For. Path. 33 (2003) 191–203

ISSN 1437–4781

Swiss stone pine trees and spruce stumps represent an important habitat for *Heterobasidion* spp. in subalpine forests

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Summary

In the Western Italian Alps (WIA), the three European species of the forest pathogen *Heterobasidion* spp. can coexist in the same area. *Heterobasidion parviporum* Niemelä & Korhonen and *Heterobasidion abietinum* Niemelä & Korhonen are normally found in areas with a significant presence of their respective primary hosts, spruce (*Picea* spp.) and fir (*Abies* spp.). The host/niche occupied by *Heterobasidion annosum* (Fr.) Bref. in the region still remains unclear. Although Scots pine (*Pinus sylvestris*), a major host for this fungal species in other parts of Europe, is abundant in the region, little or no evidence of disease caused by *H. annosum* is visible in this tree species. Two different, but not mutually exclusive, hypotheses can explain the presence of *H. annosum*: (1) Scots pines are infected but largely asymptomatic and (2) *H. annosum* has adapted to different hosts. An analysis of *Heterobasidion* species was performed in two natural, mixed-conifer forests using traditional isolation techniques and novel direct molecular diagnosis from wood. In a subalpine stand of mixed spruce (*Picea abies*), larch (*Larix* spp.), and Swiss stone pine (*Pinus cembra*), 18 naturally infected spruces and larches only yielded *H. parviporum.* A Swiss stone pine in the same stand was extensively colonized by both *H. parviporum* and *H. annosum*. In a second subalpine stand, an analysis of 18 spruce stumps and nine Swiss stone pine stumps yielded both *H. parviporum* and *H. annosum* isolates. Pine stumps had been mostly colonized by *H. parviporum* prior to tree felling, suggesting that this species may be secondarily infected by the locally predominant *Heterobasidion* species (i.e. *H. parviporum*). Results of our analysis also indicated that primary colonization of spruce stumps (e.g. through basidiospores) was caused by both *H. parviporum* and *H. annosum*, while secondary infection of such stumps was mostly because of *H. parviporum*.

1 Introduction

In the subalpine forests of the Western Alps, timber production is marginal, and management guidelines are directed to protect these delicate ecosystems, which offer habitat for abundant wildlife and represent important recreational sites both in the winter and summer. Most notably, subalpine forests are known to protect against soil erosion and avalanches (Oswald et al. 1998). These multiple purposes of the subalpine forests can be sustained only by preserving their biological and functional diversity (Bernier and André 1998).

Subalpine forests consist of a mosaic of stands, single trees and glades. Survival and establishment of trees at high elevations in the Alps is controlled by temperature parameters and by a range of additional environmental factors (Holtmeier 1993). Tree pathogens are also key factors determining tree establishment and survival (Senn 1999).

The *Heterobasidion* species complex includes at least five taxa known to significantly affect the structure and composition of forests in several regions of the world (Korhonen et al. 1998). In the Western Italian Alps (WIA), both Norway spruce [*Picea abies* (L.)

Received: 27.3.2002; accepted: 3.3.2003; editor: C. G. Shaw

Karst.] and silver fir (*Abies alba* Mill.) stands can be significantly affected by *Heterobasidion* spp. (Anselmi and Minerbi 1989; Capretti 1998; Cellerino et al. 1998). While damage caused by *Heterobasidion* spp. tends to be less at higher altitudes in other parts of the world (Maraite and Meyer 1966; Korhonen and Stenlid 1998), we have observed levels of infection up to 95% in high-elevation (1800–2300 m a.s.l.) stands in the WIA. Because most studies on diseases caused by *Heterobasidion* spp. have focused on spruce and fir stands, there is little understanding of the impacts of these pathogens in subalpine forests, where spruce is codominant with European larch (*Larix decidua* Mill.) and Swiss stone pine (*Pinus cembra* L.).

The commercial value of wood from Swiss stone pine is heightened by its sporadic distribution and its overall limited availability. Its animal-dispersed mode of seed transport largely accounts for such sporadic distribution. Nevertheless, Swiss stone pine and European larch are extremely important ecologically, as they are the only two species capable of thriving near the alpine tree line. Although in the interior Alps, either Swiss stone pine/larch or spruce may locally constitute climax coenosis at high altitudes (*Larici-Cembretum, Picetum subalpinum*, respectively), mixed transitory forests of the three species are very common in those ecozones (Kral 1989; Bernetti 1998).

