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Identification of *Heterobasidion* spp. in Poland by RFLP analysis of laccase and manganese dependent peroxidase

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Abstract: Laccase and manganese dependent peroxidase (MnP) genes in *H. annosum* s.l. were studied by PCR-RFLP. The peroxidase genes MNP1a and MNP2 showed *Heterobasidion* species specific length differences. Among eighteen monomorphic markers which were found for investigated genes, three were characteristic for *H. abietinum* and five for *H. annosum* s.s. The remaining specific markers were characteristic for *H. parviporum* and *H. abietinum* (nine markers) or for *H. parviporum* and *H. annosum* s.s. (one marker). No specific marker for *H. parviporum* was detected. On the basis of monomorphic markers, intersterility groups of eighty one strains isolated from eleven forest tree or bush species in south Poland were identified. Fifty-three belonged to *H. annosum* s.s., twenty-five to *H. parviporum*, and three to *H. abietinum*. Strains belonging to *H. annosum* s.s. were isolated from *Pinus nigra, P. strobus*, *P. sylvestris*, *Larix decidua*, *Picea abies,* as well as from *Alnus incana, Betula pendula*, *Padus avium, Quercus robur* and *Q. rubra*. *H. parviporum* was isolated from *Abies alba, Picea abies*, *Pinus nigra, P. sylvestris, Larix decidua* and from *Alnus incana*. This is the first report of *H. parviporum* occurrence on *Alnus incana* in Poland. The ascertained *H. abietinum* strains derived from *Abies alba*. Our results demonstrate a new method of *Heterobasidion* species identification based on the different length of MNP1a and MNP2 peroxidase genes and RFLP markers obtained for laccase and Mn^{2+} dependent peroxidase genes.

Additional key words: root rot, intersterility groups, laccase, manganese peroxidase, PCR-RFLP markers

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Introduction

Heterobasidion annosum s.l. plays an important role in causing root and butt rot of trees in the boreal and temperate zone of the northern hemisphere (Vainio and Hantula 1999; Asiegbu et al. 2005). In Poland it occurs within an area of 200,000 hectares (Sierota 1998). The greatest losses are caused in first and second generation stands established on post-farming lands and in tree stands in areas subjected to industrial pollution (Domański 1976; Sierota 1998). In Europe, three intersterility groups (ISG) of *Heterobasidion annosum* s.l. were distinguished: P, S and F, named after their main host species (Korhonen 1978; Capretti et al. 1990; Niemelä and Korhonen 1998). They have been separated into three biological species: *H. annosum* (Fr.) Bref. s.s., *H. parviporum* Niemelä & Korhonen and *H. abietinum* Niemelä & Korhonen (Niemelä and Korhonen 1998). They display different host preferences (Korhonen 1978; Capretti et al. 1990; Łakomy and Werner 2003) and are characterised by various virulence (Werner and Łakomy 2002). All three *Heterobasidion* species are present in Poland (Żółciak 1992; Łakomy 1996; Kowalski and Łakomy

1998; Łakomy et al. 2000; Łakomy and Werner 2003). The identification at the species level is performed mainly by sexual compatibility tests, by checking the ability of isolates to dikaryotize homokaryotic tester strains (Żółciak 1992; Łakomy and Werner 2003). However, this method has several disadvantages including the long time to complete a definite pairing and the high variability of the interaction morphology (Schulze and Bahnweg 1998). Attention is also drawn to the fact that all the fungi species show varying degrees of inter-fertility in the laboratory (La Porta et al. 1997). Mating tests clearly differentiated *H. annosum* s.s. from *H. parviporum* and *H. abietinum*, but there were some difficulties discriminating *H. abietinum* from *H. parviporum*, because they are partially compatible (Łakomy et al. 2000).

Molecular methods can be used for the identification of intersterility groups of *Heterobasidion*. In order to differentiate *H. parviporum* isolates, Garbelotto et al. (1998) used Taxon Specific Competitive Priming (TSCP) PCR in the ML5-ML6 DNA region of mt LrRNA, whereas the identification of *H. abietinum* and *H. annosum* s.s. was possible due to the different lenghts of intron in the listed region. Other authors used DNA fingerprinting (RAPD, RAMS, AP-PCR) (Garbelotto et al. 1998; Vainio and Hantula 1999; Dai et al. 2003) and PCR-RFLP (Gonthier et al. 2001) of ITS region for the identification of *Heterobasidion* species.

