion of each region were derived in a random selection. Two *P. sylvestris* and two *P. mugo* individuals were chosen for the restriction analyses. In case of detecting the interspecies specific differences of the investigated regions the remaining individuals used in the study were also analysed. 10 μl of PCR product underwent digestion, following the reaction conditions recommended by the enzyme provider (Fermentas, Lithuania). After digestion, the samples were separated in 8 % polyacrylamide gel (Sambrook et al. 1989), stained with ethidium bromide and analysed by UV light.

The PCR product of *orf*25 and *cox*1 *mt*DNA were purified with the use of QIAquick™PCR Purification Kit (Qiagen) and sequence characterized in one *P. sylvestris* (S12) and one *P. mugo* (M8) individuals. Sequencing was performed through automatic sequencer (PerkinElmer) with the use of Big Dye Terminator DNA Sequencing Kit (Applied Biosystems) according to a protocol by Liepelt et al. (2001).

Results

The PCR products of *orf*25 and *cox*1 *mt*DNA regions have a length of about 500 bp and 700 bp, respectively. The sequences of *orf*25 *mt*DNA region were submitted to Gene Bank database (http://www.ncbi.nlm.nih.gov) and they have the following accession numbers: AF361058 for *P. mugo* and AF361059 for *P. sylvestris*. The *P. sylvestris* sequences of *cox*I and *orf*25 regions have proved no difference comparing to the corresponding sequence of *P. mugo*. The *cox*I sequences of both species were identical with the *cox*I sequence of *P. sylvestris* from Gene Bank database (AJ000354).

The amplification products of *trn*V-*rbc*L, *trn*S-*trn*T, *trn*K1-*trn*K2, *trn*C-*trn*D *cp*DNA regions have the length of about 4000 bp, 1700 bp, 2200 bp and 2000 bp, respectively. The PCR-RFLP analyses of *trn*V-*rbc*L *cp*DNA region digested with the use *Mva*I endonuclease indicated the length differences of the obtained DNA fragments between *P. sylvestris* and *P. mugo*. The length of fragments differentiating both species is about 400bp and 500bp for *P. sylvestris*, and 440bp and 460bp for *P. mugo*. All the analysed *P. sylvestris* individuals (Table 1) have displayed type S

Table 1. The origin and number of the analysed *P. sylvestris* and *P. mugo* individuals

No.	The origin	Country	Latitude	Longitude	Meters above sea level	Number of analysed trees
			Pinus sylvestris			
S 1	Roshchinskaya Dacha	Russia	60°15'	29°54'	80	1
S 2	Kondezhskoe	Russia	59°58'	33°30'	70	1
S 3	Serebryanskoe	Russia	58°50'	29°07'	80	1
S 4	Silene	Latvia	55°45'	26°40'	165	1
S 5	Miłomłyn	Poland	53°34'	20°00'	110	1
S 6	Supraśl	Poland	53°12'	23°22'	160	1
S 7	Spała	Poland	51°37'	20°12'	160	1
S 8	Rychtal	Poland	51°08'	17°55'	190	1
S 9	Uścikuwiec	Poland	52°40'	16°50'	90	1
S 10	Neuhaus	Germany	52°24'	13°54'	40	1
S 11	Betzhorn	Germany	53°02'	10°30'	650	1
S 12	Lampertheim	Germany	52°30'	10°00'	95–100	1
S 13	Ardennes	Belgium	50°46'	4°26'	110	1
S 14	Haguenau	France	48°49'	7°47'	130–180	1
S 15	Sumpberget	Sweden	60°11'	15°52'	185	1
S 16	Zahorie	Slovenia	48°46'	17°03'	160	1
S 17	Pornoapati	Hungary	47°20'	16°28'	400	1
S 18	Maocnica	Serbia	43°10'	19°30'	1200	1
S 19	Prusacka Rijeka	Bosnia	44°05'	17°21'	800–970	1
S 20	Catacik	Turkey	40°00'	31°10'	1380–1420	1
			Pinus mugo			
M1-M14	Tatra Mts.	Poland	58°35'	14°45'	1700	14
Śn 1–3	Sudet Mts.	Poland	50°49'	15°46'	1500	3
U 1–2[1]	Urlea	Romania	24°80'	45°70'	2470	2