While larch and spruce are well-documented hosts for *Heterobasidion* spp. (Korhonen and Stenlid 1998), no information is available on the susceptibility of Swiss stone pine to this pathogenic species complex. In Europe, Norway spruce is the main host for *H. parviporum* (Korhonen et al. 1998), and a strict host specificity of this species on spruce has been recently confirmed in lower elevation, pure and mixed forests in WIA (Gonthier et al. 2001; Gonthier 2001). *Heterobasidion annosum* and *H. abietinum* have been occasionally found on spruce (Capretti et al. 1994; Korhonen and Piri 1994; Vasiliauskas and Stenlid 1998). Although larch is not reported as a main host for *Heterobasidion* (Korhonen and Stenlid 1998), all the three European species of this pathogen have been isolated from European larch (Capretti et al. 1994; Vollbrecht et al. 1995; Munda et al. 1998).

Little is known about the incidence of the disease and the behaviour of *Heterobasidion* on Swiss stone pine. This tree species is rarely felled in the WIA, and to date, there is only a single report of one Swiss stone pine infected by *H. annosum* and *H. parviporum* (Nicolotti et al. 1999; Gonthier et al. 2002). The finding of *Heterobasidion* on *P. cembra* is significant because (a) it is generally believed that this tree species is resistant to most decay agents, (b) the pathogen was causing an extensive butt rot in the infected tree, while it is normally reported as a cause of root decay in other pine species and (c) *H. parviporum* is generally considered a pathogen of spruce trees or pine saplings but not of adult pine trees.

Recent studies on *Heterobasidion* spp. in the WIA (Gonthier et al. 2001; Gonthier 2001) show that all the three species of *Heterobasidion* are often present in the same stand, and that *H. parviporum* and *H. abietinum* are generally found on their preferential hosts, spruce and fir, respectively. The host/niche occupied by *H. annosum* in the WIA is unclear. This fungus has been found in forests either with or without Scots pine, one of the major hosts for this species in other parts of Europe (Korhonen et al. 1998). Even in stands where both *H. annosum* and Scots pine coexist, there is no evidence of disease caused by this root rot fungus. Two different, but not mutually exclusive, hypotheses can explain the presence of *H. annosum* in the WIA: (1) Scots pines are infected, but largely asymptomatic and/or (2) *H. annosum* has adapted to different hosts/niches.

The goals of this study were to (a) describe the symptoms caused by *Heterobasidion* spp. in Swiss stone pine and determine how relevant the pathogen may be for this species and (b) contrast the nature of primary (e.g. through basidiospores) and secondary (e.g. through vegetative colonization via root contacts) infections especially in spruce stumps, by looking at the genetic structure of populations of *Heterobasidion* spp. in two subalpine mixed forests.

2 Materials and methods

2.1 Sites and stand descriptions

The two study sites were located in two naturally regenerated subalpine forests on parallel mountain slopes in the Aosta Valley (Graie Alps). One stand was in Cogne (45°35'57.34" N; 7°22'7.57" W; Heyford ED50), extending from about 1800 m a.s.l. up to the timberline (2050 m), while the other one (45°41'40.93" N; 7°19'35.92" W; Heyford ED50) was in Charvensod (1800–1900 m a.s.l.). Both forests have northern aspects with mean annual rainfall of approximately 750 mm. Both sites are classified as *P. subalpinum* on Ochrepts/Umbrepts soils (USDA Natural Resources Conservation Service 1998). The two forests have similar tree species composition and structure. They both comprise several large, even-aged groups of trees, resulting in a stand that, as a whole, can be considered uneven-aged. Spruce is the dominant species, mixed with larch and Swiss stone pine (Table 1). Regeneration of Swiss stone pine is present in groups, but the adult trees were found to be coetaneous, with ages ranging between 85 and 90 years. The dominant larches were about twice as old, while spruce trees belonged to different age classes, indicating a continuous regeneration of this species through time.

At both sites, we performed a study on the frequency of infection by the different *Heterobasidion* spp. on larch, Swiss-stone pine, and Norway spruce, including an overall analysis of symptoms and patterns of wood decay. In Cogne, we also investigated the spatial distribution of individual *Heterobasidion* genotypes in spruce, Swiss stone pine, and larch trees and stumps, all of which had clearly been infected through root contacts prior to tree felling. In Charvensod, this analysis was not performed because most spruce stumps had been infected primarily by airborne inoculum landed on the stump.

2.2 Sample of trees and stumps in Cogne

Our plot at Cogne was centred on the first Swiss stone pine individual reported as a host for *Heterobasidion* spp. (Nicolotti et al. 1999; Gonthier et al. 2002). Several stem sections and roots were sampled from this tree. All trees and stumps with diameter over 4 cm, growing not further than 30 m from the infected pine tree, were carefully mapped. The average diameter at breast height (d.b.h.) was measured for trees and estimated for stumps. General health conditions of live trees were described using indicators, such as foliage colour and crown density. Each tree was sampled by extracting two to three cores from the root collar. Stumps were sampled by obtaining a 6–7-cm-thick cross-section at the root collar.