H. annosum s.l. is a necrotrophic wood decay fungus that secretes a wide range of extracellular enzymes which degrade structural and soluble host constituents such as sugars, starch, pectin, cellulose and lignin (Asiegbu et al. 1998; Johansson 1988). Thanks to these enzymes, the fungi digests plant cell wall components providing nutrients and aids in the penetration of cells, allowing for its survival and spread through woody tissues. *H. annosum* s.l. is able to degrade lignin and detoxify endogenous phenols produced in response to infection thanks to the secretion of the laccase enzyme cooperating with lignin peroxidases (LiP) and Mn^{2+} dependent peroxidase (MnP) (Asiegbu et al. 1998; Haars et al. 1981). Individual intersterility groups of *H. annosum* s.l. produce laccase with various intensity, which is correlated with their wood decay ability in trees. *H. annosum* s.s. which possess significantly greater wood degrading ability than *H. parviporum*, secrete 5–6 times more laccase than the *H. parviporum* (Daniel et al. 1998; Asiegbu et al. 2004). The activity of MnP peroxidase was studied in homocaryotic isolates of European strains of *H. annosum* s.l. in decomposed wood, and three isoenzymes (MnP1a, MnP2, MnP3) were identified for it in Europe (Maijala et al. 2003). The presence and biochemical features of these enzymes is connected with different pathogenicity and host specialization of fungi species (Asiegbu et al. 1998).

The objective of this study was to find PCR-RFLP markers, based on laccase and MnP peroxidase genes, useful in the identification of *Heterobasidion* species with the use of strains isolated from various tree species in southern Poland. On the basis of these markers, it will be possible to prepare a method of quick and correct identification of intersterility groups, as well as obtain further data regarding the association between *Heterobasidion* species and various tree species in Poland.

Material and Methods

Isolates

Eighty-one strains of *Heterobasidion annosum* s.l. were taken into account (Table 1). They were isolated between 1993 and 2004 from roots or butts of eleven forest tree or bush species displaying symptoms of root rot or butt rot and sporadically from sporocarp trama produced at the trunk base. Wood fragments for fungi isolation were collected from 23 to approx. 80 year old trees. They derived from six Forest Districts, three National Parks and one suburban park located in southern Poland (Fig. 1).

Isolations were performed within 24 hours of the collection of samples in the field. The samples were surface sterilized with 96% ethanol and then the bark was removed from the wood. Pieces of 5 × 2 × 2 mm of decaying wood and from the zone adjoining the healthy wood were cut and placed in Petri dishes filled with 2% malt extract (MEA; 20 g l^{-1} malt extract Difco, Sparks, MD, USA, 15 g l^{-1} agar Difco supplemented with 100 mg l^{-1} streptomycin sulphate). From each sample, 6–24 fragments of wood were used for isolation. The cultures were then incubated at room temperature in the dark. Among numerous colonies of other fungi, 1–24 colonies of *H. annosum* s.l. grew. One colony from the sample was randomly collected each time. Growing mycelium was transferred to new MEA plates and incubated at 20°C in the dark.

Molecular analysis

The research was conducted in two stages: a) the first was aimed at finding PCR-RFLP markers characteristic for *Heterobasidion* species. They were based on the laccase and Mn^{2+} dependent peroxidase genes amplified for twenty-nine strains (Table 1, no. 1–29) whose fungi species were previously determined on the basis of mating tests (Łakomy et al. 2000); b) the second was aimed at *Heterobasidion* species determination of fifty-two strains (Table 1, no. 30–81) based on PCR-RFLP markers obtained during stage 1. Extraction of genomic DNA was conducted by means of a method by Carlson et al. (1991). Laccase (GenBank Y16951) and Mn^{2+} dependent peroxidase genes for *H. annosum* s.s., *H. parviporum*, *H. abietinum*: MnP1a

