Specific Primers for the Differentiation of *Heterobasidion annosum* (s.str.) and *H. parviporum* Infected Stumps in Northern Europe

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Two separate amplification products from random amplified microsatellite fingerprints of *Heterobasidion annosum* (s.str.) and *H. parviporum* were converted to specific markers. The markers were tested to be species specific and combined to a single PCR-reaction, which allowed the detection and identification of the two fungi directly from wood samples.

Keywords IS group S, IS group P, molecular identification, root and butt rot
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1 Introduction

Heterobasidion annosum (Fr.) Bref. sensu lato is a pathogenic basidiomycete, which causes root and butt rot in conifers of northern temperate and boreal forests. The species has been divided to intersterility groups (IS groups), two of which (S and P) occur in northern Europe (Korhonen 1978). Although few natural intergroup hybrids have been reported between IS groups S and P in North America (Garbelotto et al. 1996), and compatible matings have been observed in pairing experiments (Korhonen 1978, Olson and Stenlid 2001, Stenlid and Karlsson 1991), the two IS groups do not seem to hybridize naturally in Europe. Therefore the fungi occurring in Europe were recently named as *H. parviporum* (IS group S) and *H. annosum* (Fr.) Bref. sensu stricto (Niemelä and Korhonen 1998). *H. parviporum* (Niemelä and Korhonen) attacks mainly *Picea abies* (L.) Karst., and *H. annosum* s.str. prefers *Pinus sylvestris* (L.), but occurs also on several other tree species including *P. abies*.

Heterobasidion sp. spreads to new sites by basidiospore infections to fresh stumps, from which the mycelium grows vegetatively to living

trees via root contacts. This can be controlled by conducting fellings only during the wintertime when no basidiospores are formed, or by treating the fresh stumps by biological or chemical control agents (Risbeth 1952). In sites where infection has been established, the fungus may grow vegetatively from old infected stumps to the next tree generation using root contacts (Stenlid 1987, Piri 1996, Piri and Korhonen 2001). This kind of dispersal can be avoided by using a non-host tree species for forest regeneration.

In Finland, regeneration of heavily contaminated spruce stand with spruce is not recommended if the site supports growth of any other tree species. If the pathogen on the site belongs to *H. parviporum*, then pine, birch or aspen can all be planted. However, if *H. annosum* has contaminated the site, then both pine and spruce should be avoided. Therefore, it should be confirmed that *H. annosum* is not present on the site when Scots pine is planned to be used for the regeneration.

The two *Heterobasidion* species can be differentiated by pectic isozyme profiles (Karlsson and Stenlid 1991), fatty acid and sterol (FAST) profiles (Müller et al. 1995), as well as using several DNA-based techniques, including randomly amplified DNA (RAPD) analysis (Fabritius and Karjalainen 1993, Garbelotto et al. 1993, Karjalainen 1996), M13 minisatellite fingerprinting (Karlsson 1994, Stenlid et al. 1994), ribosomal DNA sequencing (Kasuga et al. 1993) and random amplified microsatellite (RAMS or ISSR) fingerprints (Vainio and Hantula 1999). All these methods as well as traditional pairing tests (Korhonen 1978) are based on mycelial cultures. However, in northern Europe a method allowing identification of *H. annosum* directly from infected wood would be ideal for practical purposes. It would also be useful if the occurrence of *H. parviporum* could be recognized in the same test.

PCR-based identification methods have been described for various fungi. The methods may be based on Internal Transcribed Spacer (Lindqvist et al. 1998), ribosomal RNA genes (Edel et al. 2000) or other DNA (Paavolainen et al. 2000). In this report we describe the development and testing of a PCR based tool for the identification of *H. annosum* and *H. parviporum*.

2 Material and Methods

2.1 DNA Isolations

DNA from both cultured isolates (Table 1) and wood chips (Table 2) was used. For most mycelial samples previously isolated DNA was applied (Table 1), and the DNA from new isolates was isolated basically as described in Vainio et al. (1998). DNA from *Heterobasidion*-infected wood chips (Table 2) was isolated with either the method described in Vainio and Hantula (2000a) (early part of this investigation) or using the UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories Inc.).

2.2 RAMS-Reactions

For the cloning of *Heterobasidion*-specific fragments we amplified RAMS markers from fungal DNA as described in Vainio and Hantula (2000b). The RAMS-primers used were CT-primer: $VDV(CT)_7C$ and CCA-primer: 5´$DDB(CCA)_5$, where D = A/G/T, B = C/G/T and V = A/C/G. Amplification products were separated by electrophoresis and visualised using UV light (Vainio and Hantula 1999).