Levels and patterns of decay and discoloration were described for each sampled wood disc and wood core. Samples were then sprayed with a benomyl solution (0.010 g benomyl, 500 μl methanol, 1 l sterile water) and incubated, for about 10 days at room temperature in a moist chamber. Larger discs were placed in plastic bags containing moist paper towels; smaller discs were placed in 15-cm Petri dishes containing moist filter paper. Colonies of *Heterobasidion* spp. were recognized by their *Spiniger* stage. Zone lines on the discs were used to identify distinct fungal genotypes. For each discrete colonized wood patch, isolations were made under a dissecting microscope (20× magnification), by transferring infected wood or fungal hyphae onto 5-cm Petri dishes filled with Pentachloronitrobenzene (PCNB)-based selective medium (Kuhlman and Hendrix 1962). In order to discriminate between colonies caused by potential airborne *Heterobasidion* spores and isolates truly colonizing the sampled wood, transfers were done only from colonies that were sporulating on both cut surfaces of sampled discs. All isolates were subsequently subcultured and stored at 5°C on malt extract agar (MEA) (20 g glucose, 20 g malt extract,

Table 1. **Characteristics of the forests where the two study sites were selected**

Forests	Elevation (m a.s.l.)	Spruce % (butt rot incidence %)[1]	Swiss stone pine % (butt rot incidence %)[1]	Larch % (butt rot incidence %)[1]	Basal area (m^2 ha^{-1})	Density (trees ha^{-1})	Average d.b.h.[2]	Dominant height (m)
Cogne	1800–2050	60 (90)	15 (?)	25 (30)	25.48	512	25	26
Charvensod	1800–1900	60 (50)	20 (?)	20 (10)	26.31	400	29	24

[1]This estimate was based on the frequency of stumps displaying the typical laminated white rot caused by *Heterobasidion* (following thinning in 1997–1999).
[2]Diameter at breast height.

2 g peptone, 20 g agar, 1 l distilled water). Context isolations were attempted from basidiocarps, when they were available.

A larch tree growing 40 cm uphill from the infected pine, was felled in 1999. The tree was 156 years old, 18 m tall, and its d.b.h. was 36 cm. An area of the stem approximately 7 cm in diameter showed typical incipient decay potentially caused by *Heterobasidion*. Because all isolations were unsuccessful, confirmation on the presence of the pathogen was obtained by direct polymerase chain reaction (PCR), and subsequent DNA sequencing from wood samples. For this process, a block ($1.5 \times 1.5 \times 1.5$ cm) from the discoloured area was excised and stored at −20°C prior to DNA extraction.

2.3 Sampling in Charvensod

A total of 15 randomly selected spruce stumps were sampled in July 1998 in Charvensod. Stumps were cut at a height of 50 cm, and only those without any visible symptoms of discoloration or decay were sampled a year after felling. Exclusion of stumps displaying signs of advanced or incipient decay was aimed at providing information on primary stump infection by airborne basidiospores rather than on secondary infection through root contacts. This distinction was made because the study was originally linked to a broader study investigating the abundance of each *Heterobasidion* spp. in the air spora. A 2–3-cm-thick cross-section of each stump was taken 20 cm below the top surface. Isolations occurred as described above for the Cogne site. Upon the discovery of symptomatic Swiss stone pines at Charvensod, nine pine stumps and three spruce stumps were also sampled. The pattern of wood decay in these stumps indicated that they had been infected prior to the felling of these trees in the summer of 2002. The area of this second sampling covered approximately 3 ha. Stump discs were incubated as described above. Five symptomatic pines did not yield any isolates. Wood blocks were collected from these trees as described for the larch at Cogne, and direct DNA typing of the putative pathogen was attempted.

2.4 Mitochondrial and nuclear typing for the diagnosis of *Heterobasidion* species

To determine the ploidy of the isolates, presence or absence of clamp connections was noted after careful microscopic observations of cultures at 300 × magnification. Isolates displaying clamps at the septa were considered to be heterokaryotic (n + n), while clampless isolates were regarded as homokaryons (n).

Three methods were employed to classify the isolates. First, a taxon-specific competitive-priming (TSCP)–PCR (Garbelotto et al. 1996) combined with a PCR-mediated detection of species-specific DNA insertions in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA) gene was used, as described by Gonthier et al. (2001). To type all three European species of *Heterobasidion* by a single PCR amplification and gel, the method was modified as follows: (1) a mix of four primers (MLS, MLF, Mito 5 and Mito 7) (Garbelotto et al. 1998; sequence for Mito 7: 5'-GCC AAT TTA TTT TGC TAC C-3') was used for DNA amplifications and (2) PCR products were electrophoresed in 2.5% Metaphor agarose gels (FMC Bioproducts, Rockland, ME) in 1 × Tris-borate buffer (TBE) at 5 V cm^{-1} for 3 h (Fig. 1).