Table 1. *Heterobasidion annosum* s.l. isolates examined

No.	Strain No.	Host	Substrate type*	For. District	Date	*Heterobasidion* species**
1	16193/1	*Abies alba*	D	ONP	05.10.1995	*H. abietinum*
2	17076	*Abies alba*	A	ONP	24.04.2003	*H. abietinum*
3	15744	*Betula pendula*	A	Świerklaniec	19.09.1993	*H. annosum* s.s.
4	17018	*Betula pendula*	A	Świerklaniec	22.09.2000	*H. annosum* s.s.
5	17017	*Betula pendula*	A	Świerklaniec	22.09.2000	*H. annosum* s.s.
6	16488	*Padus avium*	A	Kraków	26.11.1996	*H. annosum* s.s.
7	15699	*Quercus rubra*	A	Świerklaniec	09.07.1993	*H. annosum* s.s.
8	17021/1	*Larix decidua*	A	Świerklaniec	22.09.2000	*H. annosum* s.s.
9	17020/1	*Larix decidua*	A	Świerklaniec	22.09.2000	*H. annosum* s.s.
10	16073	*Larix decidua*	C	ONP	13.05.1995	*H. parviporum*
11	15739	*Larix decidua*	A	Świerklaniec	19.09.1993	*H. annosum* s.s.
12	17021/2	*Larix decidua*	A	Świerklaniec	22.09.2000	*H. annosum* s.s.
13	17004	*Larix decidua*	A	ONP	20.08.2000	*H. parviporum*
14	17020/2	*Larix decidua*	A	Świerklaniec	22.09.2000	*H. annosum* s.s.
15	15996	*Pinus nigra*	A	Świerklaniec	08.10.1994	*H. parviporum*
16	16686	*Pinus sylvestris*	C	Koniecpol	10.06.2000	*H. annosum* s.s.
17	16682	*Pinus sylvestris*	C	Koniecpol	10.06.2000	*H. annosum* s.s.
18	17044	*Pinus sylvestris*	C	Koniecpol	02.02.2001	*H. annosum* s.s.
19	15679	*Pinus sylvestris*	A	Świerklaniec	08.07.1993	*H. annosum* s.s.
20	16096	*Pinus strobus*	A	Świerklaniec	30.05.1995	*H. annosum* s.s.
21	16010	*Pinus strobus*	A	Świerklaniec	08.10.1994	*H. annosum* s.s.
22	15719	*Pinus strobus*	A	Świerklaniec	19.09.1993	*H. annosum* s.s.
23	17043	*Picea abies*	A	GNP	07.12.2000	*H. parviporum*
24	16188	*Picea abies*	D	ONP	04.10.1995	*H. parviporum*
25	17079	*Picea abies*	A	Myślenice	24.04.2003	*H. parviporum*
26	16597	*Picea abies*	C	Myślenice	06.06.1998	*H. parviporum*
27	16131	*Picea abies*	D	GNP	20.06.1995	*H. parviporum*
28	17078	*Picea abies*	A	TNP	24.04.2003	*H. parviporum*
29	17040	*Picea abies*	A	GNP	07.12.2000	*H. parviporum*
30	17084	*Abies alba*	B	ONP	20.04.2004	*H. abietinum*
31	18305	*Abies alba*	A	ONP	02.10.2004	*H. parviporum*
32	18304/1	*Abies alba*	A	ONP	02.10.2004	*H. parviporum*
33	18304/2	*Abies alba*	A	ONP	02.10.2004	*H. parviporum*
34	18306/1	*Abies alba*	A	ONP	02.10.2004	*H. parviporum*
35	18306/2	*Abies alba*	A	ONP	02.10.2004	*H. parviporum*
36	18307/1	*Abies alba*	A	ONP	02.10.2004	*H. parviporum*
37	18309/1	*Abies alba*	B	ONP	02.10.2004	*H. parviporum*
38	18309/2	*Abies alba*	B	ONP	02.10.2004	*H. parviporum*
39	18105/2	*Alnus incana*	A	Świerklaniec	15.06.2004	*H. annosum* s.s.
40	18357	*Alnus incana*	A	Świerklaniec	23.10.2004	*H. parviporum*
41	18149	*Betula pendula*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
42	18011	*Betula pendula*	A	Świerklaniec	14.06.2004	*H. annosum* s.s.
43	18015	*Betula pendula*	A	Świerklaniec	14.06.2004	*H. annosum* s.s.
44	18156	*Betula pendula*	A	Świerklaniec	17.06.2004	*H. annosum* s.s.
45	18345	*Betula pendula*	A	Świerklaniec	23.10.2004	*H. annosum* s.s.
46	18347	*Betula pendula*	A	Świerklaniec	23.10.2004	*H. annosum* s.s.
47	18355	*Betula pendula*	A	Świerklaniec	23.10.2004	*H. annosum* s.s.
48	18103/2	*Betula pendula*	A	Świerklaniec	15.06.2004	*H. annosum* s.s.
49	17072	*Larix decidua*	B	ONP	18.03.2002	*H. annosum* s.s.

