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European aspen (*Populus tremula* L.) genetic diversity assessed by molecular methods correlation with *P. tremulae* infection incidence

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This study was based on different susceptibility of *Populus tremula* clones to *Phellinus tremulae*, the major pathogen of this tree species. As the conventional resistance genes detection is time consuming and expensive, it is important to find other applicable methods to recognize resistance genes of the plants. The aim of this study was the assessment of correlation between various genetic population parameters and phenotipic, resistance or highly heritable traits of individual *P. tremula* trees.

In this study we investigated all Lithuanian *P. tremula* plus trees from 16 populations in 3 provenance regions. Investigated trees were analyzed using RAPD, SSR and PCR-RFLP methods, and their individual and population genetic parameters as well as infection incidence with *P. tremulae* were assessed.

Determined genetic parameters were examined for correlation with *P. tremulae* infection. Only two out of 292 RAPD loci were significant for damaged and undamaged by *P. tremulae* groups of trees.

The resistance of investigated European aspen plus trees with *P. tremulae* showed no dependency on the population, provenance region or individual tree heterozygosity. RAPD loci linked to infection should be investigated further, to confirm or reject their value in assesing individual tree susceptibility to *P. tremulae* and the genetic mechanisms underlying such correlation.

Key words: RAPD, PCR-RFLP, *Populus tremula*, *Phellinus tremulae*

INTRODUCTION

Wood decay fungi are capable to decompose lignified cell walls (Blanchette, 1991). Such decay leads to structural deterioration of the woody tissues, which may lead to the loss of wood mechanical strength (Guglielmo et al., 2007). A common practice to ascertain stem decay affected trees is often restricted to visuall assessment, thus such inspection alows to identify only trees with an advanced stage of decay. To complement visual inspection, decay in standing trees can be detected by

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instrumental analyses, allowing to detect fungi even at incipient stages (Tomikawa et al., 1990; Mattheck, Breloer, 1993; Habermehl et al., 1999; Müller et al., 2001).

European aspen (*Populus tremula* L.) is a highly ecologically valuable tree species, associated with numerous leaf, bark and wood inhabitants like insects, fungi, birds and even mammals (Wühlisch, 2009). Phytosanitaric conditions of aspen in Lithuania are foremost associated with true heart-rot fungus *Phellinus tremulae* Bond. Et Boriss (*Hymenochatataceae*). *P. tremulae* is widely distributed in temperate and boreal Eurasia and North America (Niemelä, 1974), and in many countries this fungus is considered as the most destructive pathogen of *Populus* spp., which often almost totally destroys the timber of aspen and limits timber crop rotation in managed forest sites to merely 40–50 years (Manion, 1991). The presence of fruiting bodies of *P. tremulae* on European aspen usually implies that the larger part or entire trunk of the tree has already been transformed to the cull status (Allen et al. 1996). As the typical true heart-rot fungus, *P. tremulae* is a very host-specific species and in Europe occurs predominantly on living *P. tremula* (Niemelä, 1974).

Small twigs are considered to be the primary infection route of *P. tremulae* for stems of aspen, although very fresh wounds perhaps can serve as infection foci as well (Holmer et al., 1994). Thus, the opportunities for infection by true heart-rot fungi (as *P. tremulae*) are quite restricted, limiting establishment in the stem of tree of only a few number of fungal genotypes (Rayner, Boddy 1988). For instance, Holmer et al. (1994) reported only one to four genets of *P. tremulae* per tree (mostly one). Such small numbers of *P. tremulae* genotypes per stem suggest that high genetic variation of aspen in resistance to heart-rot decay should exist. Such significant differences in the amount and position of decay among clones of aspen have been reported decades ago (Wall, 1971). Thus, as aspen clones seem to differ in susceptibility to *P. tremulae*, investigation of genetic diversity and breeding of this tree species against the pathogen could be considered and may result in future stands with the increased resistance (Manion, 1991).

Plant resistance to pathogens consists of recognition of the attacking organism and induction of various defense responses. Infection can induce systemic acquired resistance, thus protecting the plant against subsequent attacks of the same or other pathogen species (McDowell, Woffenden, 2003). Plant resistance at the genetic level is determined by single genes or quantitative trait loci (McDowell, Woffenden, 2003). To puzzle out the genetic mechanism of such resistance, consensus linkage maps saturated with molecular markers are produced for various plant species. To establish segregating progeny population for QTL analysis resistant (or in many cases more resistant) and susceptible parent plant individuals or populations are hybridized. Subsequent steps are the following: 1) infection of plants with respective pathogens and 2) detection of the QTL, according to the results of infection. However, screening of QTL is problematic due to the expenditure of progeny population establishment, especialy concerning tree species. Thus it is important to find other applicable methods to recognize resistance genes of the plants.

RAPD is a relatively simple and inexpensive molecular marker system, used to reveal DNA polymorphisms of unknown origin (Williams et al., 1990). Although RAPD amplifies anonymous loci, some of them might be located in genes, responsible for various functions of the investigated organisms. RAPD loci might be as well located in genes responsible for resistance to pathogens only by chance.

The aim of this study was the assessment of correlation between various genetic population parameters and phenotipic, resistance or highly heritable traits of individual *P. tremula* trees.

MATERIALS AND METHODS

Plant material. *P. tremula* plus trees are an important share of national Lithuanian forest genetic resources. Plus trees are selected as the exceptional representatives of autochtonous tree species by the Lithuanian Forest State Service, and later managed by local forest enterprises. In

this study all existing (in year 2007) 137 *P. tremula* plus trees were included. These trees were selected in 16 forest enterprises and 18 forest districts (Table 1).

