

## Porodaedalea (Phellinus pini group, Basidiomycetes) in Europe: a new species on Larix sibirica, P. niemelaei

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Two specimens of *Porodaedalea* (*Phellinus pini* group, Basidiomycetes), collected from *Larix sibirica* in Finland, were compared with the European-based species *P. pini* and *P. chrysoloma*, and other taxa originating from North America, Russia, and China. While differentiation based on fruiting-body characters was difficult, pairing tests and sequence data of the nuclear ribosomal ITS region (ITS-1, 5.8S, ITS-2) showed the collections from *Larix sibirica* to be distinct and they are suggested as a new species, *Porodaedalea niemelaei* M. Fischer.

Key words: Pairing tests, *Phellinus pini* group, *Porodaedalea*, *Porodaedalea niemelaei*, sequence analysis

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### Introduction

"*Phellinus*, with its many complexes of closely related species, seems to be still in a strong evolutionary stage" (Ryvarden 1991). One of these still unresolved, problematic groups is that of *Phellinus pini* (Brot. : Fr.) Ames and its relatives, usually referred to as the *P. pini* group. In a comprehensive study on the European poroid Hymenochaetales, the *P. pini* group was raised by Fiasson and Niemelä (1984) to generic level, as *Porodaedalea* Murrill. This taxonomic status was accepted by, for instance, Nuss (1986) and Jahn and Jahn (1986); it was not followed by Gilbertson and Ryvarden (1987), Parmasto (1988), Larsen and Cobb-Pouille (1990), and Ryvarden and Gilbertson (1994). Recently, an intermediate concept has been developed by Dai (1999), who attributes a subgeneric rank to the *P. pini* group, i.e., *Phellinus* subg. *Porodaedalea* (Murrill) Y.C. Dai. The concept of Fiasson and Niemelä, which is derived from an array of 20 different characters of the fruiting-body and the mycelium, correlates well with several sets of molecular data (Fischer 1996; unpubl. data); therefore, the designation

*Porodaedalea* instead of *P. pini* group is used in this paper.

*Porodaedalea* has a worldwide distribution, having been reported from a variety of conifers in Europe, North America, Asia, and Africa. A number of closely related taxa, most of them not separable by morphological and/or anatomical features of the fruiting-body, are known to exist worldwide. However, only the type species, *P. pini* (Brot. : Fr.) Murrill, and *P. chrysoloma* (Fr.) Fiasson & Niemelä, are generally recognized and included in the respective handbooks. The possible existence of additional taxa in North America and Asia has been suggested by Percival (1933), Owens (1936), Overholts (1953), and Parmasto (1985). As a consequence, several taxa related to *Porodaedalea* have been introduced during the last years: *Porodaedalea piceina* (Peck) Niemelä (Niemelä 1985), *Phellinus vorax* (Harkness) Cerný (Cerný 1985), *P. cancriformans* (M.J. Larsen et al.) M.J. Larsen & Lombard (Larsen & Cobb-Pouille 1990), as well as *P. himalayensis* Y.C. Dai and *P. yamanoi* (Imazeki) Parmasto (Dai 1999).

Pairing tests of single-spore isolates from 54 samples collected in Europe, North America, Asia, and Africa demonstrated the existence of numerous intersterile taxa within *Porodaedalea*, most of them probably host-specific (Fischer 1994). Apart from *P. pini* and *P. chrysoloma*, both restricted to Europe, seven intersterility groups, including *P. piceina* (Niemelä 1985), were demonstrated for North America, three intersterility groups for Asia, and one for North Africa (Morocco). Within the non-European intersterility groups of *Porodaedalea*, a distinct morphological and/or anatomical differentiation is preceded by the formation of reproductive barriers. Later on, the results obtained by pairing tests were essentially confirmed by RFLPs (restriction fragment length polymorphisms) of an enzymatically amplified portion of the nuclear RNA genes (Fischer 1996).

Both pairing tests and RFLPs support the traditional view that only two taxa of *Porodaedalea* exist in Europe. While *P. pini* seems restricted to species of *Pinus* (Jahn 1963; Kreisel of *P. chrysoloma* from *Abies*, *Larix*, and *Pinus mugo* or *P. sylvestris* (Bourdot & Galzin 1928; Jahn 1967; Cerný 1985) may be questionable. In Europe, I have found only species of *Pinus* and *Picea abies* as hosts for *P. pini* and *P. chrysoloma*, respectively.

