ARTICLES

Detection of *Heterobasidion annosum* in Scots Pine Trees Using a Polymerase Chain Reaction Based Method

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Abstract

The fungal complex *Heterobasidion annosum* s.l. (*sensu lato*) is the major cause of root and butt rot of conifers in the Northern Hemisphere. There is evidence of a genetic component in the resistance of conifers to this fungus. In order to study the genetic basis of resistance in Scots pine, information about the infection level of trees growing in forest stands is important, in combination with data about genotypes or gene expression profiles. Symptoms of *H. annosum* root rot in living trees are not unique and can be very similar to symptoms caused by other diseases. Additionally, infected *P. sylvestris* trees can stay asymptomatic for long periods. Therefore, in order to assess the levels of *H. annosum* infection in forest stands, a direct method for the detection of this pathogen in living Scots pine trees is required. In this paper we present the results of detection of *H. annosum* DNA in living Scots pine trees using a polymerase chain reaction (PCR) assay. A preliminary assay of 300 trees, analysing one sample per individual, revealed 119 trees positive for *H. annosum* DNA. A more detailed examination of two excavated tree trunks and partial root systems revealed a heterogeneous distribution of *H. annosum* within pine. Twenty five trees were further chosen for more detailed determination of infection level analysing five samples per individual. The distribution of *H. annosum* around the stem base of Scots pine trees was found to be heterogeneous, however, the determination of the infection level was found to be reproducible. This study provides a method for rapid, PCR-based determination of *H. annosum* infection levels both in pine stands and individuals.

Key words: *Heterobasidion annosum*, root rot, species-specific PCR detection

Introduction

The fungal complex *Heterobasidion annosum* s.l. (*sensu lato*) is the major cause of root and butt rot of conifers in the Northern Hemisphere. Within this complex, multiple intersterility groups have been identified (Korhonen 1978, Capretti et al. 1990), and more recently, two of these groups have been defined as separate species– *H. annosum* (Fr.) Bref. *sensu stricto*, which predominantly infects pine, and *H. parviporum* Niemelä and Korhonen, which predominantly infects spruce (Niemelä and Korhonen 1998). Scots pine is the major forest tree species for Latvia, accounting for 37 % of the total forest area (Anon 2007). Economic losses related to *H. parviporum* infection in spruce are estimated to be 800 – 4,790 EUR/ha in Latvia (Gaitnieks et al. 2007) which suggests that significant economic losses are also caused by *H. annosum* infection in Scots pine forest stands.

There is evidence of a genetic component in the resistance of *Picea abies* to *H. parviporum* and *Pinus sylvestris* to *H. annosum* (Kvaalen and Solheim 2000; Korshikov and Demkovich 2008). In order to study the genetic basis of resistance in Scots pine, information about the infection level of trees growing in forest stands is important, in combination with data about genotypes or gene expression profiles. Furthermore, data about natural infection levels are needed for artificial inoculation experiments of *P. sylvestris* stems with *H. annosum*. *Picea abies* can express a systematic response against *H. parviporum* (Swedjemark et al. 2007), and therefore detection of natural infection levels is required in order to establish the infection status of trees prior to inoculation. In addition, a method to quantify levels of natural infection in pine stands would be useful for detection of individuals or families with differing resistance to *H. annosum*.

It has been shown that identification of *H. annosum* infected trees by visual characteristics does not give accurate results (Rönnberg et al. 2006).

Symptoms of *H. annosum* root rot in living trees are not unique, and cannot be distinguished from those caused by other root diseases (Greig 1998). Additionally, an infected Scots pine tree can remain asymptomatic even if two thirds of the root system has been damaged by the root rot fungus (Greig 1998). In contrast to Norway spruce, root and butt rot does not cause a cavity in the centre of the tree in Scots pine. In Scots pine *H. annosum* prefers sapwood because of fungistatic substances in heartwood (Korhonen and Stenlid 1998). This makes identification of the disease in Scots pine more difficult than in Norway spruce, where a single drill probe to the centre of the tree is sufficient to detect infection. Therefore, in order to assess the levels of *H. annosum* infection in forest stands, as well as individual trees, a method for the direct detection of this pathogen in living Scots pine trees is required.