Wood and leaf samples of additional 7 trees exhibiting clear *P. tremulae* infection were collected from Biržai, Ignalina, Kretinga, Raseiniai, Šalčininkai, Tauragė and Utena forest

Table 1. *Populus tremula* plus tree numbers, locality and coordinates, age, soil typology group (Vaičys, 2006) and the presence of *P. tremulae* assessed using molecular means. *P. tremula* plus tree data were obtained from the Lithuanian Forest State Service forest genetic resources digest

Tree No.	Forest enterprise	Provenance region	Latitude	Longitude	Forest soil type	Tree age	Presence of *P. tremulae*	Individual tree heterozygosity
130	Anykščiai	2	55°34’20.5”	24°50’08.9”	Lds	62	+	100%
131	Anykščiai	2	55°34’22.0”	24°50’09.1”	Lds	62	+	100%
132	Anykščiai	2	55°34’21.0”	24°50’09.0”	Lds	62	+	100%
133	Anykščiai	2	55°34’21.5”	24°50’09.3”	Lds	62	+	100%
134	Anykščiai	2	55°34’21.8”	24°50’09.9”	Lds	62	+	80%
135	Anykščiai	2	55°34’20.9”	24°50’10.1”	Lds	62	–	100%
136	Anykščiai	2	55°34’21.5”	24°50’09.7”	Lds	62	–	100%
137	Anykščiai	2	55°34’20.6”	24°50’11.0”	Lds	62	+	100%
138	Anykščiai	2	55°34’20.7”	24°50’09.6”	Lds	62	+	100%
139	Anykščiai	2	55°34’21.3”	24°50’09.2”	Lds	62	+	100%
140	Anykščiai	2	55°34’22.4”	24°50’09.3”	Lds	62	+	80%
141	Anykščiai	2	55°34’22.5”	24°50’09.8”	Lds	62	–	100%
142	Anykščiai	2	55°34’22.7”	24°50’11.6”	Lds	62	–	100%
143	Anykščiai	2	55°34’21.6”	24°50’09.5”	Lds	62	+	100%
144	Anykščiai	2	55°34’21.6”	24°50’09.4”	Lds	62	–	100%
042	Biržai	2	56°21’32.4”	24°52’16.9”	Lcp	62	–	80%
043	Biržai	2	56°21’25.6”	24°52’17.8”	Lcp	67	+	100%
158	Ignalina	3	55°19’33.3”	26°28’08.8”	Ldp	67	–	80%
159	Ignalina	3	55°19’33.2”	26°28’11.2”	Ldp	37	–	80%
160	Ignalina	3	55°19’33.7”	26°28’11.1”	Ldp	37	–	100%
161	Ignalina	3	55°19’33.8”	26°28’09.9”	Ldp	37	+	80%
162	Ignalina	3	55°19’34.7”	26°28’07.1”	Ldp	37	+	80%
084	Jurbarkas	2	55°09’15.3”	23°19’16.9”	Lfp	37	+	40%
085	Jurbarkas	2	55°09’15.7”	23°19’18.2”	Lfp	52	–	100%
086	Jurbarkas	2	55°09’14.9”	23°19’18.5”	Lfp	52	+	100%
087	Jurbarkas	2	55°09’14.6”	23°19’16.9”	Lfp	52	–	100%
088	Jurbarkas	2	55°09’14.8”	23°19’19.1”	Lfp	52	+	100%
089	Jurbarkas	2	55°09’15.4”	23°19’19.0”	Lfp	52	–	100%
090	Jurbarkas	2	55°09’13.8”	23°19’17.2”	Lfp	52	+	100%
091	Jurbarkas	2	55°09’14.1”	23°19’19.2”	Lfp	52	–	80%
092	Jurbarkas	2	55°09’13.0”	23°19’18.7”	Lfp	52	–	80%
093	Jurbarkas	2	55°09’13.4”	23°19’19.5”	Lfp	52	+	100%
074	Kaišiadorys	2	54°53’35.1”	24°19’57.8”	Pdn	52	+	80%
075	Kaišiadorys	2	54°53’36.0”	24°20’00.3”	Pdn	62	+	80%
076	Kaišiadorys	2	54°53’34.5”	24°20’00.8”	Pdn	62	+	80%
077	Kaišiadorys	2	54°53’34.1”	24°19’58.3”	Pdn	62	+	80%
078	Kaišiadorys	2	54°53’33.8”	24°20’00.5”	Pdn	62	+	100%
079	Kaišiadorys	2	54°53’33.6”	24°19’59.1”	Pdn	62	–	60%
080	Kaišiadorys	2	54°55’43.5”	24°13’13.3”	Lcp	62	+	80%

Table 1 (continued)