Fungus-host relationships seem to be of considerable importance in speciation of parasitic lignicolous basidiomycetes, a prominent case being that of *Heterobasidium annosum* (Fr.) Bref., where the existing intersterility groups have been demonstrated to be largely host-specific (Chase & Ullrich 1985; Korhonen et al. 1989; Niemelä & Korhonen 1998). In *Phellinus*, three intersterile taxa, two of them host specific, were found to exist within *P. igniarius* as defined by Niemelä (1975). So, host range of lignicolous basidiomycetes deserves special attention.

In 1994 and 1995, Tuomo Niemelä sent two fruiting-bodies of *Porodaedalea*, both collected on the same tree of *Larix sibirica* at different times in Finland. These collections, 94–617 and TN 5877, representing different ontogenetic stages, were examined as follows: An integrative approach was used, which was based on the fruiting-body characters, pairing tests with selected tester strains, and a molecular sequence analysis of the nuclear ribosomal ITS region (ITS-1, 5.8S, ITS-2). Special emphasis was on the

comparison with well defined material of *P. pini* and *P. chrysoloma*. Two more specimens originating from *Larix*, collected in China and Russia, and representing intersterility groups As-II and As-III (Fischer 1994), as well as specimens from *Picea glauca* (Canada; representing *P. piceina*) and *Pinus muricata* (U.S.A.; representing intersterility group N-VI), were also included.

#### Materials and methods

*Specimens examined:* *Porodaedalea pini* (Brot. : Fr.) Murrill: Germany, Bavaria, on *Pinus sylvestris*, 12.VII.1984 – 84–71. Finland, Satakunta prov., on *Pinus sylvestris*, 24.IV.1994 – TN 5749.

*Porodaedalea chrysoloma* (Fr.) Fiasson & Niemelä: Finland, on *Picea abies*, 24.VI.1987 – TN 4008. Germany, Bavaria, on *Picea abies*, 8.IV.1989 – 89–67.

*Porodaedalea piceina* (Peck) Niemelä: Canada, Quebec, on *Picea glauca*, 24.IX.1987 – 87–001 (≡ N-I in Fischer 1994).

*Specimens of uncertain affinity:* Finland, Pohjois-Häme prov., on *Larix sibirica*, 17.VI.1994 – 94–617; 20.VIII.1995 – TN 5877. U.S.A., California, on *Pinus muricata*, 22.III.1991 – 91–109 (≡ N-VI in Fischer 1994). Russia, Siberia, on *Larix dahurica*, IX 1990 – 151400 (≡ As-II in Fischer 1994). China, Jilin prov., Chanbaishan, on *Larix olgensis*, 12.X.1991 – P-86 (≡ As-III in Fischer 1994).

*Cultural conditions:* All cultures were grown on ME medium (2% agar, 2% malt extract, 0.05% yeast extract) at 23°C and 65% humidity.

*Isolation of single spores:* A section of the hymenium was attached to the lid of a Petri plate. Discharged spores were dispersed with Ringer's solution (NaCl, 0.25%; KCl, 0.01%; CaCl<sub>2</sub>, 0.005%; NaHCO<sub>3</sub>, 0.005% in distilled H<sub>2</sub>O) and aseptically isolated after germination.

*Pairing tests and comparative microscopy:* Pairing tests and microscopy were performed as described in Fischer (1994). Three distinct types of reactions were distinguished in intra-strain pairings: 1) Selfing resulted in intermingling of mycelia; 2) formation of a line of demarcation was observed in pairings of incompatible isolates (A=); 3) development of a secondary mycelium was observed in pairings of compatible isolates (A ≠).

*DNA isolation:* DNA was isolated from fresh and lyophilized mycelium. Isolation was essentially as described by Lee and Taylor (1990). DNA pellets were resuspended in 10 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Concentration of DNA was examined in 1% agarose gels.