Traditionally, the presence of fungal pathogens has been determined by culturing samples from plants followed by visual identification of fungi. The disadvantages of this approach are the time required to grow fungal cultures, the possibility that not all fungi will be able to be cultured, and the difficulty of distinguishing fungi morphologically. More recently, PCR-based methods have been developed for the identification of all fungi present in a sample using universal primers (Krüger et al. 2009), or detection of particular fungi using species-specific PCR primers (Garbelotto et al. 1996, Bahnweg et al. 2002, Hantula and Vainio 2003). Most reported studies have utilised only one sample from each individual in order to survey for the presence of a particular pathogen or fungus. This can lead to under-reporting of the presence of a particular fungus, particularly if visual symptoms are absent, or if the distribution of the fungus within the individual is discontinuous. We chose a PCR based method for detection of *H. annosum* DNA in wood utilising primers from Hantula and Vainio (2003). Several PCR methods for detection of *H. annosum* DNA have been reported (Garbelotto et al. 1996, Bahnweg et al. 2002, Hantula and Vainio 2003), but none of these methods are reported to have been used to study a forest stand phytosanitary situation as extensively as reported here. The aim of this study was to utilise *H. annosum* specific PCR primers in order to detect the presence of the fungus in living trees, as well as quantifying the level of infection within individual trees.

Materials and methods

Plant material

Samples were collected from a 29 year old experimental *P. sylvestris* plantation near Kalsnava, Latvia where a root rot outbreak was detected. Four experiments were performed as part of this study. In the initial experiment, for preliminary testing and adaptation of the method, one sample from each of 300 trees was analysed. In conjunction with this preliminary experiment, a second experiment was performed, in which the trunks and partial root systems of two trees were excavated in order to determine the distribution of fungal DNA within an individual. Samples were taken from various points in the excavated trunk and root system, and analysed for the presence of fungal DNA using the PCR method. A third, more detailed experiment was performed, analysing five samples from each of 25 trees included in the preliminary experiment. Finally, in order to determine the repeatability of the determination of infection level using a PCR detection method, a fourth experiment was performed, in which an additional five samples from each of these 25 individuals were analysed. Samples were taken at intermediate points between the initial sampling points, approximately 11 months after the initial multiple sample experiment.

The sampling points were located at the base of the stem where *H. annosum* fruiting bodies usually occur. In the third and fourth experiments, five wood samples from each tree were taken from the stem circumference with approximately equal distances between each sampling point. A round-end chisel was used to collect wood samples. Sample dimensions were approximately 2 × 1.5 cm (diameter × depth). Sampling dates were – September 2008 for the preliminary single sample per tree experiment and trunk and partial root system excavation, November 2008 for the initial multiple sample per tree experiment, and October 2009 for the repeat of the multiple sample per tree experiment.

DNA extraction

DNA was isolated using the Genomic DNA purification kit (Fermentas, Lithuania). Total DNA was extracted from wood from which the bark was removed. Wood samples were cut into small chips, placed into 2 ml centrifuge tubes and a 5 mm diameter steel ball was added to each tube. Samples were frozen in liquid nitrogen and homogenised twice (with repeated freezing) in a ball mill (Retch, model MM400) at 30 Hz for two minutes. After that the procedure described in the DNA isolation kit manual was performed with the following modifications: PVP25 (polyvinylpyrrolidone) was added to the lysis buffer provided in the kit to a final concentration of 4 %, centrifugation speed and time in all steps were increased to 16,100 g and 15 minutes. Incubation with RNase A (Fermentas, Lithuania) was added at the NaCl precipitation step by adding 4 mg of RNase A to each sample together

with 100mL 1.2 M NaCl solution. Incubation was carried out at 37 °C for 30 minutes.

DNA was extracted from *H. annosum* and *H. parviporum* cultures for use as positive controls.

Polymerase Chain Reaction (PCR)

We used PCR primers MJ-F and MJ-R to detect *H. annosum* and primers KJ-F and KJ-R to detect *H. parviporum* (Hantula and Vainio 2003). The MJ primer pair amplifies a fragment of approximately 100 base pairs (bp) from *H. annosum* DNA while the KJ primer pair amplifies an approximately 350 bp long fragment from *H. parviporum*. Both primer pairs are proven to be species specific (Hantula and Vainio 2003). PCR reactions for each primer pair were performed separately, using the following PCR conditions: initial denaturation 10 min, 95°C; 40 cycles of amplification (30 s denaturation, 95°C; 35 s annealing, 67°C; 1 min extension, 72°C); final extension 7 min, 72°C. PCR composition: 0.2 mM each deoxynucleotide, 0.5 mM each primer, 0.5 U/reaction of recombinant *Taq* DNA polymerase (Fermentas, Lithuania), 1.5 mM MgCl2, 2.5 ml of 10x PCR buffer with KCl (included in polymerase kit), 3 ml DNA. PCR reaction volume was 25 ml. DNA concentration in samples was not measured as it would not give information about the proportion of fungal DNA in the sample. A positive control for successful DNA extraction and PCR was performed utilising a pine chloroplast DNA specific primer pair Pt71936 (Vendramin et al 1999). Products were analysed by 2 % agarose gel electrophoresis and visualised using ethidium bromide staining. Each sample was analysed with the fungal specific primer pairs MJ and KJ as well as with the chloroplast primer pair Pt71936 as a positive control. Results from the fungal PCR assay were only taken into account if the positive control was successful.