081	Kaišiadorys	2	54°55’43.3”	24°13’12.9”	Lcp	57	–	80%
082	Kaišiadorys	2	54°55’43.5”	24°13’13.3”	Lcp	57	–	100%
083	Kaišiadorys	2	54°55’43.3”	24°13’13.5”	Lcp	57	–	80%
109	Kėdainiai	2	55°19’43.6”	23°46’36.3”	Lds	57	–	100%
110	Kėdainiai	2	55°19’44.2”	23°46’38.4”	Lds	62	+	100%
111	Kėdainiai	2	55°19’42.9”	23°46’37.3”	Lds	62	+	100%
112	Kėdainiai	2	55°19’41.4”	23°46’37.2”	Lds	62	–	100%
113	Kėdainiai	2	55°19’33.6”	23°46’39.0”	Lds	62	+	100%
114	Kėdainiai	2	55°19’37.7”	23°46’38.7”	Lds	62	–	100%
115	Kėdainiai	2	55°19’37.5”	23°46’37.8”	Lds	62	+	80%
116	Kėdainiai	2	55°19’36.0”	23°46’36.3”	Lds	62	+	80%
117	Kėdainiai	2	55°19’36.0”	23°46’39.5”	Lds	62	+	60%
118	Kėdainiai	2	55°19’35.1”	23°46’37.8”	Lds	62	–	80%
119	Kėdainiai	2	55°19’31.1”	23°46’40.2”	Lds	62	–	100%
163	Kretinga	1	55°55’36.8”	21°35’21.9”	Lcl	62	–	80%
164	Kretinga	1	55°55’37.4”	21°35’24.0”	Lcl	67	–	100%
165	Kretinga	1	55°55’36.4”	21°35’23.0”	Lcl	47	–	60%
166	Kretinga	1	55°55’35.9”	21°35’21.5”	Lcl	37	–	60%
167	Kretinga	1	55°55’36.7”	21°35’26.2”	Lcl	47	–	80%
168	Kretinga	1	55°55’35.6	21°35’25.2”	Lcl	47	+	60%
039	Kuršėnai	1	55°53’42.1”	22°41’32.2”	Ncs	67	–	100%
040	Kuršėnai	1	55°53’40.6”	22°41’34.5”	Ncs	72	–	100%
041	Kuršėnai	1	55°53’46.2”	22°41’31.3”	Ncs	67	–	100%
145	Kuršėnai	1	55°53’44.2”	22°41’38.8”	Lcs	67	+	80%
146	Kuršėnai	1	55°53’42.4”	22°41’39.3”	Lcs	67	+	80%
104	Marijampolė	2	54°38’03.2”	23°37’30.9”	Lds	67	+	80%
105	Marijampolė	2	54°38’03.3”	23°37’30.4”	Lds	47	–	80%
106	Marijampolė	2	54°38’04.6”	23°37’32.9”	Lds	47	+	80%
107	Marijampolė	2	54°38’04.3”	23°37’31.3”	Lds	47	+	100%
108	Marijampolė	2	54°38’05.1”	23°37’32.8”	Lds	47	–	80%
044	Pakruojis	1	56°17’10.6”	24°02’02.1”	Nfs	47	+	60%
045	Pakruojis	1	56°17’11.1”	24°02’01.9”	Nfs	57	–	60%
046	Pakruojis	1	56°17’11.3”	24°02’02.2”	Nfs	57	+	80%
047	Pakruojis	1	56°17’12.0”	24°02’04.0”	Nfs	57	+	80%
048	Pakruojis	1	56°17’11.3”	24°02’04.2”	Nfs	57	–	60%
049	Pakruojis	1	56°16’01.4”	24°03’44.5”	Lfs	57	–	40%
050	Pakruojis	1	56°16’02.0”	24°03’43.3”	Lfs	67	+	100%
051	Pakruojis	1	56°16’01.4”	24°03’43.8”	Lfs	67	–	80%
052	Pakruojis	1	56°16’00.7”	24°03’43.0”	Lfs	67	+	100%
053	Pakruojis	1	56°16’01.1”	24°03’43.3”	Lfs	67	+	40%
054	Pakruojis	1	56°16’00.2”	24°03’42.2”	Lfs	68	+	60%
055	Pakruojis	1	56°16’00.8”	24°03’45.5”	Lfs	67	–	80%
056	Pakruojis	1	56°15’55.8”	24°03’44.1”	Lfs	67	–	80%
057	Pakruojis	1	56°15’55.2”	24°03’43.7”	Lfs	71	–	60%
058	Pakruojis	1	56°15’59.0”	24°03’46.7”	Lfs	67	+	100%
059	Pakruojis	1	56°15’58.5”	24°03’45.3”	Lfs	67	+	80%
060	Pakruojis	1	56°15’58.0”	24°03’43.0”	Lfs	71	+	80%
061	Pakruojis	1	56°15’58.0”	24°03’43.8”	Lfs	67	–	80%
062	Pakruojis	1	56°15’58.4”	24°03’43.2”	Lfs	67	+	80%
063	Pakruojis	1	56°15’56.5”	24°03’41.9”	Lfs	67	+	80%
064	Pakruojis	1	56°15’57.8”	24°03’45.9”	Lfs	67	–	80%

Table 1 (continued)

Tree No.	Forest enterprise	Provenance region	Latitude	Longitude	Forest soil type	Tree age	Presence of *P. tremulae*	Individual tree heterozygosity
065	Pakruojis	1	56°15’56.3”	24°03’42.3”	Lfs	68	–	80%
066	Pakruojis	1	56°15’57.3”	24°03’43.2”	Lfs	57	+	80%
067	Pakruojis	1	56°15’59.7”	24°03’45.2”	Lfs	68	+	80%
068	Pakruojis	1	56°15’55.8”	24°03’42.9”	Lfs	69	–	60%
069	Pakruojis	1	56°15’58.7”	24°03’42.5”	Lfs	67	–	100%
070	Pakruojis	1	56°15’57.1”	24°03’47.2”	Lfs	72	–	0%
071	Pakruojis	1	56°15’55.7”	24°03’41.5”	Lfs	72	–	60%
072	Pakruojis	1	56°15’56.5”	24°03’41.9”	Lfs	66	+	80%
073	Pakruojis	1	56°15’56.8”	24°03’46.1”	Lfs	67	+	80%
038	Raseiniai	2	55°11’48.5”	22°53’33.6”	Udp	72	+	60%
094	Raseiniai	2	55°14’52.6”	22°59’47.8”	Lds	47	–	60%
095	Raseiniai	2	55°14’51.7”	22°59’47.2”	Lds	47	+	60%
096	Raseiniai	2	55°14’51.3”	22°59’48.6”	Lds	47	+	60%
097	Raseiniai	2	55°14’50.7”	22°59’46.7”	Lds	47	+	60%
098	Raseiniai	2	55°14’50.3”	22°59’48.9”	Lds	47	–	40%
099	Raseiniai	2	55°14’49.2”	22°59’47.5”	Lds	47	–	60%
100	Raseiniai	2	55°14’48.0”	22°59’49.3”	Lds	47	–	60%
101	Raseiniai	2	55°14’47.0”	22°59’47.5”	Lds	47	–	60%
102	Raseiniai	2	55°14’44.3”	22°59’46.8”	Lds	47	–	60%
103	Raseiniai	2	55°14’44.8”	22°59’48.2”	Lds	47	+	60%
020	Rokiškis	2	55°48’04.4”	25°47’17.8”	Ndp	47	–	60%
021	Rokiškis	2	55°48’04.4”	25°47’17.7”	Ndp	62	+	60%
022	Rokiškis	2	55°47’59.9”	25°48’13.1”	Nds	62	–	80%
023	Rokiškis	2	55°48’01.7”	25°48’13.4”	Nds	67	–	100%
120	Šakiai	2	55°01’48.9”	23°05’01.7”	Lfs	67	+	100%
121	Šakiai	2	55°01’48.4”	23°05’01.5”	Lfs	67	+	60%
122	Šakiai	2	55°01’47.6”	23°05’01.2”	Lfs	67	+	100%
123	Šakiai	2	55°01’45.2”	23°05’04.8”	Lfs	67	–	100%
124	Šakiai	2	55°01’44.4”	23°05’00.2”	Lfs	67	+	60%
125	Šakiai	2	55°01’44.3”	23°05’02.1”	Lfs	67	+	60%
126	Šakiai	2	55°01’45.3”	23°05’01.5”	Lfs	67	–	80%
127	Šakiai	2	55°01’41.9”	23°0502.3’”	Lfs	67	+	80%
128	Šakiai	2	55°01’47.8”	23°05’02.5”	Lfs	67	+	80%
129	Šakiai	2	55°01’48.1”	23°05’01.6”	Lfs	67	+	80%
036	Šalčininkai	3	54°16’02.2”	25°44’09.6”	Ncp	67	–	80%
152	Šalčininkai	3	54°16’05.0”	25°43’58.8”	Ncp	72	–	80%
153	Šalčininkai	3	54°16’05.0”	25°43’58.8”	Ncp	57	+	20%
154	Šalčininkai	3	54°16’05.0”	25°43’58.2”	Ncp	57	+	20%
155	Šalčininkai	3	54°16’05.8”	25°43’57.9”	Ncp	57	+	20%
156	Šalčininkai	3	54°16’05.8”	25°43’57.9”	Ncp	57	–	40%
157	Šalčininkai	3	54°16’06.2”	25°43’58.2”	Ncp	57	+	80%
037	Tauragė	2	55°23’35.4”	22°09’26.7”	Ncp	57	+	60%
147	Utena	3	55°07’17.9”	25°32’28.5”	Ldp	67	–	100%
148	Utena	3	55°07’17.4”	25°32’28.9”	Ldp	62	+	60%
149	Utena	3	55°07’21.7”	25°32’28.7”	Ldp	62	–	80%
150	Utena	3	55°07’19.3”	25°32’30.1”	Ndp	62	–	80%
151	Utena	3	55°07’21.7”	25°32’30.0”	Ndp	62	+	80%