2.3 The Design of Specific Primers and PCR-Conditions

Amplification products CT1350 and CCA1210 specific to *H. annosum* and *H. parviporum* (Vainio and Hantula 1999) were cloned from isolates 36 and 29, respectively, to *Escherichia coli* as described previously (Vainio and Hantula 2000b). The selection of fragments was based on standard agarose gel electrophoresis. The cloned inserts were sequenced by A.L.F. DNA Sequencer™ (Pharmacia Biotech, Uppsala, Sweden) using M13 reverse and forward primers and the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech, England). Primers MJ-F and MJ-R (Table 3) were designed based on the sequence of CT1350 (Gen Bank accession number AY211523) in order to amplify an approximately 100 bp fragment from isolates belonging to *H. annosum*, and primers

Table 1. Fungal isolates used.

Species	Isolate	Original collector[a)]	Species	Isolate	Original collector[a)]
H. parviporum	1	KK[b)]	*Cylindrobasidion evolvens*	Var1/45	AMH[c)]
H. parviporum	2	KK[b)]	*Penicillium* sp.	M71/A1	EV[c)]
H. parviporum	3	KK[b)]	*Panellus mitis*	Ref1/75	AMH[c)]
H. parviporum	4	KK[b)]	*Coniophora arida*	6C7	AMH[c)]
H. parviporum	5	KK[b)]	*Verticicladiella procera*	Ref2/24	AMH[c)]
H. parviporum	6	KK[b)]	*Exophiala* sp.	Var3/10	AMH[c)]
H. parviporum	7	KK[b)]	*Ischnoderma benzoinum*	28-75	AMH[d)]
H. parviporum	8	KK[b)]	*Phialophora* sp.	Var7/27	AMH[c)]
H. parviporum	9	KK[b)]	*Amylostereum chailletii*	Ref2/34	AMH[c)]
H. parviporum	10	KK[b)]	*Amylocystis lapponica*	11715/1	RP[c)]
H. parviporum	13	KK[b)]	*Stereum sanguinolentum*	Var1/57	AMH[d)]
H. parviporum	18	KK[b)]	*Polyporus brumalis*	15a8	AMH[d)]
H. parviporum	22	HM[b)]	*Phlebiopsis gigantea*	K31/7	EV[c)]
H. parviporum	29	OH[b)]	*Peniophora pithya*	Ref1/31	AMH[c)]
H. annosum	32	KK[b)]	*Merulius lacrymans*	10a10	AMH[c)]
H. annosum	33	KK[b)]	*Bjerkandera adusta*	Ref1/8	AMH[c)]
H. annosum	34	KK[b)]	*Sistotrema brinkmannii*	72-3	AMH[d)]
H. annosum	35	KK[b)]	*Hypholoma capnoides*	1iA11	AMH[c)]
H. annosum	36	KK[b)]	*Chondrostereum purpureum*	Ref1/16	AMH[c)]
H. annosum	37	KK[b)]	*Armillaria cepistipes*	92159/VE	KK[c)]
H. annosum	38	KK[b)]	*Fomitopsis pinicola*	7C8	AMH[c)]
H. annosum	39	KK[b)]	*Phaeolus schweinitzii*	Ref2/25	AMH[c)]
H. annosum	40	KK[b)]	*Gliocladium* sp.	M84/1	EV[c)]
H. annosum	44	KK[b)]	*Armillaria ostoyeae*	94276/2	KK[c)]
H. annosum	45	KK[b)]	*Armillaria borealis*	92146/4	KK[c)]
H. annosum	52	OH[b)]	*Neobulgaria premnophila*	11d9	AMH[c)]
H. annosum	53	HM[b)]	*Resinicium bicolor*	12a7	AMH[c)]
Polyporus borealis	Ref1/36	AMH[c)]	*Trichoderma* sp.	K62/11	EV[c)]
Trametes hirsuta	Ref3/7	AMH[d)]	*Ascocoryne* sp.	M15/3	EV[d)]
Trametes zonata	Ref1/20	AMH[c)]	*Rhinocladiella* sp.	MU7/5	EV[c)]
Merulius serpens	11a4	AMH[d)]	*Amylostereum areolatum*	Ref2/36	AMH[c)]

[a)] KK, Kari Korhonen; HM, Helga Marxmüller; OH, Ottmar Holdenrieder; AMH, Anna-Maija Hallaksela; EV, Eeva Vainio; RP, Reijo Penttilä.
[b)] More detailed information about these isolates has been given previously (Vainio and Hantula 1999).
[c)] More detailed information about these isolates has been given previously (Vainio and Hantula 2000a).
[d)] Unpublished.