Results

Initially, detection of fungal DNA was performed using one sample from each tree. Of the 300 individuals tested with one sample per tree, *H. annosum* DNA was detected in 119 trees, while the results were negative in 181 trees. All samples gave negative results in PCR reactions with KJ-F and KJ-R primers for detection of *H. parviporum*. Assays negative for the detection of fungal DNA were not repeated, as all samples were positive for the DNA extraction and PCR amplification control using the pine chloroplast primers.

Four trees with *H. annosum* fruiting bodies on their stem bases were included in this analysis but *H. annosum* DNA was detected in only two of these. Therefore, in order to investigate the distribution of *H. annosum* DNA within infected trees, the stumps and partial root systems of two additional infected trees (where a fruiting body was detected) were extracted from the soil. One tree was still living at the time of extraction, while the other tree was completely wilted and dead. Various parts of the stumps and roots were analysed using the described PCR method to detect *H. annosum* DNA (Table 1). Analysing the living tree, there were two positive and one negative PCR assay for samples from the trunk and one positive and one negative sample from the tap root. Both assays from one lateral root were positive, while a single sample from a second lateral root was negative. Analysing the wilted and dead tree, both samples from the trunk were negative, as well as a single sample from the tap root. From the lateral roots, one root had two positive samples, a second root one positive and one negative sample, and a third lateral root had one positive sample.

Table 1. Distribution of *H. annosum* DNA within the stumps and partial root systems of two excavated trees

	Sample number	Sample location	*H. annosum* DNA (+/-)
Living tree	1	Trunk, outside (1cm from bark)	+
	2	Trunk, 5cm from centre	-
	3	Trunk, 2cm from centre	+
	4	Lateral root #1, adjacent to trunk, outside	+
	5	Lateral root #1, adjacent to trunk, centre	+
	6	Lateral root #2, 10 cm from trunk	-
	7	Taproot, adjacent to trunk	-
	8	Taproot, 20 cm from trunk	+
Wilted tree	9	Trunk, outside (1cm from bark)	-
	10	Trunk, 1cm from centre	-
	11	Lateral root #1, adjacent to trunk, outside	+
	12	Lateral root #1, adjacent to trunk, centre	+
	13	Lateral root #2, 5 cm from trunk, outside	-
	14	Lateral root #2, 5 cm from trunk, centre	+
	15	Tip of lateral root #3, 30 cm from trunk	+
	16	Taproot, 5 cm from trunk	-

To further investigate the heterogeneous distribution of *H. annosum* within pine stems, a subset of trees was analysed using multiple samples from each individual. Twenty trees where no *H. annosum* DNA was detected and five trees testing positive for *H. annosum* DNA were chosen for further analysis using five samples per tree (a total of 125 samples). Each analysed tree had an infection level value from "0/5" to "5/5" assigned (number of positive *H. annosum* PCR assays/ number of positive control PCR assays). Only four of the 25 tested trees were assigned with a 0/5 score, seven trees were assigned with 1/5, five trees 2/5, eight trees 3/5 and one tree 5/5. No trees were scored as 4/5 (Table 2, Fig. 1). The four trees scored as 0/5 were from individuals that were also negative in the preliminary screening.

To determine the repeatability of the results, the 25 trees were resampled, analysing five new samples from each previously analysed tree. Results of the repeated experiment are given in Table 1 and Figure 1 where they are compared to the results of the original

Table 2. Comparison of results obtained in both infection level determination experiments. * – individuals where positive PCR amplification controls were negative for some samples. NA – not analysed. Infection level – (number of positive H. annosum PCR assays/ number of positive control PCR assays)

Sample name	Infection level in original experiment	Infection level in repeated experiment
Du12-III-4	0/5	3/3*
Ja15-V-6	3/5	0/2*
Ja18-III-2	3/5	5/5
Ja21-III-2	1/5	3/4*
Ja21-V-1	5/5	4/5
Ja21-V-5	1/5	4/4*
Ja2-III-4	2/5	4/4*
Ja3-II-5	3/5	4/4*
Ja3-III-5	0/5	1/5
Ja3-V-1	3/5	2/2*
Ja3-V-3	1/5	3/5
Ja3-V-5	3/5	3/5
Ja4-III-2	1/5	4/5
Je7-III-5	0/5	5/5
Lub1-III-1	2/5	2/5
Ma21-III-3	0/5	2/5
Ma6-V-2	3/5	2/4*
Sm3-II-1	2/5	NA
Sm3-II-2	2/5	3/5
Sm3-V-2	1/5	1/4*
Sm6-V-2	3/5	1/4*
Sm9-III-2	2/5	5/5
Str17-I-1	1/5	2/4*
Str17-I-2	3/5	2/4*
Str17-III-3	1/5	4/5