enterprises. Two samples representing sound looking wood samples were taken from Biržai and Rokiškis forest enterprises. These 9 trees were selected from the same forest stands as plus trees as a reference material.

One wood and leaf sample per each plus and reference tree were collected for molecular analysis. Wood samples were taken using an 8 mm Pressler's drill. Each plus tree was drilled to the stem core once at the breast height from the ground. Before each sampling event the drill was sterilized with 96% ethanol. Collected wood samples were placed in sterile plastic containers. Leaf samples for DNA extraction were collected from the upper part of the tree crown, using a SherrillTree BigShot® line launcher (SherrillTree Inc.). Only sound looking, fully developed and mechanically intact leafs were collected. Fresh leaf samples were immediately placed in plastic sample bags with self-indicating silica gel (approximately 10–15 g of silica gel for 1 g of plant material). The sampling of both wood and leaf tissue was performed May–July 2007.

DNA extraction. DNA for European aspen plus tree genetic polymorphism and infection with the *P. tremulae* analysis was extracted using a Nucleospin Plant mini kit (Macherey-Nagel, Düren, Germany). DNA from the *P. tremulae* fruiting body was extracted using the same extraction kit (Nucleospin Plant mini kit, Macherey-Nagel).

RAPD analysis. In order to establish European aspen RAPD profiles we used DNA extracted from *P. tremula* leaf and wood samples simultaneously. For the RAPD analysis we have selected 15 most informative primers out of 60 tested (selected primers are given in Table 2). Primer informativeness was evaluated using several criteria: (a) overall DNA amplification quality, (b) amplification of polymorphic fragments, (c) size differences of polymorphic fragments and (d) DNA fragments reproducibility (Pivorienė, 2008). PCR conditions and fragment separation was performed as described in Žvingila et al. (2002). Three independent PCR amplifications were performed using separately extracted DNA from wood and leaf samples. Independent amplification was performed in order to avoid any inacuracies and mistakes, as well to be sure that all amplified bands are of tree origin. The PCR results were analysed using only reliably reproducible DNA bands. A DNA fragment of particular size (locus) was scored as present or absent and marked as 1 or 0, respectively. DNA fragment sizes were aproximately evaluated according to the MassRuler™ DNA Ladder Mix (Fermentas, Lithuania) and DNA Ladder 100 bp plus (Applichem, Maryland Heights, USA).

The DNA band of the same mobility in 1.5% agarose gel was considered identical. Gel places, which because of the multiple banding pattern were hard to evaluate, were excluded from scoring, and left unanalyzed. The RAPD fragment was considered polymorfic when its frequency was lower than 99%.

SSR analysis. To infer individual tree heterozigosity five fluorescently labeled SSR primer pairs were used (Table 3): PMGC2607, GCPM1532, GCPM1608 (information on these SSR primers was taken from International *Populus* Genome Consortium IPGC (http://www.ornl.gov./sci/ipgc/ssr_resource.htm)), WPMS14 and

Table 2. RAPD primers used for *P. tremula* analysis

Primer	Primer sequence 5'–3'	Primer	Primer sequence 5'–3'
Roth A-01	CAGGCCCTTC	Roth B-12	CCTTGACGCA
Roth A-03	AGTCAGCCAC	Roth B-13	TTCCCCCGCT
Roth A-04	AATCGGGCTG	Roth B-17	AGGGAACGAG
Roth A-05	AGGGGTCTTG	Roth 170-02	CAGGGTCGAA
Roth A-09	GGGTAACGCC	Roth 170-05	GAGATCCGCG
Roth A-19	CAAACGTCGG	Roth 170-09	TGCAGCACCG
Roth B-01	GTTTCGCTCC	Roth 370-01	TCCCTGTGCC
Roth B-03	CATCCCCCTG		

Table 3. Primers used for microsatellite analysis of *P. tremula*

Primer	Primer sequence	Fluorescence label	Annealing temperature	Chromosome No.
PMGC2607	F3'TTAAAGGGTGGTCTGCAAGC5' R3'CTTCTTGCACCTCGTTTTGAG5'	PET	47.5 °C	8
GCPM1532	F3'ATGCTTTGCTTGCTCTTAAC5' R3'ACTATTGCTTGTCTTGGCAT5'	PET	43 °C	16
GCPM1608	F3'GCTCCTGGTTTTACCACAT5' R3'GAACAGCAGGATCATAGAGC5'	VIC	47 °C	15
WPMS14	F3'CAGCCGCAGCCACTGAGAAATC5' R3'GCCTGCTGAGAAGACTGCCTTGAC5'	NED	56 °C	5
WPMS16	F3'CTCGTACTATTTCCGATGATGACC5' R3'AGATTATTAGGTGGGCCAAGGACT5'	FAM	51 °C	7

WPMS16 (Tuskan et al., 2004). All utilized primers were tested for transferability to *P. tremula* and heterozigosity by Pakull et al. (2009). For the microsatellite analysis we selected SSR primers that amplify regions in different chromosomes (Pakull et al., 2009; Yin et al., 2008).