Table 2. The PCR primer sequences of the analyzed *cp*DNA and *mt*DNA regions

cpDNA region	**5'–3' sequence of primer 1**	**5'–3' sequence of primer 2**	**References**
*trn*V-*rbc*L	cga acc gta gac ctt ctc gg	gct tta gtc tct gtt tgt gg	Dumolin et al. 1997
*trn*S-*trn*T	cga ggg ttc gaa tcc ctc tc	aga gca tcg cat ttg taa tg	Demesure et al. 1995
*trn*K1-*trn*K2	ggg ttg ccc ggg act cga ac	caa cgg tag agt act cgg ctt tta	Demesure et al. 1995
*trn*C-*trn*D	cca gtt caa atc tgg gtg tc	ggg att gta gtt caa ttg gt	Demesure et al. 1995
mtDNA region	**5'–3' sequence of primer 1**	**5'–3' sequence of primer 2**	**References**
*orf*25	atg cta ttt gct gct att cc	agg act atc aag cct tct cg	Wang et al. 1996
*cox*I	tta tta tca ctt ccg gta ct	agc atc tgg ata atc tgg	Glaubitz and Carlson 1992

haplotype, whereas all the *P. mugo* individuals have possessed type M haplotype (Fig. 1). PCR-RFLP analysis of *trn*V-*rbc*L region, which applied the other restriction enzymes, as well as the analysis of the *trn*S-*trn*T, *trn*K1-*trn*K2, *trn*C-*trn*D *cp*DNA regions presented in Table 3, have not shown any differences of the restriction patterns between *P. sylvestris* and *P. mugo*.

Discussion

The high homology of the plastid genome of higher plants, which has been supported by the comparative analyses of the complete *cp*DNA sequences of several species, has enabled to describe some universal PCR primers allowing the amplification of the corresponding DNA regions even in the group of phylogenetically distinct taxa (Taberlet et al. 1991; Demesure et al. 1995). The four previously described pairs of PCR primers designed for the amplification of non-coding regions between *t*RNA genes have been applied in the presented study. Since the level of mutation observable in these regions is significantly higher than in conservative coding sequences, these regions have been regarded as particularly useful ones in the analyses of species variability (Dumolin-Lapegue et al. 1997).

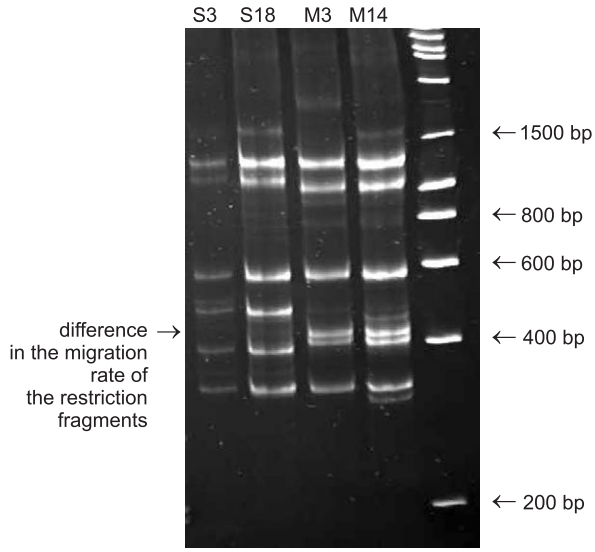

Fig. 1. 8.0 % polyacrylamide gel of PCR products as amplified from the chloroplast DNA region *trn*V-*rbc*L and digested with the restriction enzyme *Mva*I. *P. sylvestris* individuals (S3, S18) exhibit a distinct different banding pattern (haplotype S) as compared with the *P. mugo* individuals (M3, M14) (haplotype M). Right line of the gel – molecular size standard (Smart-Ladder, Eurogentec, Germany)

The PCR-RFLP analyses conducted with the application of different restriction enzymes have resulted in detecting the polymorphism of *trn*V-*rbc*L region differentiating *P. sylvestris* and *P. mugo*. The observable differences in the electrophoresis migration rate of the obtained DNA fragments may be correlated with the presence of point mutations in two sites of the species sequences which cause the loss/gain of the restriction site for *Mva*I enzyme. The existence of the restriction sites in different parts of the amplified regions of both species leads to DNA fragment length polymorphism and does not influence the number of obtained fragments.