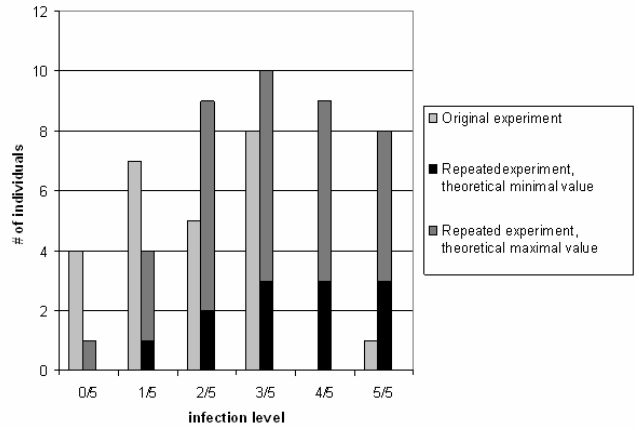

Figure 1. Results of infection level evaluation by PCR and comparison of results of original and repeated experiments. Infection level – (number of positive *H. annosum* PCR assays/ number of positive control PCR assays). Theoretical minimum and maximum values in the repeated experiment were calculated by assuming *H. annosum* assays in samples where positive control failed were either all negative or positive, respectively

experiment. In the repeated experiment, not all samples returned positive results for the DNA extraction and PCR amplification positive control. These samples came from 12 trees, which are marked with an asterisk in Table 1. Presumably, the failure of the positive control was due to poor quality DNA, or to the presence of PCR-inhibiting substances. Unfortunately, it was not possible to resample from these individuals due to the small circumference of the tree stems. Therefore, in order to compare the results of the two infection level experiments, the theoretical minimum and maximum number of individuals in each infection level class was calculated, assuming that the unsuccessfully amplified samples (where the positive control failed) would be respectively all negative or all positive for *H. annosum* DNA. In the repeated experiment, there is a decrease in the lower infection level classes (0/5 and 1/5), and a subsequent increase in the higher infection level classes (4/5 and 5/5) (Fig. 1). Comparing the same individuals between experiments, in most cases, the infection level increased or remained the same (taking into account the possible maximum infection level in individuals where it was not possible to analyse all samples). In the second experiment, the infection level decreased in two individuals (Ja21-V-1 and Sm6-V-2) (Table 2).

Discussion and conclusions

Prior to the start of this experiment, the presence of *H. annosum* within the analysed plantation was already established by the detection of fruiting bodies on some individual trees. Results of the preliminary testing (300 trees, one sample per tree) confirmed that the infection level in the plantation was high, with almost 40% of trees testing positive for *H. annosum* DNA. The negative PCR amplification results with *H. parviporum* specific primers KJ-F and KJ-R supports the fact that disease seen in pines is more likely to be caused by *H. annosum* and not by *H. parviporum.*

One surprising result was that of the four trees sampled where fruiting bodies were observed, only two were positive for *H. annosum* DNA. This indicated that the distribution of *H. annosum* DNA within infected trees was not homogeneous. More detailed analysis of two excavated tree trunks and root systems showed that the distribution of *H. annosum* DNA was highly heterogeneous. PCR results from sampling sites very close to each other differed, for example from the inside and outside of lateral roots, as well as at a distance of ~3cm within the tree trunk (Table 1).

Given that the excavation of root systems is a very labour-intensive process, we decided to further investigate the presence and heterogeneity of *H. annosum* DNA within the trunks of living trees, analysing multiple samples from a single individual. In the more detailed analysis using five samples per tree, the proportion of infected trees was markedly higher (86%), indicating that analysis of one sample per tree returns a high rate of false – negative results. In the initial infection level determination experiment, 16 samples had an infection level 0/5 to 2/5 and 9 samples had an infection level 3/5 to 5/5. The reason for this is unclear. It could indicate that the samples with a lower infection level became infected later than the nine samples with a higher infection level. There was how-

ever, no spatial correlation of sampled individuals with infection level (data not shown), so it was not possible to detect the directional spread of *H. annosum* within the plantation. Another possibility is that these individuals possess resistance mechanisms which inhibit the spread of *H. annosum* within the tree.