The polymerase chain reaction for the microsatellite analysis was performed in total of 15 µl, containing 50 ng of template DNA, 5 pmol of forward fluorescence labelled and 5 pmol reverse primers, 0.2 units of Dream Taq DNA Polymerase (Thermo Fisher Scientific), 30 µM of dNTP mix (Thermo Fisher Scientific), 1× DreamTaq buffer (Thermo Fisher Scientific). Thermocycling was performed in the GeneAmp PCR system 9700, under the following conditions: 4 min at 94 °C, 38 cycles of 94 °C for 30 s, primer annealing temperature (Table 3) for 45 s, 72 °C for 1 min, and the final extension of 10 min at 72 °C.

The selected primers were possible to use in multiplex, determining their amplified fragments length. The obtained PCR products were mixed together. 1 µl of the PCR products mix was diluted in 10 µl Formamid, 0.3 µl of an internal fragment sizes solution (Gene Scan 500 LIZ standart, ABI), and separated using an ABI-PRISM Genetic Analyzer 310. The data analysis was carried out using the Gene-Mapper software.

Detection of *P. tremulae*. The presence of *P. tremulae* in the collected wood samples was determined using fungal DNA sequences. For fungal DNA amplification we have used ITS1-F (5' CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') primers, which are known to amplify the ITS (internal transcribed spacer) region. Amplification conditions were used as described by Jasalavich et al. (2000). The obtained PCR fragments were separated on 3% agarose gel with ethidium bromide. Next to the European aspen wood DNA samples DNA extracted from the *P. tremulae* fruiting body and amplified with the same ITS primers were loaded. Fungal DNA fragments were excised from agarose gel and without subsequent cleaning steps used in the restriction analysis in order to determine if the obtained fungal DNA belongs to *P. tremulae*. The restriction analysis was performed as described in Jasalavich et al. (2000).

The identification of other fungal species obtained in *P. tremula* wood samples has not been performed.

Statistical analysis. The European aspen RAPD data analysis was performed using the GenAlEx 6.5 computer software (Peakall, Smouse, 2006; 2012).

The European aspen trees genetic analysis data, obtained using the RAPD method, was used to assess the corelation between RAPD fragments and tree infection with *P. tremulae*.

For the correlation assessment the binary data matrix was used. The SAS procedure GENMOD (generalized linear models) with model options of the link function 'logit' and the binomial distribution variance function was used for estimation of the locus band presence or absence effect for wood infection with *P. tremulae*. Only polymorphic fragments were used for the evaluation of data (SAS 9.4 package, 2002–2012 by SAS Institute Inc., Cary, NC, USA). In order to check which loci were the most discriminating for the sites or populations, the stepwise discriminant analysis was done to select a subset of the fragments for use in discrimination among the classes. The STEPDISC SAS procedure was applied to select RAPDs contributing most to the differentiation of individuals grouped by wood infection with *P. tremulae* properties. The least significance level 0.05 was used for the selection of single RAPD. The detrended correspondence analysis (in PAST version 2.17c) was applied to detect the pattern in wood infection in the groups of trees (damaged and undamaged) by the most group discriminating RAPD markers.

RESULTS

RAPD analysis. After runing European aspen wood DNA amplification of 137 trees with 15 random decamer primers, which previously were chosen as polymorphic and best suited for *P. tremula* research (Table 2), we obtained a total of 292 DNA bands. The results of the RAPD amplification are presented in Table 4. 282 out of 292 amplified bands (96.6%) were polymorphic. On the average, the outcome of one primer amplification comprised 19.5 bands. The most informative primer was Roth A-05 (27 bands), followed by primers Roth A-01 and Roth B12 both resulting in 25 bands (Table 4). The least amount of RAPD locus was determined using primers Roth B-13 and Roth B-17 (14 and 12 bands, respectively). The amplified fragment sizes varied from 250 to 3 500 bp. The broadest size range was obtained with primer Roth 170-05 (Table 4).

Different number of RAPD fragments were obtained in RAPD profiles of the examined European aspen trees. The amount of DNA fragments characteristic to one genotype varied from 93 (tree No. 155 from Šalčininkai) to 160 (tree No. 094 from Raseiniai). On the average 132.32 loci per genotype were obtained.

The RAPD analysis data were used to calculate mean genetic diversity indices (Table 5). Evidently, an effective number of alleles exceeds the number of different alleles for populations with a low investigated individual number (5 or less) (Table 5). The Shannon information index varies from 0.192 in Kuršėnai up to 0.401 in

Table 4. RAPD results of amplification with 15 random primers

Primer	No. of amplified bands	No. of polymorphic bands	Amplified band size range
Roth A-01	25	23	380–3 000 bp
Roth A-03	23	23	550–3 500 bp
Roth A-04	20	19	470–2 500 bp
Roth A-05	27	27	380–3 000 bp
Roth A-09	22	21	400–3 000 bp
Roth A-19	20	20	400–2 500 bp
Roth B-01	15	15	470–2 000 bp
Roth B-03	18	16	460–3 000 bp
Roth B-12	25	23	480–3 000 bp
Roth B-13	14	14	520–2 500 bp
Roth B-17	12	11	400–1 900 bp
Roth 170-02	20	20	250–3 000 bp
Roth 170-05	18	18	390–3 500 bp
Roth 170-09	15	14	800–3 000 bp
Roth 370-01	18	18	520–3 000 bp

Kėdainiai and 0.402 in Pakruojis populations. The lowest expected heterozygosity values were calculated for Kuršėnai (0.127), Tauragė (0.136), and Anykščiai (0.196) populations. Kuršėnai and Tauragė populations were represented by merely 5 and 2 trees, respectively (Table 5).