No.	Strain No.	Host	Substrate type*	For. District	Date	*Heterobasidion* species**
50	18030	*Larix decidua*	A	Świerklaniec	14.06.2004	*H. annosum* s.s.
51	18037	*Larix decidua*	A	Świerklaniec	14.06.2004	*H. annosum* s.s.
52	17074	*Picea abies*	B	ONP	24.04.2003	*H. parviporum*
53	17077	*Picea abies*	A	TNP	24.04.2003	*H. parviporum*
54	17080	*Picea abies*	A	BNP	24.04.2003	*H. parviporum*
55	17087	*Picea abies*	B	ONP	20.04.2004	*H. annosum* s.s.
56	17086	*Picea abies*	A	ONP	20.04.2004	*H. annosum* s.s.
57	18244	*Pinus nigra*	A	Świerklaniec	18.06.2004	*H. annosum* s.s.
58	18241	*Pinus nigra*	A	Świerklaniec	18.06.2004	*H. annosum* s.s.
59	18121	*Pinus strobus*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
60	18005	*Pinus strobus*	A	Świerklaniec	14.06.2004	*H. annosum* s.s.
61	18117/1	*Pinus strobus*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
62	18199	*Pinus strobus*	A	Świerklaniec	17.06.2004	*H. annosum* s.s.
63	18120	*Pinus strobus*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
64	18153	*Pinus strobus*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
65	18123/1	*Pinus strobus*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
66	18123/2	*Pinus strobus*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
67	17075	*Pinus sylvestris*	B	Opoczno	24.04.2003	*H. annosum* s.s.
68	18042	*Pinus sylvestris*	A	Świerklaniec	14.06.2004	*H. parviporum*
69	18107/1	*Pinus sylvestris*	A	Świerklaniec	15.06.2004	*H. parviporum*
70	18126/1	*Pinus sylvestris*	A	Świerklaniec	16.06.2004	*H. parviporum*
71	18122	*Pinus sylvestris*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
72	18361	*Pinus sylvestris*	A	Mielec	23.10.2004	*H. annosum* s.s.
73	18108	*Quercus robur*	A	Świerklaniec	15.06.2004	*H. annosum* s.s.
74	18296	*Quercus robur*	A	Świerklaniec	02.07.2004	*H. annosum* s.s.
75	18298	*Quercus robur*	A	Świerklaniec	02.07.2004	*H. annosum* s.s.
76	18303	*Quercus robur*	A	Świerklaniec	02.07.2004	*H. annosum* s.s.
77	18101/3	*Quercus rubra*	A	Świerklaniec	15.06.2004	*H. annosum* s.s.
78	18101/1	*Quercus rubra*	A	Świerklaniec	15.06.2004	*H. annosum* s.s.
79	18152/1	*Quercus rubra*	A	Świerklaniec	16.06.2004	*H. annosum* s.s.
80	18289	*Quercus rubra*	A	Świerklaniec	02.07.2004	*H. annosum* s.s.
81	18288	*Quercus rubra*	A	Świerklaniec	02.07.2004	*H. annosum* s.s.

* – substrate type: A – killed tree, B – stump, C – diseased tree, D – laying log
** – *Heterobasidion* species identified on the basis of PCR-RFLP markers during the present investigation
BNP – Babia Góra National Park, GNP – Gorce National Park, ONP – Ojców National Park, TNP – Tatra National Park

(GenBank respectively AJ507472, AJ507469, AJ507471), MnP2 (AJ507479, AJ507475, AJ507478) and MnP3 (AJ507484, AJ507485, AJ507482) (Maijala et al. 2003) were amplified. Primers were designed on the basis of the above-listed sequences with the use of the DNASTAR Lasergene programme (DNASTAR, Inc.) or it was used primers previously designed by Maijala et al. (2003) (Table 2).