The identical restriction patterns for the other analysed *cp*DNA regions indicate the absence of deletion/insertion differences which could be detected by the applied DNA electrophoresis in polyacrylamide gel. In these regions, however, there can still occur nucleotide substitution that have not been discovered. The comparative analyses of *cp*DNA sequences of numerous *Pinaceae* representatives indicate the relatively high rate of nucleotide substitution in the inter genic regions in relation to the parts of coding regions subjected to stronger selective effects (Ziegenhagen and Fladung 1997; Wang et al. 1999).

In spite of the conservative character of genes and a relatively low level of the sequence polymorphism in the group of even phylogenetically distinct taxa, the cases of intra-species variability of *cp*DNA regions have been detected, among others, for *Abies alba* (Ziegenhagen et al. 1995), *Quercus robur* (Dumolin et al. 1995), *Fagus sylvatica* (Demesure et al. 1996) or *Nothofagus nervosa* (Marchelli et al. 1998). In the present study, the restriction analysis of the *trn*V-*rbc*L region carried out with the use of *Mva*I enzyme for individuals from different geographical regions has proved the presence of S haplotype in all *P. sylvestris* individuals and M haplotype in all *P. mugo* individuals. This result indicates that the observed differences are species-specific and allow for distinguishing *P. sylvestris* from *P. mugo* based on plastid DNA.

In spite of the highly conserved character of the *mt*DNA sequences in higher plant, the examples of nucleotide substitutions in the coding regions were indicated. Due to the observable mechanism of RNA editing these differences do not effect the changes of the peptide amino acids component and do not undergo the high selection influences (Glaubitz and Carlson 1992; Wang and Szmidt 2001). Therefore, the PCR-RFLP analyses of the *orf*25 and coxI *mt*DNA regions conducted before, which have not indicated the sequence differences between *P. sylvestris* and *P. mugo*, have not excluded the existence of undetected point mutations (Wachowiak et al. 2000). Nevertheless, the presented results indicate that the *P. sylvestris* sequences of these regions are identical with the corresponding sequences of *P. mugo*. Thus, these regions are not useful for developing the species specific *mt*DNA markers.

The differences in the *trn*V-*rbc*L region detected in this study together with previously described for *P. sylvestris* and *P. mugo cp*DNA species-specific markers proved to be useful in the hybridisation analyses of both species. Paternally transmitted *cp*DNA markers particularly allow indicating the present episodes of hybridisations in case of the detected inconsistency of the species-specific *cp*DNA haplotypes of the parental tree and its F1 progeny. Nevertheless, it is still necessary to develop species specific *mt*DNA markers, which would allow for more precise analyses of the species hybridisation and identification of hybrid trees.

Table 3. *cp*DNA regions conducted to the PCR-RFLP analyses

The amplified *cp*DNA regions	The applied restriction enzymes
*trn*V-*rbc*L	*Mbo*I, *Msp*I, *Mva*I, *Pst*I, *Sma*I
*trn*S-*trn*T	*Alu*I, *Bsh*1236I, *Bsu*RI, *Dra*I, *Hinf*I, *Hin*6I, *Rsa*I *Sau*3A
*trn*K1-*trn*K2	*Bsp*120I, *Eco*32I, *Hind*III, *Mbo*I, *Msp*I, *Mva*I, *Pst*I
*trn*C-*trn*D	*Alu*I, *Apa*I, *Bsp*120I, *Bsu*RI, *Dra*I, *Eco*RI, *Eco*32I, *Eco*471, *Hae*III, *Hin*6I, *Hind*III, *Hinf*I, *Kpu*I, *Mbo*I, *Msp*I, *Pst*I, *Rsa*I, *Sac*I, *Sma*I