The presence of *P. tremulae* mycelium and main genetic diversity indices were used to assess the correlation between them.

SSR analysis. All five microsatellite loci used in this study were polymorphic and revealed 7 to 14 alleles, on the average 10.6 alleles per locus. The locus GCPM 1532 was least polymorphic,

Table 5. The within population genetic diversity indexes over the loci, for European aspen assessed using RAPD data

Population	Statistics	N	Na	Ne	I	He	uHe
Anykščiai	Mean	15	1.426	1.331	0.298	0.196	0.203
	±SE		0.047	0.021	0.016	0.012	0.012
Biržai	Mean	4	1.313	1.344	0.303	0.203	0.231
	±SE		0.050	0.022	0.017	0.012	0.013
Ignalina	Mean	6	1.451	1.400	0.348	0.232	0.253
	±SE		0.049	0.023	0.017	0.012	0.013
Jurbarkas	Mean	10	1.504	1.393	0.343	0.229	0.241
	±SE		0.045	0.022	0.017	0.012	0.012
Kaišiadorys	Mean	10	1.613	1.415	0.377	0.248	0.261
	±SE		0.044	0.021	0.015	0.011	0.011
Kėdainiai	Mean	11	1.722	1.442	0.401	0.264	0.277
	±SE		0.038	0.021	0.015	0.011	0.011
Kretinga	Mean	7	1.521	1.395	0.352	0.233	0.251
	±SE		0.046	0.022	0.016	0.011	0.012
Kuršėnai	Mean	5	1.039	1.217	0.192	0.127	0.141
	±SE		0.050	0.020	0.016	0.011	0.012
Marijampolė	Mean	5	1.158	1.330	0.278	0.188	0.209
	±SE		0.053	0.023	0.018	0.012	0.014
Pakruojis	Mean	30	1.835	1.439	0.402	0.262	0.267
	±SE		0.030	0.021	0.014	0.011	0.011
Raseiniai	Mean	12	1.549	1.403	0.360	0.239	0.249
	±SE		0.045	0.021	0.016	0.011	0.012
Rokiškis	Mean	5	1.342	1.353	0.316	0.209	0.232
	±SE		0.052	0.022	0.016	0.012	0.013
Šakiai	Mean	10	1.655	1.424	0.379	0.250	0.264
	±SE		0.040	0.022	0.015	0.011	0.012
Šalčininkai	Mean	8	1.577	1.409	0.368	0.242	0.258
	±SE		0.046	0.022	0.015	0.011	0.012
Tauragė	Mean	2	0.975	1.232	0.198	0.136	0.181
	±SE		0.049	0.020	0.017	0.012	0.015
Utena	Mean	6	1.454	1.386	0.346	0.229	0.250
	±SE		0.049	0.022	0.016	0.011	0.012
Total	Mean	9.125	1.446	1.369	0.329	0.218	0.236
	±SE	0.094	0.012	0.005	0.004	0.003	0.003

N – sample size; Na – No. of different alleles; Ne – effective number of alleles; I – Shannons information index; He – expected heterozigosity; uHe – unbiased expected heterozigosity; ±SE standart error.

while the locus WPMS 14 had the highest number of alleles. The SSR analysis of 137 European aspen plus trees were used to measure tree individual heterozigosity (Table 1).

***P. tremulae* detection.** The presence of *P. tremulae* mycelium in European aspen wood samples was assessed using PCR-RFLP, described by Jasalavich et al. (2000). PCR was performed using ITS region primers ITS-1F and ITS4 three times with each DNA sample obtained from European aspen wood. This region is most widely used to identify fungal species because it is extremely variable among species and conservative within. The ITS region in different fungal species is of different length and its DNA sequence is also variable. The results of amplification are shown in Fig. 1.

According to the amplification results, the identical to *P. tremulae* amplification bands allowed to preliminary identify which of the inspected wood samples was colonized with this parasitic fungus. All amplified bands with the same mobility as *P. tremulae* control were subjected for excision from the gel, subsequent reamplification and digestion with restriction enzymes (AluI, HaeIII, TaqI and RsaI), resulting in identification of all aspen wood samples infected with *P. tremulae* (Table 1).

The amplification of wood extracted DNA revealed that 73 (53.28%) out of 137 *P. tremula* trees were infected with *P. tremulae*. The average age of infected and healthy trees was almost identical and attained 59.9 and 59 years, respectively. The highest proportion of infected European aspen trees was found in Šakiai (80%), Anykščiai (66.7%), Kaišiadorys (60%) and Marijampolė (60%) stands (see Table 1). The Tauragė stand was represented by the single infected tree, thus not included in the analysis. According to the DNA test, the healthiest stands were found in Kretinga and Rokiškis (16.7% and 25% infected trees, respectively), followed by Ignalina and Kuršėnai stands, both with 40% infected trees (Fig. 2).

Statistical analysis. The relation between the presence of *P. tremulae* in European aspen wood samples and RAPD profiles was determined using the SAS procedure GENMOD.

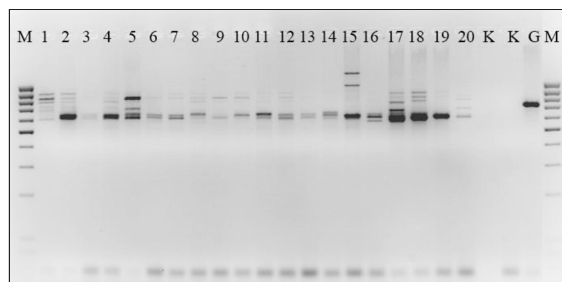

Fig. 1. Amplified fungal fragments from European aspen wood DNA. M – molecular size marker, 1 to 20 – amplified wood DNA samples, K – PCR control samples (sample without DNA and sample with plant DNA), G – amplified *P. tremulae* DNA sample

In the present study, just 5 loci of European aspen were considered important in differentiation of trees by infection by *P. tremulae* (A5_850, B13_1100, B17_850, 17002_1750 and 17009_1600). Only fragments A5_850 ($p = 0.0278$) and B13_1100 ($p = 0.0222$) were significant in group differentiation. Loci B17_850, 17009_1600 and 17002_1750 were close to 5% of statistical significance.