The amplification was performed in 100 ml of reaction mixture consisted of: Taq buffer, 2 μM $MgCl_2$, 0.2 mM dNTP, 0.1 μM each primer (Proligo), 12 U polymerase (Fermentas), 40 ng DNA. Thermocycler was programmed as follows: initial denaturation – 94°C, 5 minutes, annealing – 56°C (laccase) or 54°C (MnP1a, MnP2, MnP3), 2 minutes, elongation – 72°C, 5 minutes, subsequently 36 (laccase) or 33 (MnP1a, MnP2, MnP3) cycles comprising denaturation – 94°C, 1 minute, annealing 56°C (laccase) or 54°C (MnP1a, MnP2, MnP3), 2 minutes and elongation 72°C, 3 minutes. The elongation of the last cycle was prolonged to 10 minutes. The result of the amplification and the length of its products was visualised in a 2% agarose gel. Before digestion, PCR products were purified by acetate buffer precipitation. The obtained DNA was dissolved in 20 μl of TE buffer.

Ten restriction enzymes were used for the digestion of the DNA fragments: AluI, BcnI, Bme1390I, Bsp143I, BsuRI, Cfr13I, Csp6I, Hinf1, Taq1, Tru1I (Fermentas). Digestion was conducted in 5 μl of a reaction mixture consisting of: 1 μg DNA, 1U of restriction enzyme and the buffer. The digestion reactions were conducted at a temperature recommended by

Fig. 1. Schematic map of Poland with marked locations of isolates origin of *Heterobasidion*. BNP – Babia Gora National Park, GNP – Gorce National Park, ONP – Ojcow National Park, TNP – Tatra National Park

the manufacturer for two hours. The obtained DNA fragments were resolved electrophoretically in 6–8% of polyacrylamide gel at a voltage of 3V/cm for 4 hours. The GeneRuler 100 bp Ladder Plus (Fermentas) was used as a marker of the length of DNA fragments. The gels were stained for 10 minutes in 1 μg/ml ethidium bromide.

Results

All strains were characterised by the presence of the laccase gene with a length of 1520 bp. The applied primers produced Mn^{2+} peroxidase fragments with a length depending on the gene (MNP1a, MNP2, MNP3) and *Heterobasidion* species. Amplification of the MnP1a peroxidase gene produced fragments of two lengths: 548 bp for *H. abietinum* and *H. parviporum*, and 580 bp for *H. annosum* s.s. MnP2 gene fragments had a length of 539 bp for *H. abietinum* and 547 bp for *H. annosum* s.s. and *H. parviporum*. In the case of the MnP3 gene, two different amplification fragments were obtained: 562 and 577 bp; however, no dependence was ascertained between the length of obtained DNA fragments and the pathogen species.

The digestion of PCR products enabled the obtaining of 184 PCR-RFLP markers. For the laccase and peroxidase genes: MnP1a and MnP2 eighteen monomorphic markers for *Heterobasidion* species were found. In the case of MnP3 peroxidase gene no monomorphic DNA fragments were found. Nine markers were common for *H. parviporum* and *H. abietinum*, whereas only one was common for *H. parviporum* and *H. annosum* s.s. The remaining markers were characteristic for *H. abietinum* (three markers) or *H. annosum* s.s. (five markers) (Table 3, Fig. 2). The presence of the above-listed monomorphic markers enabled the identification of strains belonging to *H. abietinum* and *H. annosum* s.s. Identification of *H. parviporum* strains was more difficult; no marker characteristic for only this species was observed. In this case two digestions of laccase gene were needed for the identification: a 208 bp fragment after Csp6I digestion together with 562 bp fragment after HinfI digestion classified a given strain as *H. parviporum*.

The identification of *Heterobasidion* species of twenty-nine strains (stage I) was agreed with the results of mating tests. This enabled the determination of fungi species of the remaining strains solely on the basis of the presence of PCR-RFLP markers (Table 1). Out of eighty-one analysed strains, fifty-three belonged to *H. annosum* s.s., twenty-five to *H. parviporum*, and three to *H. abietinum*. Strains belonging to *H. annosum* s.s. were isolated from *Pinus nigra, P. strobus, P. sylvestris, Larix decidua, Picea abies,* as well as from *Alnus incana, Betula pendula, Padus avium, Quercus robur* and *Q. rubra*. *H. parviporum* were isolated from *Abies alba, Picea abies, Pinus nigra, P. sylvestris, Larix decidua* and from *Alnus incana*. The ascertained *H. abietinum* strains derived from *Abies alba* (Table 4).