Conclusions

1) The PCR-RFLP analyses allow indicating the differences between *P. sylvestris* and *P. mugo* in the *trn*V-*rbc*L *cp*DNA region digested with the use of *Mva*I restriction enzyme
2) The analyses of the species representatives from different geographical regions have proved that the observed differences in the *trn*V-*rbc*L region are species-specific and useful in the studies of hybridisation between *P. sylvestris* and *P. mugo*
3) No deletion/insertion or nucleotide substitutions were detected in the *trn*S-*trn*T, *trn*K1-*trn*K2 and *trn*C-*trn*D *cp*DNA regions of *P. sylvestris* and *P. mugo*
4) No differences were detected in the corresponding sequence of *orf*25 and *cox*I *mt*DNA of *P. sylvestris* and *P. mugo*

Acknowledgements

Thanks are given to Sascha Liepelt from Nature Conservation Division, Faculty of Biology, Philipps-University Marburg, Germany for his help in DNA sequencing. The research was supported by the State Committee for Scientific Research, Poland (KBN Grant no. 0306/P04/2001/21).

Referencess

Bobola M.S., Eckert R.T., Klein A.S. 1996. Hybridization between Picea rubens and Picea mariana: differences observed between mountain and costal island populations. Canadian Journal of Forest Research 26: 428–432.

Bobowicz M.A. 1990. Mieszańce *Pinus mugo* Turra × *Pinus sylvestris* L. z rezerwatu „Bór na Czerwonem" w Kotlinie Nowotarskiej. Wydawnictwo Naukowe UAM, Poznań.

Boratyński A. 1991. Range of natural distribution. In: M. Giertych, C. Matyas (ed.) Genetics of Scots Pine. Akademia Kiado, Budapest.

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.

Demesure B., Sodzi N., Petit R.J. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Molecular Ecology 4: 129–131.

Demesure B., Comps B., Petit R.J. 1996. Chloroplast DNA phylogeography of the common beetch (*Fagus sylvatica* L.) in Europe. Evolution 50: 2515–2520.

Dumolin S., Demesure B., Petit R.J. 1995. Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. Theoretical and Applied Genetics 91: 1253–1256.

Dumolin-Lapegue S., Pemonge M.H., Petit R.J. 1997. An enlarged set of consensus primers for the study of organelle DNA in plants. Molecular Ecology 6: 393–397.

Germano J., Klein A.S. 1999. Species-specific nuclear and chloroplast single nucleotide polymorphism to distinguish *Picea glauca*, *P. mariana* and *P. rubens*. Theoretical and Applied Genetics 99: 37–49.

Glaubitz J.C., Carlson J.E. 1992. RNA editing in the mitochondria of a conifer. Current Genetics 22: 163–165.

Isoda K., Shiraishi S., Watanabe S., Kitamura K. 2000. Molecular evidence of natural hybridization between *Abies veitchii* and *A. homolepsis* (Pinaceae) revealed by chloroplast, mitochondrial and nuclear DNA markers. Molecular Ecology 9: 1965–1974.

Liepelt S., Kuhlenkamp V., Anzidei M., Vendramin G.G., Ziegenhagen B. 2001. Pitfalls in determining size homoplasy of microsatellite loci. Molecular Ecology Notes 1: 332–335.

Marchelli P., Gallo L., Scholz F., Ziegenhagen B. 1998. Chloroplast DNA markers reveal a geographical divide across Argentinean southern beech *Nothofagus nervosa* (Phil.) Dim. et Mil. distribution area. Theoretical and Applied Genetics 97: 642–646.

Marczewski A. 1993. Populacja kosodrzewiny z Tatrzańskiego Parku Narodowego w Ogrodzie Botanicznym PAN w Warszawie – Powsinie. Wydawnictwo Muzeum Tatrzańskiego w Zakopanem, pp. 47–52.

Neale D.B., Wheeler N.C., Allard R.W. 1986. Paternal inheritance of chloroplast DNA in Douglas-fir. Canadian Journal of Forest Research 16: 1152–1154.