PCA based on the detrended correspondence analysis using 5 important RAPD loci were performed and revealed the distribution in two axes of the infected and non-infected trees. The obtained results are presented in Fig. 3. B13_1100 locus explained 7% of differentiation by wood infection. The convex hulls of two groups are overlapping, but the frequencies in two loci (Fig. 3) indicate quite good possibilities for using these RAPDs loci in tree breeding at early ontogenesis stages.

Additionaly, we calculated the correlation whether tree individual heterozygosity, RAPD marker polymorphism, provenance region, population or forest soil type had any effect on *P. tremula* infection with *P. tremulae*. The Pearson correlation between tree individual heterozigosity by 5 SSR loci and the presence or absence of wood rot was estimated close to zero (–0.04). The same correlation between the tree individual RAPD marker polimorphism and the presence of stem heart rot also resulted in a similar estimate (–0.03). The percentage of

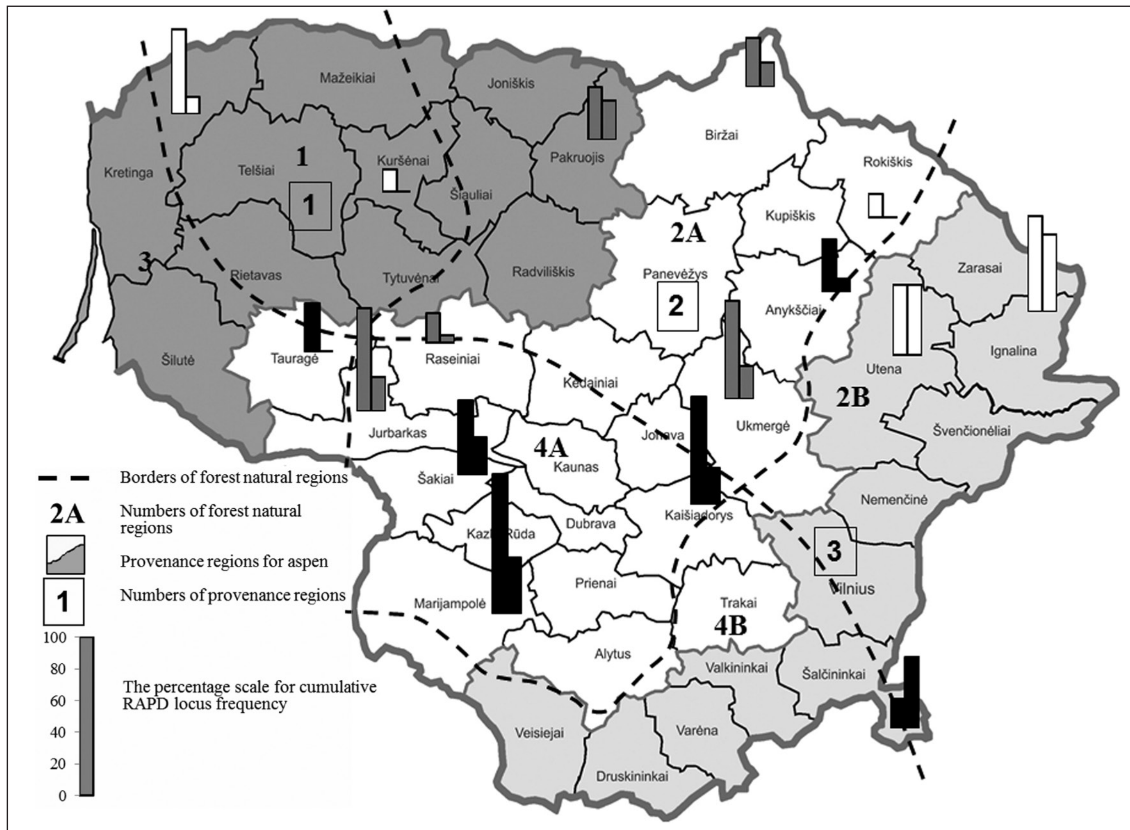

Fig. 2. Distribution of European aspen populations with indication of *P. tremulae* heart rot (black colour indicates that DNA of *P. tremulae* was detected in >50% of investigated aspen trees, grey colour shows that DNA is detected in 50%, and white colour indicates that it is detected in <50%) and cumulative frequency per category (%) in RAPD loci B13-1100 (on the left side) and A5-850 fragments

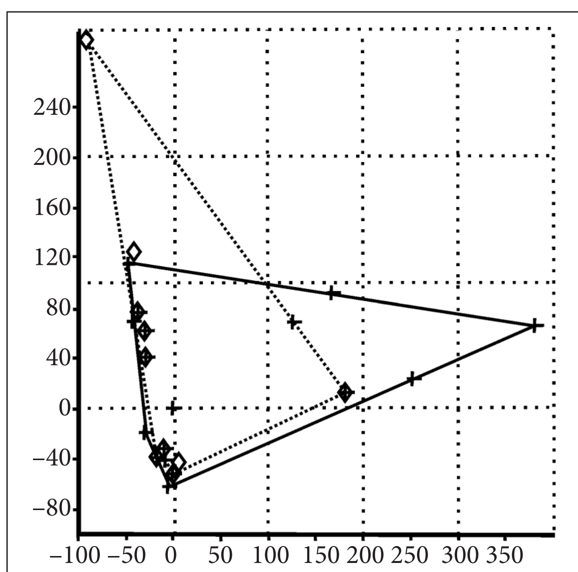

Fig. 3. Distribution in two axes of European aspen uninfected (indicated by a dashed line and a diamond shape) and infected by *P. tremulae* (indicated by a solid line and a cross shape) trees based on the results of detrended correspondence analysis using RAPD loci B13-1100, A5-850, B17-850, 17002-1750, 17009-1600

P. tremulae affected trees was slightly lower on temporarily moistured or overmoistured and eutrofic sites.

The Provenance region and population influence on heart rot was tested using the SAS procedure MIXED (REML option), but the results

showed that these two factors had no effect on rot distribution.