Secondly, a PCR Restriction Fragment Length Polymorphism (RFLP) on the Internal Transcribed Spacer (ITS) was used to distinguish between *H. parviporum* and *H. annosum* (Gonthier et al. 2001). ITS RFLPs can also be used to verify the presence of hybrid heterokaryons (Garbelotto et al. 1996). Results of molecular typing from mitochondrial and nuclear markers were compared with each other to check for the occurrence of nuclear-mitochondrial chimeras across species.

Thirdly, sexual compatibility tests with homokaryon testers of the three species (T4, T5, T6 - kindly provided by Prof. P. Capretti and A2r, A27r and A66r), as described by

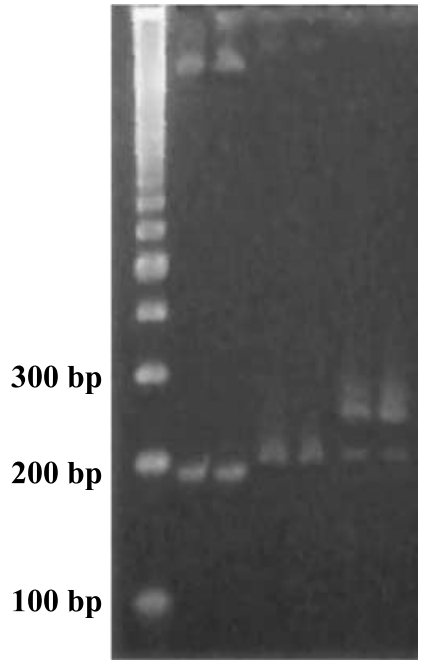

Fig. 1. Diagnosis of the European species of *Heterobasidion* by taxon-specific competitive-priming–polymerase chain reaction (TSCP–PCR) combined with PCR-mediated detection of species-specific insertions in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA) gene. First lane is the DNA molecular standard (100-bp ladder); lanes 2 and 3 are *H. parviporum* isolates; lanes 4 and 5 are *H. abietinum* isolates; lanes 6 and 7 are *H. annosum* isolates

Stenlid and Karlsson (1991), were employed. The Buller phenomen was used to identify heterokaryon isolates, and in that case, the occurrence of clamp connections was verified in the tester thallus, at least 3 cm from the interaction zone.

To characterize the *Heterobasidion* species on larch and pines that had symptomatic wood but did not yield isolates, direct DNA extractions from wood samples were performed. Sawdust was obtained by drilling in the wood blocks. The sawdust was placed in a 1.5 ml Eppendorf tube with 300 ml of 2XCTAB extraction buffer. DNA was extracted from the sawdust by adding an equal volume of 24 : 24 : 1 phenol : chloroform : isoamyl alcohol and vortexing vigorously for 2 min. After 15 min centrifugation at 9500 g, the supernatant was collected and the extraction was completed using the GeneClean II Kit (Qbiogene, Carlsbad, CA), following the manufacturer's instructions. DNA was diluted 1 : 100 in PCR water and a nested PCR approach was utilized to obtain amplification of *Heterobasidion* template DNA. It should be noted that the pathogen was no longer culturable and was expected to be present in very small titre. A first round of PCR was performed with primers ITS-1F and ITS4 (White et al. 1990; Gardes and Bruns 1993), using parameters described by Garbelotto et al. (1996). The PCR product was then diluted 1 : 100 in PCR water and a second PCR amplification was performed using two internal primers (ITS-S1 and ITS4) (White et al. 1990; Garbelotto et al. 1996). Sequences of the complimentary strands of the PCR product were obtained using an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA). Sequence alignments were obtained by using Sequencer software (GeneCodes, Ann Arbor, MI) and optimized

by manual alignment. Using the consensus sequences, a BLAST search of the GenBank database (NCBI) was performed.

2.5 Somatic incompatibility tests

To study the presence and the patterns of colonization of *Heterobasidion* genotypes, all conspecific isolates from Cogne were paired at least twice in all possible combinations (Stenlid and Karlsson 1991). Plates were incubated at room temperature for 4–5 weeks and examined periodically.

3 Results

3.1 Symptoms and signs of *Heterobasidion* in the infected Swiss stone pine at Cogne

The infected Swiss stone pine tree at Cogne was externally an asymptomatic tree. Tree crown colour and density were average for the local population. Other typical symptoms associated with infection by *Heterobasidion* such as collar or root resinosis were also absent.