Neale D.B., Sederoff R.R. 1988. Inheritance and evolution of conifer organelle genomes. In: Genetic Manipulation of Woody Plants. Hanover J.T., Keathley D.E. (ed.). Plenum Press, New York, pp. 251–254.

Neet-Sarqueda C., Plumettaz Clor A.C., Becholey I. 1988. Mise en evidence de l'hybridation introgressive entre *Pinus sylvestris* L. et *Pinus uncinata* DC. en Valais (Suisse) par deux methods multivariees. Botanica Helvetica 98: 161–169.

Perron M., Bousquet J. 1997. Natural hybridization between black spruce and red spruce. Molecular Ecology 6: 725–734.

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. Bulletin De La Societe Des Amis Des Sciences et Des Lettress De Poznań 22: 107–122.

Sambrook J., Fritsch E.F., Maniatis T. 1989. Molecular Cloning: A Laboratory Manual (2nd edn). Cold Spring Harbor Laboratory Press, New York.

Siedlewska A., Prus-Głowacki W. 1994. Allozyme variability of putative hybrid swarm population (*Pinus mugo* Turra × *P. sylvestris* L.) from Topielisko peat – bog near Zieleniec. Genetica Polonica 35: 285–302.

Staszkiewicz J., Tyszkiewicz M. 1969. Naturalne mieszańce *Pinus mugo* Turra x *Pinus silvestris* L. w Kotlinie Nowotarskiej. Fragmenta Floristica et Geobotanica 15 (2): 187–212.

Staszkiewicz J. 1993. Variability of *Pinus mugo* × *P.sylvestria* (*Pinaceae*) hybrid swarm in the Tisovnica nature reserve (Slovakia). Polish Botanical Studies 5: 33–41.

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.

Viewegh J., Cambalowa H. 1993. Variableness of hybrid swarm *Pinus celekovskiorum* A. et Gr. processed with some cluster analyses methods. Biologia Bratislava 48: 39–44.

Wachowiak W., Leśniewicz K., Odrzykoski I., Augustyniak H., Prus-Głowacki W. 2000. Species specific *cp*DNA markers useful for studies on the hybridization between *Pinus mugo* × *P.sylvestris*. Acta Societatis Botanicorum Poloniae 69: 273–276.

Wagner D.B., Furnier G.R., Saghai-Maroof M.A., Williams S.M., Dancik B.P., Allard R.W. 1987. Chloroplast DNA polymorphism in lodgepole and jack pines and their hybrids. Proceedings of the National Academy of Sciences of the USA 84: 2097–2100.

Wang X.R., Szmidt A.E. 1990. Evolutionary analysis of *Pinus densata* (Masters), a putative Tertiary hybrid. Theoretical and Applied Genetics 80: 641–647.

Wang X.R., Szmidt A.E., Lu M.Z. 1996. Genetic evidence for the presence of cytoplasmic DNA in pollen and megagametophytes and maternal inheritance of mitochondrial DNA in Pinus. Forest Genetics 3 (1): 37–44.

Wang X.R., Tsumura Y., Yoshimira H., Nagasaka K., Szmidt A.E. 1999. Phylogenetic relationship of Eurasian pines (Pinus, Pinaceae) based on chloroplast *rbc*L, *mat*K, *rps*20-*rps*18 spacer and *trn*V intron sequences. American Journal of Botany 86: 1742–1753.

Wang X.R., Szmidt A. 2001. Molecular Markers in Population Genetics of Forest Trees. Scandinavian Journal of Forest Research 16: 199–220.

Ziegenhagen B., Kormutak A., Schauerte M., Scholz F. 1995. Restriction site polymorphism in chloroplast DNA of silver fir (*Abies alba* Mill.). Forest Genetics 2: 99–107.

Ziegenhagen B., Fladung M. 1997. Variation in the *psb*C gene region of the gymnosperms and angiosperms as detected by a single restriction site polymorphism. Theoretical and Applied Genetics 94: 1065–1071.