*Polymerase chain reaction (PCR):* The PCR was used to amplify a portion of the nuclear ribosomal DNA unit defined by the primer combination ITS1 and ITS4 (for primer sequences, see White et al. 1990). The fragment spans the entire region of the internal transcribed spacers, i.e., ITS-1 and ITS-2, as well as the 5.8S rRNA gene.

The PCR reactions were set up in 100 µl volumes and were overlaid with two drops of mineral oil. Hot start PCR was applied throughout (d'Aquila et al. 1991). Thirty-seven cycles were performed on a Biometra TRIO-Thermoblock, using the following parameters: 94°C denaturation step (1 min 30 s), 53°C annealing

step (45 s), 72°C primer extension (1 min 30 s). A final incubation step at 72°C (7 min) was added after the final cycle. Five µl of each PCR reaction were electrophoresed on 1% agarose gels. DNA molecular weight marker VI (Boehringer, Mannheim) was used as standard. The amplified products were purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instructions. DNA was suspended in 50 µl Tris-HCl buffer (10 mM, pH 8.0).

**Sequencing:** Fragments were sequenced with the AmpliTaq DNA Polymerase FS Dye Terminator Cycle Sequencing kit (Perkin Elmer), using 2 µl of premix, 1 µl of the primers (8 pmol of ITS1 and ITS4, respectively), and 3.5 µl of the PCR products. The reactions were set up in 11 µl volumes, and were overlaid with a drop of mineral oil.

Sequences were generated in two directions and twenty-five amplification cycles were carried out, using the following parameters: 96°C denaturation step (30 s), 59°C annealing step (15 s) for ITS1, 53°C annealing step (15 s) for ITS4, 60°C primer extension (4 min). DNA was precipitated by addition of 2 µl of NaAc (3 M, pH 4.8) and 55 µl of EtOH 100%, and was then washed with 150 µl of EtOH 70%. The DNA pellet was resuspended in EDTA (50 mM, pH 8.0) : formamide = 1 : 4.

The electrophoresis was done with an ABI 373A Automatic Sequencer (Perkin Elmer). After processing the raw data with SeqEd (version 3.0), the sequences were aligned using the CLUSTAL W (version 1.6) program (Thompson et al. 1994). A final alignment was performed by eye. Alignment gaps were treated as missing data.

For neighbor joining analysis, a distance matrix was generated using DNA DIST, a program from the PHYLIP 3.5c package (Felsenstein 1993). The calculation was performed using the Kimura 2 model and a transition-transversion ratio of 1.5. Bootstrap values for internal nodes were calculated by 1000 replications using the programs SEQBOOT and CONSENSE.

## Results

### Pairing tests

Strains 94–617 and TN 5877 originate from the same tree of *Larix sibirica*; the mycelia isolated from the fruiting-bodies intermingled when paired and so were assignable to one single individual.

A sufficient number of single spore isolates were obtained from TN 5877, and intra-strain pairing tests using eight single spore isolates demonstrated a unifactorial pattern of sexuality. Corresponding results were obtained for the other strains under study (for details, see Fischer 1994).

For inter-strain pairings two different isolates per mating type factor were selected from each strain as testers; these were paired in all combinations. The results of the inter-strain pairings showed TN 5877 to be reproductively isolated and only intersterile results were obtained in

pairings involving single spore testers of TN 5877 (Table 1).

### Sequencing

The size of the rDNA portion defined by the primer combination ITS1 and ITS4 was slightly variable and ranged between 681 (strain 151400) and 691 (*P. chrysoloma*) nucleotides. No intra-specific variation was observed. Length variations between taxa were due to several small length mutations (insertions and deletions) in the ITS-1 and ITS-2 region; the size of these mutations ranged between one and three nucleotides. The total length of the alignment was 694 nucleotides.