Decay occupied about 29% of the stem section at d.b.h. and it was entirely confined to the heartwood. The butt rot appeared fibrous, soft, pale to dark brown, and quite dry. Peripheral areas of the decay were stained either brown or lilac, with blue-grey border lines. A hollow decay pocket was noticed from the collar zone up to 3 m of height, but isolations were successful for 1 m past the hollowed pocket. Decay was also present in the central core in the seven main roots of the tree. Decay was observed until the average root diameter reached approximately 7 cm. Rot and the staining associated with root decay were comparable with rot and staining in the main stem. The fungus was also successfully isolated from 30% of the fine roots (0.5–7 cm diameter). Fine roots displayed no external disease symptoms such as resinosis, despite the fact that *Heterobasidion* was present on their entire cross-section. No *Heterobasidion* mycelium was ever observed between the bark and the wood.

All isolates from the pine were heterokaryotic. Isolates from the stem and from one root were typed as *H. parviporum* and belonged to the same somatic compatibility group. The other six main roots were colonized by a single *H. annosum* genet. In the stump, a distinct dark line, about 2 mm wide, was present between wood occupied by either of the two fungal individuals. When isolates from the two sides of the dark line were paired in somatic incompatibility tests, a demarcation line characterized by dense mycelium was produced rather than the regular barrage zone.

3.2 *Heterobasidion* infestation in the subalpine mixed forest at Cogne

A total of 35 trees was sampled around the infected Swiss stone pine, including 27 spruce trees, four larches and four Swiss stone pines. Typical *Heterobasidion* decay was observed in 21 trees (60%), and the fungus was isolated from 17 (48.6%). The pathogen was present in 17 of 27 sampled spruce tree and stumps (63%). Infected trees and stumps ranged from 6 to 35 cm in diameter. Decay columns occupied between 10 and 40% of the trees' cross-sections.

A single *Heterobasidion* basidiocarp was found in a decay pocket of a spruce stump. Cores from larch and other Swiss stone pines were healthy in appearance and did not yield any isolates. All spruce isolates were clamped and typed as *H. parviporum*. With the exception of the infected Swiss stone pine, all other trees or stumps were colonized by single fungal genotypes. *H. annosum* was isolated only from the Swiss stone pine. Although no isolates were obtained from the larch tree growing in close proximity to the

Swiss stone pine tree, the pathogen's DNA was amplified directly from the wood. The BLAST search resulted in a perfect match between the fungal DNA found in the larch wood and *H. parviporum*.

Root contacts were detected between two roots of the infected Swiss stone pine and roots of two spruce stumps at 5.60 m and 6.40 m from the pine tree, respectively. *Heterobasidion parviporum* isolates were obtained from both stumps. The same genet was isolated from the one of the pine roots and roots of the spruce stump, indicating secondary vegetative spread of the isolate between spruce and pine.

Using somatic incompatibility tests, 14 genets of *H. parviporum* were identified on a total of 18 infected trees and stumps (average of 1.29 trees/stumps per genet) (Fig. 2). The largest genet had colonized three trees within a distance of 7 m, while two smaller genets occupied two trees each. The remaining 11 genets (61.1%) were detected only in single trees.

3.3 Stump colonization by *Heterobasidion* spp. at Charvensod

A total of 25 *Heterobasidion* isolates was collected from 15 randomly selected spruce stumps in Charvensod. All isolates were obtained from the sapwood. Eleven (44%) were typed as *H. parviporum* and 14 (56%) as *H. annosum*. Three stumps were colonized by isolates of both species of the fungus, and homokaryotic strains were more frequent than those of heterokaryotic (16 vs. 9, respectively).

A total of nine isolates was obtained from three spruce stumps growing in proximity of symptomatic pine stumps. Based on the decay pattern, stumps had been infected by the pathogen through secondary root-to-root infection. Mitochondrial TSCP and nuclear ITS analysis indicated that they all were *H. parviporum*. A total of six *Heterobasidion* isolates were obtained from nine pine stumps. Pine stumps had also been infected by secondary fungal spread through root contacts. Five isolates from pine stumps were *H. parviporum*, one isolate was *H. annosum.* It should be noted that in this case, although spruce and pine stumps had been colonized as standing trees, presumably through the roots, it can't be excluded that some of the isolates may have been the result of subsequent primary infections.