Discussion

The correct identification of fungal species and races has a fundamental significance for proper procedures aimed at the control and restriction of the epi-

Table 2. Primers used in laccase and peroxidase genes amplification of *Heterobasidion annosum* s.l.

No.	Gene	Primer name	Sequence (5'→3')	Tm [°C]
1	Laccase	LAC-F[1]	CTGTCTGCTCAATACTGTGAC	44
		LAC-R[1]	GGTGTAGAAGCGGATGGT	56
2	MnP1a	MnP1-F[2]	GATGGGTCCATCATCGTA	54
		MnP1-R[2]	GAGTTCCTGGGATCGTCAC	60
3	MnP2	MnP2-F[2]	TGCCGATGGGTC(G/T/A)ATATC	54
		MnP2-R[1]	AACGGCGTTCCAGGGATGGTT	61
4	MnP3	MnP3-F[2]	GATGGGTCCCT(C/T)AT(C/T)GTG	60
		MnP3-R[1]	GGGGTGGAGTCGAACGGTGTCC	63

[1] – primers designed with the use of DNASTAR Lasergene, [2] – primers designed by Maijala et al. (2003)

Table 3. Monomorphic fragments for *Heterobasidion* species

No.	Gene	Restriction enzyme	Length [bp]	*Heterobasidion* species
1	Laccase	BcnI	274	*H. abietinum*, *H. parviporum*
2		BcnI	126	*H. abietinum*, *H. parviporum*
3		BcnI	79	*H. abietinum*, *H. parviporum*
4		Csp6I	562	*H. abietinum*
5		Csp6I	208	*H. parviporum*, *H. annosum* s.s.
6		Hinf1	562	*H. abietinum*, *H. parviporum*
7		Tru1I	76	*H. annosum* s.s.
8	MnP1a	BcnI	319	*H. abietinum*
9		BsuRI	266	*H. abietinum*, *H. parviporum*
10		BsuRI	158	*H. abietinum*, *H. parviporum*
11		Cfr13I	260	*H. abietinum*, *H. parviporum*
12		Cfr13I	144	*H. annosum* s.s.
13		Taq1	580	*H. annosum* s.s.
14	MnP2	Hinf1	373	*H. abietinum*
15		Taq1	290	*H. abietinum*, *H. parviporum*
16		Taq1	175	*H. annosum* s.s.
17		Taq1	151	*H. abietinum*, *H. parviporum*
18		Taq1	140	*H. annosum* s.s.

demic spread of fungi over large forest areas. It is particularly significant in the case of fungi which are complex species comprising numerous intersterility groups. Nowadays, the most frequently used method for identification of *Heterobasidion* species is through mating tests. However *H. parviporum* and *H. abietinum* are partially interfertile in the laboratory and this is a pitfall of mating tests (Łakomy et al. 2000). It indicates that this method of identification is not enough to separate *Heterobasidion* species, particularly *H. parviporum* and *H. abietinum* (Johannesson and Stenlid 2003). It results from a much closer affinity between *H. abietinum* and *H. parviporum* in comparison with strains belonging to *H. annosum* s.s. This dependency was proven in numerous studies based both on methods using random primers such as RAPD (Fabritius and Karjalainen 1993; La Porta et al. 1997), RAMS (Dai et al. 2003) and AP-PCR (Garbelotto et al. 1998), as well as sequence analysis of ribosomal DNA (IGS, ITS) (Harrington and Rizz 1999; Johannesson and Stenlid 2003) or isoenzymes (Otrosina et al. 1993). Johansson et al. (1999) also ascertain that the laccase isozyme pattern of *H. abietinum* was similar to that of *H. parviporum*. The same conclusions are drawn by other authors on the basis of laccase sequence analyses for strains belonging to fungi species (Asiegbu et al. 2004). In the case of *H. annosum* s.s., identification is easy both with the use of mating tests as well as molecular biology methods. In relation to this, methods of molecular biology are coming to be

Table 4. Frequency of samples belonging to the different *Heterobasidion* species on the plant species examined