The repeated infection level determination experiment was performed almost a year after the original experiment so the natural spread of *H. annosum* within the host tree would have influenced the results of the repeated experiment. It has been shown that *H. annosum* can spread up to 2m/year within roots (Rishbeth 1962). Comparing the data between the two experiments (Fig. 1), it is evident that infection level in the repeated experiment has shifted towards the higher infection level classes. This is probably a function of the growth and spread of *H. annosum* within the trees during the 11 months since the initial experiment. In most cases the infection levels increased or remained the same when comparing the same individual between experiments, however, in two individuals, the infection level decreased in the second experiment, which is probably a sampling artefact due to the heterogeneous distribution of *H. annosum* within the tree trunk.

It is clear that due to the heterogeneous distribution of *H. annosum* within Scots pine trees, multiple samples from each individual are required to assess the infection status and level, particularly in order to avoid generating false negative results. Our results show that the use of five samples per tree can give an assessment of *H. annosum* infection levels within an individual; however the determined infection level is not absolute, due to the heterogeneous distribution of the pathogen within pine trees. The fact that in the repeated experiment, the infection level increased or remained the same in the majority of individuals indicates that the analysis of five samples per tree is indicative of the actual infection level within individuals. The increased infection level within individuals in the repeated experiment is indicative of the progress of infection over time. One significant factor limiting the number of samples able to be obtained from one tree is the stem diameter. In the 29 year old plantation sampled in these experiments, increasing the number of samples per tree could have a detrimental effect on the growth of the individuals. When sampling trees with larger diameters, it would be possible to increase the sample number per tree, or alternatively, a different sampling scheme could be utilised, for example in a vertical direction along the stem of the tree. However, further investigation into the pattern of distribution of *H. annosum* within pine stems is required in order to determine the pattern of spread of the fungus within tree tissues.

In this report, we have utilised *H. annosum* specific PCR primers to assess the infection level of individual Scots pine trees, utilising multiple samples from each individual. The analysis of multiple samples from individuals allowed us to determine infection level differences between individuals, as well as to demonstrate the heterogeneous distribution of *H. annosum* within Scots pine tree stems. The application of this method to pine stands or plantations will allow for the determination of the overall phytosanitary condition of the stands, as well as for the identification of potential differences in resistance to *H. annosum* between individuals or families, which can then be utilised in further resistance studies or within breeding programs.

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ОПРЕДЕЛЕНИЕ *HETEROBASIDION ANNOSUM* В ДЕРЕВЬЯХ СОСНЫ МЕТОДОМ ОСНОВЫВАННЫМ НА ПОЛИМЕРАЗНОЙ ЦЕПНОЙ РЕАКЦИИ

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Резюме

Грибной комплекс *Heterobasidion annosum* s.l. (*sensu lato*) является главной причиной корневой губки у хвойных деревьев на северном полушарии. Резистентность хвойных деревьев к этому комплексу наряду с другими факторами имеет и генетическую основу. Для исследования генетической основы резистентности сосны, информация об уровне инфекции деревьев, растущих в местных насаждениях, важна для сравнения, как генотипов, так и для сравнения экспресии генов. Симптомы поражения *Heterobasidion annosum* в растущих деревьях не уникальны и могут быть близкими симптомам, вызванным другими болезнями. При этом инфицированные *Heterobasidion annosum* деревья могут не проявлять симптомы болезни длительное время. Поэтому, для определения уровня инфекции в лесных насаждениях, необходимо выработать метод определения этого патогена в растущих соснах.

В этой работе представлено определение ДНК *Heterobasidion annosum* в растущих соснах с использованием полимеразной цепной реакции (ПЦР). При анализе 300 деревьев, исследуя один образец каждого индивида, было найдено, что 119 из них содержат ДНК *Heterobasidion annosum*. При более детальном исследовании двух выкопанных стволов деревьев и их корневой системы, было найденно гетерогенное распределение грибка *Heterobasidion annosum* в сосне. Поэтому, далее, для более детального исследования уровня инфекции деревьев, растущих в лесных насаждениях, были отобранны двадцать пять деревьев и проанализированы пять образцов каждого индивида. Несмотря на найденное гетерогенное распределение *Heterobasidion annosum* по периметру основания ствола сосны, определение уровня инфекции является воспроизводимим.

В этой работе представлен быстрый метод, основанный на ПЦР, для определения уровня инфекции *Heterobasidion annosum* как в лесных насаждениях сосны, так и на отдельных деревьях.

Ключевые слова: *Heterobasidion annosum*, корневая губка, видоспецифическое определение с ПЦР