KJ-F and KJ-R (Table 3) were designed based on the sequence of CCA1210 (accession number AY211522) in order to amplify an approximately 350 bp fragment from isolates belonging to *H. parviporum*. The amplification conditions for these primers were optimised using pure cultures so that both primer pairs could be used in the same PCR-reaction.

As a result, the amplification was carried out using a 'hot start' protocol where DNA samples and primers (each in a concentration of 0.5 uM) were denaturated at +95 °C for 10 minutes, after which a dNTP-mix (each deoxynucleotide in a concentration of 0.2 mM) and 20 U/ml of Dynazyme thermostable DNA-polymerase (Finnzymes Ltd, Espoo, Finland) were added. Then 40 cycles of amplification (30 s denaturation at 95 °C, 35 s annealing at 67 °C, 1 min extension at 72 °C) and a final extension of 7 minutes were carried out. The buffer conditions were the same as suggested by the manufacturer of the Dynazyme polymerase.

2.4 Tests for the Specificity of the Primers

The specificity of the primers was tested using DNA from 14 isolates of *H. parviporum*, 13

Table 2. Information about the wood chips, and amplification products obtained.

Wood chip	Wood species	*Heterobasidion species*/ basis of the determination	Length of the amplification product
KK1 [a)]	Norway spruce	*H. annosum*/pairing tests [a)]	100 bp
KK2	Norway spruce	*H. annosum*/pairing tests	100 bp
KK3	Norway spruce	No *Heterobasidion*	No amplification
KK4	Norway spruce	*H. parviporum*/pairing tests	350 bp
KK5	Norway spruce	*H. annosum*/pairing tests	100 bp
KK6	Norway spruce	*H. parviporum*/pairing test	350 bp
KK7	Norway spruce	*H. annosum*/pairing tests	100 bp
KK8	Norway spruce	*H. annosum*/pairing tests	100 bp
TP 3.1.5	Norway spruce	*H. annosum*/pairing tests	100 bp
TP 3.2.1	Norway spruce	*H. parviporum*/pairing tests	350 bp
TP 3.2.3	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.2.3/140	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.2.5	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.3.2/10	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.3.2/140	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.4.7	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.4.9	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.5.11	Norway spruce	Probably *H. parviporum*/stand	350 bp
TP 3.5.16	Norway spruce	Probably *H. parviporum*/stand	350 bp
KL9	Scots pine	*H. annosum*/tree species	100 bp
KL10	Scots pine	*H. annosum*/tree species	100 bp
KL11	Scots pine	*H. annosum*/tree species	100 bp
KL12	Scots pine	*H. annosum*/tree species	100 bp
KL13	Scots pine	*H. annosum*/tree species	100 bp
KL14	Scots pine	*H. annosum*/tree species	100 bp
KL15	Scots pine	*H. annosum*/tree species	100 bp
KL16	Scots pine	*H. annosum*/tree species	100 bp
KL17	Scots pine	*H. annosum*/tree species	100 bp
KL18	Scots pine	*H. annosum*/tree species	100 bp
K42B	Norway spruce	*H. parviporum*/18S rDNA profiling [b)]	350 bp
K42C	Norway spruce	*H. parviporum*/18S rDNA profiling	350 bp
K42D	Norway spruce	*H. annosum*/18S rDNA profiling	100 bp
K61A	Norway spruce	*H. annosum*/18S rDNA profiling	100 bp

[a)] Samples with abbreviations including KK, TP or KL were provided by Kari Korhonen, Tuula Piri and Katriina Lipponen, respectively. Other samples were collected by the authors. All samples originateed from southern Finland.
[b)] Based on the principles described previously (Korhonen 1978).
[c)] Based on the principles described previously (Vainio and Hantula 2000a).

isolates of *H. annosum* and 35 isolates of other fungal species (Table 1). Mixtures of *H. annosum* and *H. parviporum* DNA were used as templates in order to test the capability of primers to detect both species simultaneously. In addition, the primers were tested on 33 wood chip samples, five of which were known to be colonised by *H. parviporum* (five samples), 18 by *H. annosum* (18 samples), and nine samples by unidentified *Heterobasidion* sp. (from a forest stand known to be infected by *H. parviporum*). In addition, one

Table 3. The sequences of the primers designed.