Four partial ITS fungal sequences were obtained from symptomatic wood of five pines. One sequence (462 bp long) perfectly matched *H. parviporum*, two (471 bp, GBAY241671)

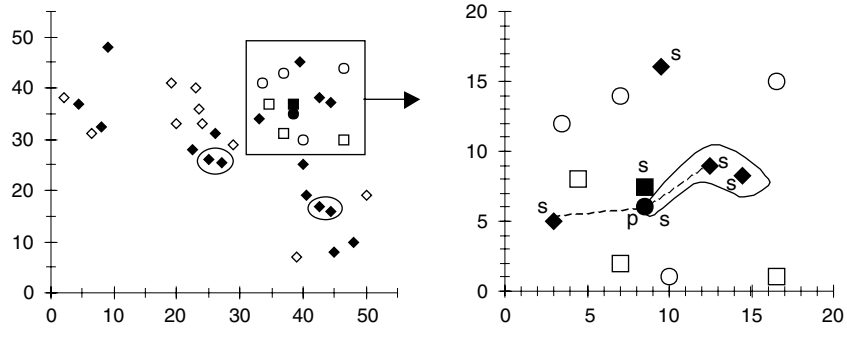

Fig. 2. Spatial distribution of Norway spruce (rhombus), larch (square) and Swiss stone pine (circle) trees or secondarily infected stumps in the forest of Cogne. Distances are in metres. Shaded symbols show trees or stumps containing *Heterobasidion*. Territorial clones including more than one tree are encircled, and root contacts among the infected pine and other trees/stumps (figure on the right) are symbolized by dotted lines. The letters S an P represent *H. parviporum* and *H. annosum* isolates, respectively. The remaining infected spruces in figure on the left were all colonized by *H. parviporum*.

were identical to each other and a 177-bp portion of them was a 98% match to *Tricholoma acerbum*, and the fourth one was 463 bp long and a 95% match to *Hypodonta radula* (GBAY241670).

Nuclear PCR RFLP typing on isolates collected from both sites indicated the absence of both heterokaryon hybrids and nuclear-mitochondrial chimeras.

4 Discussion

All previous reports of *H. annosum sensu lato* (*s.l.*) on *Pinus* spp. within the Subsection *Cembrae* of the Section *Cembra* (Debazac 1977) have originated from Eastern Europe and Siberia (Negrutskii 1963; Darozhkin and Fedarau 1976; Aref'ev 1991; Kolomiets and Bogdanova 1992). These regions are well outside the natural range of Swiss stone pine, and are populated by the closely related *P. sibirica* Du Tour (Tutin et al. 1993). While we cannot exclude that the pine trees in those reports may have been Swiss stone pines planted outside their natural range, it is more likely that those reports referred to *P. sibirica* (K. Korhonen, personal communication). We have previously reported a single infected pine tree (Nicolotti et al. 1999; Gonthier et al. 2002), but this is the first extensive report on infection of Swiss stone pine by *Heterobasidion* spp.

Heterobasidion spp. are generally reported to kill pine trees by infecting the cambium and then spreading into the root sapwood (Korhonen and Stenlid 1998). The infected Swiss stone pine at Cogne was apparently asymptomatic and displayed internal stem decay almost exclusively. All studied stumps at Charvensod displayed analogous internal stem decay. Similar symptoms have been described for *P. sibirica* (K. Korhonen, personal communication), suggesting that in these two closely related pine species, this pathogen may be responsible for a butt rot rather than a root disease. In the Cogne tree, internal decay was also observed in the heartwood portion of the roots. Heart rots in stems and primary roots may result in physiological and mechanical stress in these pine species, as opposed to the rapid decline and mortality described in other pine species, in which the pathogen actively kills the living cambium of the roots and root collar.

Both *H. parviporum* and *H. annosum* were present in the Swiss stone pines examined in this study. In the Cogne tree, both species caused a similar type of rot in the bole and roots, but, while most isolations from the bole were *H. parviporum*, most isolations from the roots were *H. annosum*. This finding may suggest a preference of each species for a different part of the tree. Roots of Swiss stone pine, rather than boles, may have to be sampled to obtain more *H. annosum* isolates. The co-existence of two *Heterobasidion* species in the same tree has been reported only rarely in Norway spruce (Delatour 1998; Vasiliauskas and Stenlid 1998) and in ponderosa pine (*P. ponderosa*) (Garbelotto et al. 1996). This tree represents the first reported colonization by two *Heterobasidion* species on a European pine (Gonthier et al. 2002).

The presence of both fungal species on the same host at both sites may indicate that host specificity for this pathogen is not strict, but may be regulated by ecological conditions and site history. It has been shown that in sites where a shift in tree species composition has occurred, a carry-over effect of the pathogen into the new rotation may occur. For instance, spruce regeneration in sites originally rich in pines (normally attacked by *H. annosum*) display a high percentage of trees infected by the pine pathogen (Korhonen et al. 1998).