No.	Host	*Heterobasidion* species			Total
		H. annosum s.s.	*H. parviporum*	*H. abietinum*	
1	*Abies alba*	0	7	3	10
2	*Larix decidua*	9	2	0	11
3	*Picea abies*	2	10	0	12
4	*Pinus nigra*	2	1	0	3
5	*Pinus strobus*	11	0	0	11
6	*Pinus sylvestris*	7	3	0	10
7	*Alnus incana*	1	1	0	2
8	*Betula pendula*	11	0	0	11
9	*Padus avium*	1	0	0	1
10	*Quercus robur*	4	0	0	4
11	*Quercus rubra*	6	0	0	6
	Total	53	25	3	81

used more and more frequently in the identification of *Heterobasidion* species. In contrast to identification based on traditional pairing tests, they are more accurate, sensitive and rapid (Garbelotto et al. 1993). It is necessary to emphasise that the amplification results of two peroxidase genes (MnP1a and MnP2) in our study displayed varied lengths of DNA fragments, which are characteristic for fungi species. These results show that the simultaneous conduct of amplification of the MNP1a and MNP2 genes may be used for *Heterobasidion* species identification.

The obtained monomorphic markers indicate that it is easy to differentiate *H. annosum* s.s. from *H. parviporum* and *H. abietinum*. Half of the obtained monomorphic markers were simultaneously found in *H. parviporum* and *H. abietinum*. *H. annosum* s.s. may also be identified with the use of monomorphic markers that are characteristic only for this group. In total, fourteen out of eighteen monomorphic markers can be used to differentiate *H. annosum* from the remaining fungi species. It is also relatively easy to identify strains belonging to *H. abietinum* thanks to the discovery of markers that are characteristic for it. Even though the research has not led to finding markers characteristic for *H. parviporum*, it is possible to identify strains belonging to it with the use of a marker combination. Monomorphic markers obtained for *H. annosum* s.s. and *H. abietinum* may not only serve as a