Primer	Sequence (starting from 5´end)
MJ-F	GGTCCTGTCTGGCTTTGC
MJ-R	CTGAAGCACACCTTGCCA
KJ-F	CCATTAACGGAACCGACGTG
KJ-R	GTGCGGCTCATTCTACGCTATC

sample from a healthy tree (both spruce and pine) was used as a negative control.

The sensitivity of the detection was tested using known amounts of *H. annosum* and *H. parviporum* DNA in PCR-reactions. We also conducted PCR-reactions, where different amounts (ratios) of both *H. annosum* and *H. parviporum* DNA was added to reactions with primer pairs detecting only one species (either MJ-F and MJ-R or KJ-F and KJ-R).

3 Results and Discussion

Initial tests with the two primer pairs were carried out separately. PCR reactions with primers MJ-F and MJ-R resulted in successful amplification of an approximately 100 bp fragment only when DNA from *H. annosum* was used as a template (not shown). In contrast, the amplification of 350 bp fragment with primers KJ-F and KJ-R was successful only using template DNA from *H. parviporum* (not shown). No unspecific fragments with unexpected sizes were observed in these reactions.

After the initial trials, both primer pairs were combined in a single PCR reaction. Amplification products were observed only if *Heterobasidion* DNA was present, and their sizes were in accordance with the origin of the template DNA (i.e. *H. annosum* or *H. parviporum*; Fig. 1). If DNA from both species was present, both bands appeared (Fig. 1). No amplification products were observed in amplifications with DNA from the other species tested (not shown).

Finally we made tests with wood chip samples known to be infected by *H. annosum* or *H. parviporum*. In all cases a positive identification was made; and in all cases the identification agreed with the pre-existing information about the fungal species (Fig. 1; Table 1). No amplification products were obtained from the negative control.

In sensitivity tests primer pair MJ-F and MJ-R was able to detect even 15×10^{-3} pg of *H. annosum* DNA. Primer pair KJ-F and KJ-R was less sensitive, and detected only amounts higher than 15 ng of *H. parviporum* DNA. In competition experiments both species were detected even if the ratio of the two templates was as skewed as 1:10.

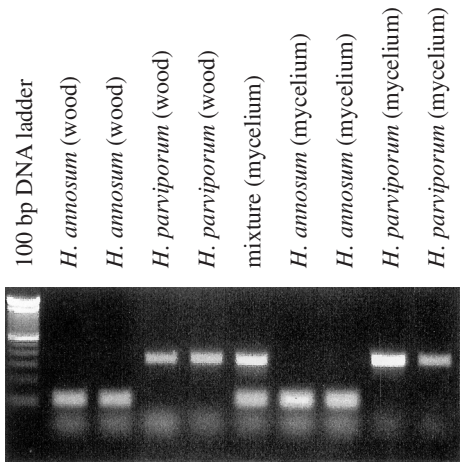

Fig. 1. Examples of amplification products obtained with the developed primers. The samples from left to the right are as follows: wood chip KL12, wood chip KK2, wood chip 3.4.9, wood chip 3.5.11, a mixture of isolates 29 and 52, isolate 52, isolate 32, isolate 29 and isolate 9. On the left is a DNA ladder, where the band sizes from the bottom to the first double intensity band are 100, 200, 300, 400 500 and 600 bp.

The primers pairs developed were based on RAMS-primers. In Blast searches no homologies exceeding 20 bp were observed for CCA1210. Therefore it was not possible to conclude on what type of DNA primers KJ-F and KJ-R are based. In contrast, Blast search conducted with CT1350 showed high levels of similarity with the ribosomal gene cluster, and therefore we could conclude that both primers MJ-F and MJ-R are based on Intergenic Spacer Region. This may explain why the detection of *H. annosum* was more sensitive, as there are several copies of ribosomal gene cluster in the genome.

There is plenty of molecular data, which would probably also enable the differentiation of the two *Heterobasidion* species directly from a wood chip (Karjalainen 1996, Kasuga and Mitchelson 2000, Kasuga et al. 1993). In this work a new set of primers was designed to allow the detection of the two species directly from decaying wood material in one PCR-reaction without any additional analyses (except electrophoresis). This makes the

detection cheap and simple, and should make it useful for even practical purposes.

As a conclusion, we developed a primer system which can be used for the detection and identification of *H. annosum* and *H. parviporum* directly from the decaying wood in northern Europe. However, our identification test was developed for research and practical usage in Finland (and northern Europe) and therefore it was not tested with *H. abietinum*, which occurs commonly in southern Europe, or with *Heterobasidion* species (IS-groups) occurring in Asia or North America. Therefore the molecular identification tool described here should only be used in northern Europe.

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