When pathogen incidence is high, interspecific root contacts may be routes used by *Heterobasidion* spp. to infect unusual hosts (Capretti et al. 1994). Interspecific contagion was proven in our study by following the secondary spread of the same *H. parviporum* genotype between a spruce and a Swiss stone pine tree at the Cogne site. Furthermore, although no isolates were obtained from the larch growing in proximity of the infected Swiss stone pine, the close proximity of the two trees (<0.5 m), and the presence of *H. parviporum* in both, may also be the result of interspecific contagion.

General observations on symptoms and patterns of decay suggest that vegetative root-to-root spread among spruce trees plays a primary role at the Cogne study site, as reported for many other locations worldwide (e.g. Stenlid and Redfern 1998). The secondary nature of the infections at Cogne was confirmed by the results of the somatic compatibility analysis, which highlighted the presence of identical genotypes in different trees. Sizes of genets were comparable with those reported by Swedjemark and Stenlid (1993): the largest genet had colonized three trees and had a diameter of approximately 7 m. On each individual tree, a single fungal genotype was retrieved. This observation is in accordance with data published by Vasiliauskas and Stenlid (1998).

Despite the extensive sampling of all spruce and larch trees, and stumps at the Cogne study site, only Swiss stone pine was found to be infected and colonized by *H. annosum*. The pathogen had efficiently colonized the pine roots, but could not be found in the roots of neighbouring spruce trees. The two root contacts uncovered between the pine and neighbouring spruces were both unavailable for the vegetative spread of *H. annosum*, because of the competitive colonization of such root contacts by *H. parviporum*. The incidence of *H. parviporum* at Cogne is extremely high, and the opportunity for establishment of *H. annosum* outside Swiss stone pine, whether through root contacts or airborne inoculum, may be extremely limited.

In Cogne, all sampled spruces were only infected by *H. parviporum*. It should be noted that in this study site, all spruce trees and stumps appeared to have been infected secondarily through root contacts, rather than primarily by basidiospores landing on stumps. Conversely, both *H. parviporum* and *H. annosum* were found in the spruce stumps at Charvensod, often coexisting in the same stump. *H. parviporum* and *H. annosum* were equally present at this site, representing 44 and 56% of the collected isolates, respectively. In contrast with Cogne, the stumps sampled at Charvensod were infected primarily by airborne inoculum, and overall spruce infestation is significantly lower here (50%) than at Cogne. The presence of available uninfected stumps and trees may allow for an establishment of *H. annosum* in this site. The high recovery of *H. annosum* isolates obtained in this study and the reported greater saprotrophic ability of *H. annosum* vs. *H. parviporum* in spruce wood (Daniel et al. 1998) indicate that spruce stumps may represent another alternate and suitable habitat for the survival of *H. annosum* in areas were Scots pine, the host generally associated with *H. annosum*, is absent or apparently unaffected.

Heterobasidion parviporum and *H. annosum* co-exist both in spruce stands (Gonthier et al. 2001) and in mixed forests growing at high elevations in the WIA. We show here that at least two niches are simultaneously shared by both species in subalpine forests. In this study, neither heterokaryon hybrids nor nuclear-mitochondrial chimeras between *H. parviporum* and *H. annosum* were found. Although hybridization may be a rare event in the *Heterobasidion* spp. complex in the WIA (Gonthier et al. 2001), niche overlaps like the one exemplified by this study enhance the possibility of gene introgression and hybridization between species, in spite of strong mating barriers.

Finally, our results show that *Heterobasidion* spp. may be locally very pervasive in subalpine forests, with high levels of infestation. Although timber production is rarely important in these environments, loss of structural integrity as a result of internal decay may have important consequences on the stability of these forests.

Acknowledgements

This research has been supported by a grant of Regione Autonoma Valle d'Aosta / *Région Autonome Vallée d'Aoste*, Assessorato Agricoltura e Risorse Naturali / *Assessorat Agriculture et Ressources Naturelles*. We are grateful to Dr Kari Korhonen for his helpful information about the epidemiology of *H. annosum* on *P. sibirica*. We also thank the lumberjacks of the 'Région Autonome Vallée d'Aoste'.