DISCUSSION

The European aspen wood sampling performed in this study is advantageous and helps to prevent cross-contamination between samples. Sampling technique using an 8 mm Pressler's drill has no impact on the health conditions of the trees. The Pressler's drill has supremacy over other type of drills as it does not produce sawdust, which is the primary cause of cross-contamination (Jasalavich et al., 2000).

RAPD markers have been used to evaluate the levels of genetic diversity within and among 16 European aspen populations representing three provenance regions. Studied *P. tremula* populations exhibit different levels of genetic diversity, with the mean Shanon's information index of 0.329. Genetic diversity is usually high in wind pollinated tree species, as indicated by other studies (Hamrick et al., 1992; Slavov et al., 2002; Žvingila et al., 2002), however, population differentiation between European aspen is low, compared to genetic variation within populations. It was shown that variation between European aspen populations in selectively neutral genetic markers is aproximatelly 1% (Lexer et al., 2005; Hall et al., 2007). Population differentiation in this study was 4%, this larger differentiation estimate could be explained by unequal and in some cases very small sample sizes in different populations (Table 1). The genetic differences obtained in this study is largely due to amplified fragment frequency differences, rather than the presence of population specific bands. The majority of amplified RAPD bands is common for most of the investigated European aspen populations. The population structure and its inter and intra variability is affected by the pattern of species distribution, pollen and seeds dispersal mode, particular biological aspects of the species etc. Tree species with a broad and continuous distribution often exhibit population differentiation below 3% (Slavov et al., 2002). The present population differentiation of aspen in Lithuania can be increased due to the scattered distribution and human impact.

In this study we have investigated European aspen plus trees. Plus trees are phenotipically elite individuals of the species they represent, and are selected in the most valuable and best evaluated stands from the breeders' point of view. Selected plus trees are tested in clone trials and/or field tests to confirm their superiority and the ability to transmit their phenotypic advantage to their offsprings. The identification of plus trees that are more resistant to *P. tremulae* than common aspen individuals is helpful determining which of the trees should be used in future breeding programmes. As literature data suggests, aspen trees are highly variable in resistance to heart rot fungi (Wall, 1971; Holmer, 1994; Manion, 1991; Eckstein et al., 1979), thus the correlation between RAPD markers and infection incidence with *P. tremulae* was assessed. RAPD markers are known to amplify anonymous regions of genome, thus allowing to catch some of the genetic variation underlying the resistance mechanisms only by chance. To increase our chances in capturing the correlation, we have used 15 highly informative primers (Table 2). However, a statistically significant correlation between infection with *P. tremulae* and RAPD loci was established for only two of them. The obtained results suggest that search for the correlation without prior resistance screening and genetic mechanisms underlying the resistance is unreliable. The chance of inferring genetic markers associated with European aspen resistance to heart rot using RAPD is negligible.

The subsequent step of our study should be the identification of genome regions, represented by B13-1100 and A5-850 fragments. The identification of these regions could lead to marker assissted breeding, or QTL involved in resistance mechanism identification. All European aspen plus trees were examined for *P. tremulae* infection by molecular means. Molecular methods were chosen after a small scale experiment where pure *P. tremulae* culture from collected wood samples were grown on fungal growth media (data not shown). The obtained results confirmed data of similar studies that in many cases false negative results because of the slow growth of *Phellinus* mycelium on the media can be

obtained (Mallett, Myrholm 1995; Pollastro et al., 2000; Charkravarty, Hiratsuka, 2007).

We have also evaluated the usefullness of ITS region samples, designed by Jasalavich et al. (2000), for detecting *P. tremulae* fungus, and confirmed their usefulness in *P. tremula*. Detection of *P. tremulae* infection in European aspen trees, used in this study, proved to be valuable in detecting this parasitic fungus at incipient stages of decay. Such screening for infection could be useful in resolving which trees should be selectivelly felled before this heart rot causing fungus transforms the tree to the cull status (Allen et al., 1996).

CONCLUSIONS

The infection of European aspen plus trees with *P. tremulae* does not depend on population, provenance region or individual tree heterozygosity. RAPD loci that are correlated to the tree infection should be investigated further, to confirm or reject their value in assesing individual tree susceptibility to the *P. tremulae* and the genetic mechanisms underlying the correlation.

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DREBULĖS (*Populus tremula* L.) GENETINĖS ĮVAIROVĖS, NUSTATYTOS MOLEKULINIAIS METODAIS, KORELIACIJA SU MEDŽIŲ UŽKRĖSTUMU *P. tremulae* PATOGENU

Santrauka
Šio tyrimo pagrindas – skirtingas *Populus tremula* klonų jautrumas *Phellinus tremulae* – pagrindiniam šios medžių rūšies patogenui. Kadangi įprastiniai atsparumą lemiančių genų nustatymo tyrimai yra brangūs ir ilgai trunkantys, svarbu surasti ir pritaikyti metodus, kurie leistų greičiau ir pigiau nustatyti augalų atsparumo genus. Tyrimo tikslas buvo nustatyti koreliaciją tarp įvairių populiacijų genetinių parametrų bei fenotipinių, atsparumo ar aukšto paveldimumo požymių, būdingų individualiems *P. tremula* medžiams.

Tyrimui atrinkti Lietuvos drebulės medžiai iš 16 populiacijų trijuose drebulės kilmės rajonuose buvo tiriami naudojant APPD, SSR (mikrosatelitų) bei PGR-RFIP metodus. Šiais metodais buvo nustatyti individualūs ir populiaciniai genetiniai parametrai bei užkrėstumas *P. tremulae* patogenu.

Atlikta molekuliniais metodais nustatytų genetinių parametrų koreliacija su užkrėstumu. Tik 2 iš 292 APPD lokusų statistiškai patikimai skyrė pažeistą ir nepažeistą puvinio medžių grupes.

Tirtų drebulės rinktinių medžių atsparumas *P. tremulae* nepriklauso nuo medžių populiacijos, kilmės rajono ar individualaus medžio heterozigotiškumo. APPD lokusai, koreliuojantys su medžių užkrėstumu, turėtų būti tiriami toliau, kad būtų galima patvirtinti jų vertę nustatant atskirų individų jautrumą *P. tremulae* bei genetinius mechanizmus, lemiančius koreliaciją.

Raktažodžiai: APPD, PGR-RFIP, *Populus tremula*, *Phellinus tremulae*