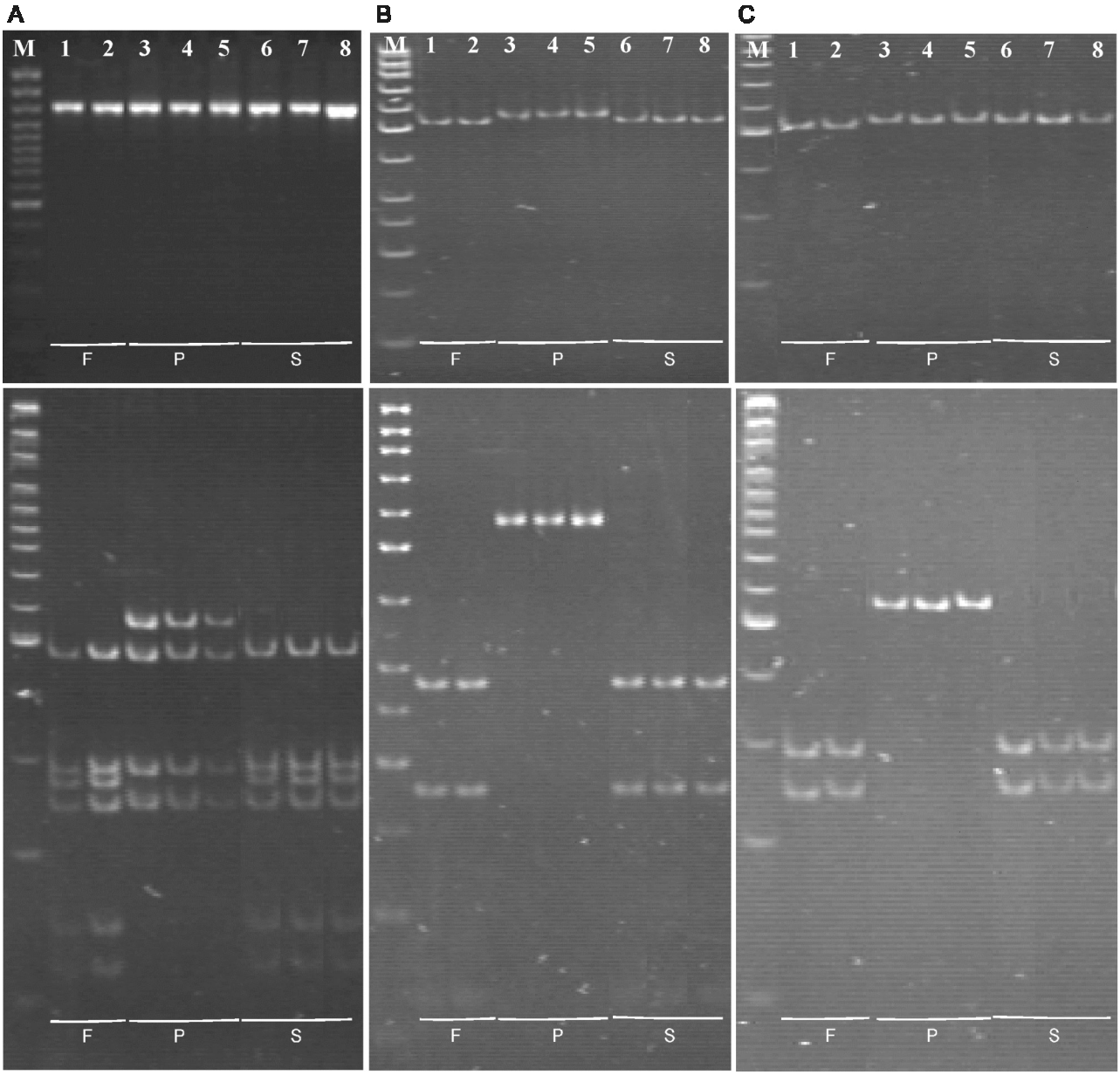

Fig. 2. PCR-RFLP for laccase (A), MNP1a peroxidase (B) and MNP2 peroxidase (C). In upper raw the length of amplicons, in lower raw electrophoretic profiles. Amplicons were digested with the restriction enzymes: BcnI (A and C) and TaqI (B) and electrophorised in 6% polyacrylamide gels. M – marker, lines 1, 2 – F ISG (*H. abietinum)* isolates, line 3–5 – P ISG (*H. annosum* s.s.) isolates, 6–8 – S ISG (*H. parviporum*) isolates

diagnostic tool in the identification of pathogen species; on the basis of their sequences, specific primers may be prepared which will be used for the identification of *Heterobasidion* species directly in the infected wood.

Recent studies of phylogeny within the *H. annosum* complex indicate that the three European (S, P, F) groups form separate clades. Several authors showed genetic differences among *Heterobasidion* species using isozymes, polymorphism in ITS regions and housekeeping genes like: glyceraldehyde dehydrogenase, elongation factor, laccase and manganese peroxidase (Asiegbu et al. 2004; Johannesson and Stenlid 2003; Chase et al. 1991). The last two genes are involved in pathogenicity by their participation in the lignin bio-degradation process. Differences in their sequences occurring in strains belonging to fungi species may reflect a selective effect of the strain growth, various pathogenicity and the mode of the infection process occurring on various host plant species (Garbelotto et al. 1998; Johansson et al. 1999; Johannesson and Stenlid 2003; Asiegbu et al. 2005). Previous researches show the existence of several genes of laccase (Asiegbu et al. 2004) and Mn^{2+} peroxidise (Maijala et al. 2003) in the *H. annosum* complex. The different length of peroxidase genes and characteristic for *Heterobasidion* species RFLP markers found in present studies also suggest a potential role of the investigated genes in host adaptation.

Our results show that the strains of *H. annosum* s.s. occur in all investigated tree species with the exception of *Abies alba*. In Poland this fungus species was found almost in the whole country (Łakomy et al. 2000). It is connected with the fact that Scots pine stands contribute to 70% of Polish forests. Among the deciduous trees *H. annosum* s.s. often occurs on birch trees which play an important role as a pathogen source (Domański 1976; Łakomy and Werner 2003). In the present research *H. parviporum* was found on *Picea abies* and *Abies alba*, and occasionally on *Larix decidua*, *Pinus nigra* and *Pinus sylvestris*. This species of fungus caused root rot on young and heart rot on older spruce individuals. It was also identified on 30-year-old pines, which showed root rot symptoms, but it was not found on pines which indicated heart rot. It should be emphasized that we also found this species on *Alnus incana*. This is the first report of occurrence of the *H. parviporum* on this species in Poland. Among ten fungal strains isolated from *Abies alba* only three belonged to *H. abietinum* and remaining to *H. parviporum*. *H. abietinum* occurs mainly in south Poland but is rather rare. Łakomy and Werner (2003) suggest that it is caused by genetic resistance of *Abies alba* provenances from central Europe. Our researches confirm that, from three investigated species of pathogen, *H. abietinum* has the most narrow host range.

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