Résumé

Les souches de pin cembro et d'épicéa représentent un habitat important pour Heterobasidion spp. dans les forêts sub-alpines

Dans les Alpes italiennes occidentales, les trois espèces européennes du pathogène forestier *Heterobasidion* spp. peuvent coexister dans la même zone. *Heterobasidion parviporum* Niemelä & Korhonen et *Heterobasidion abietinum* Niemelä & Korhonen se trouvent habituellement dans les zones avec une présence significative de leur hôte primaire respectif, l'épicéa (*Picea* spp.) et le sapin (*Abies* spp.). L'hôte (la niche) occupé(e)par *Heterobasidion annosum* (Fr.) Bref. dans la région est encore mal connu(e). Bien que le pin sylvestre (*Pinus sylvestris*), hôte très important de ce champignon dans d'autres parties d'Europe, soit abondant dans la région, il semble peu ou pas affecté par *H. annosum*. Deux hypothèses non exclusives l'une de l'autre pourraient expliquer la présence d'*H. annosum*: 1- Les pins sylvestres sont infectés mais en grande partie asymptomatiques et 2- *H. annosum* est adapté à d'autres hôtes. Une analyse des espèces d' *Heterobasidion* a été réalisée dans deux forêts naturelles mélangées conifères-feuillus, en utilisant les techniques traditionnelles d'isolement et de nouveaux outils de diagnostic moléculaire direct à partir du bois. Dans un peuplement sub-alpin mélangé d'épicéa (*Picea abies*), mélèze (*Larix* spp.) et pin cembro (*Pinus cembra*), seul *H. parviporum* a été obtenu à partir de 18 épicéas et mélèzes naturellement infectés. Un pin cembro du même peuplement était largement colonisé en même temps par *H. parviporum* et *H. annosum*. Dans un deuxième peuplement sub-alpin, des isolats de *H. parviporum* et *H. annosum* ont été obtenus conjointement de 18 souches d'épicéas et de 9 souches de pins cembro. Les souches de pins avaient été pour la plupart colonisées par *H. parviporum* avant abattage, suggérant que les pins pourraient être infectées secondairement par l'espèce localement prédominante d' *Heterobasidion* (c'est-à-dire ici *H. parviporum*). Nos résultats indiquent également que la colonization primaire des souches d'épicéas (par les basidisopores) se réalise à la fois pour *H. parviporum* et *H. annosum* tandis que l'infection secondaire de ces souches est principalement due à *H. parviporum*.

Zusammenfassung

Zirbelkiefern und Fichtenstümpfe als wichtiges Habitat für Heterobasidion spp. in subalpinen Wäldern

In den westlichen italienischen Alpen können die drei europäischen Arten von *Heterobasidion* spp. auf derselben Fläche gemeinsam vorkommen. *Heterobasidion parviporum* Niemlä & Korhonen und *Heterobasidion abietinum* Niemlä & Korhonen werden normalerweise in Gebieten mit bedeutenden Vorkommen ihrer jeweiligen hauptsächlichen Wirte *Picea* spp. und *Abies* spp. gefunden. Die von *Heterobasidion annosum* (Fr.) Bref. im Gebiet besetzte Wirtsnische ist noch unbekannt. Obwohl die Wald-Kiefer (*Pinus sylvestris*), ein wesentlicher Wirt für diese Pilzart in anderen Teilen Europas, in der Region häufig vorkommt, gibt es kaum Hinweise auf durch *H. annosum* verursachte Krankheitssymptome an *P. sylvestris*. Zwei verschiedene, sich gegenseitig nicht ausschliessende Hypothesen können aber das Vorkommen von *H. annosum* erklären: (1) Die Waldkiefern sind zwar infiziert, jedoch grösstenteils symptomfrei und (2) *H. annosum* hat sich an verschiedene Wirte angepasst. In zwei natürlichen Koniferenmischbeständen wurden die *Heterobasidion*-Arten mit klassischen Isolierungsmethoden und direkter molekularer Diagnose am Holz untersucht. In einem subalpinen Mischbestand aus Fichte (*Picea abies*), Lärche (*Larix* spp.) und Zirbelkiefer (*Pinus cembra*) waren 18 natürlich infizierte Fichten und Lärchen nur von *H. parviporum* besiedelt. Eine Zirbelkiefer aus demselben Bestand war stark mit *H. parviporum* und *H. annosum* besiedelt. An einem zweiten subalpinen Standort wurden aus 18 Fichten- und 9 Zirbelkiefernstümpfen *H. parviporum* und *H. annosum* isoliert. Die *P. cembra*-Stümpfe waren hauptsächlich vor dem Fällen von *H. parviporum* besiedelt worden, was vermuten lässt, dass diese Baumart vom örtlich vorherrschenden *H. parviporum* sekundär infiziert wurde. Die Ergebnisse weisen darauf hin, dass bei Fichtenstümpfen eine primäre Besiedlung (mit Basidiosporen) durch *H. parviporum* und *H. annosum* erfolgte, während eine sekundäre Besiedelung der Stümpfe hauptsächlich durch *H. parviporum* erfolgte.

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