The phylogenetic tree generated by the neighbor-joining analysis is subdivided into three clades (Fig. 1). The European species, *Porodaedalea pini* and *P. chrysoloma*, came out as distinctly separated. Strongly supported by a bootstrap value of 100%, *P. pini* appeared as sister group to the North American taxa, *P. piceina*, from *Picea glauca*, and intersterility group N-VI, from *Pinus muricata*. Strains originating from *Larix* were unified in a single clade (bootstrap

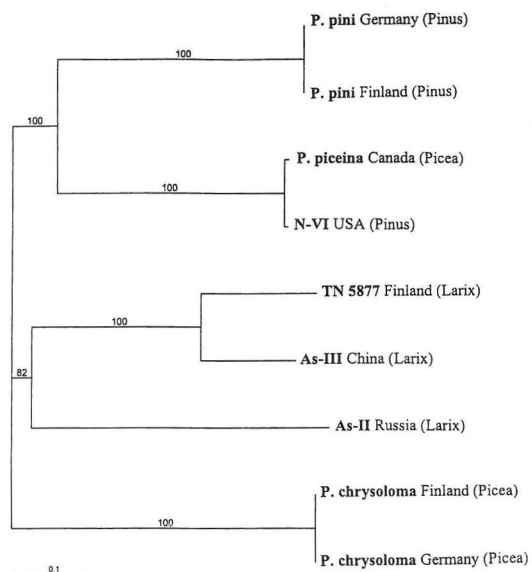


Fig. 1. Relationships between taxa of *Porodaedalea* (*Phellinus pini* group) from Europe, North America and Asia inferred from the nuclear ITS region (ITS-1, 5.8S, ITS-2) using the neighbor joining method. Support from 1000 bootstrap replications indicated above the branches.

value 82%). Within this clade, TN 5877 was positioned next to the Chinese strain, P-86, from *Larix olgensis* (bootstrap value 100%); the Russian strain, 151400, from *Larix dahurica*, was separated by a long branch length.

#### Measurements of spores and setae

Spores were ellipsoid to subglobose in *P. pini*, mostly subglobose in *P. chrysoloma*, and ellipsoid to subglobose in TN 5877 and 94-617. According to the size of basidiospores and hymenial setae, no unequivocal differentiation was possible between *P. pini* and *P. chrysoloma*. On the average, *P. pini* had somewhat larger spores,  $4.5.5(-6) \times 2.5-4.5(-5) \mu\text{m}$ , and setae,  $(14-)-16-54(-58) \times 5-15 \mu\text{m}$ , than *P. chrysoloma*, with spores of  $(3-)-3.5-4.5 \times 2.5-4 \mu\text{m}$  and setae of  $(15-)-20-42(-45) \times (3-)-4-11(-15) \mu\text{m}$ . As shown before, there is, however, some overlap between these taxa (Fischer 1996).

Even though belonging to one single individual, spore sizes were not uniform for 94-617 and TN 5877. In 94-617, representing a large and more massive fruiting body, spore dimensions were  $(5.5-)-6-7 \times (4.5-)-5-5.5 \mu\text{m}$ ; in TN 5877, representing a younger specimen, spores were smaller,  $(4.5-)-5-6 \times (3.5-)-4-4.5(-5) \mu\text{m}$ . The size of the hymenial setae was  $(15-)-20-45(-50) \times (4-)-5-11 \mu\text{m}$  for both collections. Thus, basidiospores of the Finnish strains on *Larix* are slightly larger than those of *P. pini* and *P. chrysoloma*; setal dimensions are similar to those of *P. chrysoloma*.

For the Chinese strain from *Larix olgensis*, P-86, spores were found to be distinctly smaller,  $3.5-4 \times 2.5-3.5 \mu\text{m}$ , setae resembled those of *P. chrysoloma* and were  $25-50 \times 5-10 \mu\text{m}$ . Since no fruiting-bodies were available for strain 151400, from *Larix dahurica*, no data can be provided here.

#### Taxonomic conclusions

Taken together, the characters examined in this study demonstrate a distinct taxonomic position of TN 5877 and 94-617; therefore, these collections are suggested to represent a new species:

*Porodaedalea niemelaei* M. Fischer, *spec. nov.*

*Basidiomata perennia, effuso-reflexa ad pileata; superficies rubrobrunnea ad atra, concen-*

*trice sulcata, irregulariter fracta et sero incrustata. Superficies pororum luteobrunnea ad rubiginosa, pori circulares vel plus minusve daedaleoidei, (1-)-2-3 in quoque millimetro. Contextus rubiginosus, lignosus durus, ca. 5 mm crassus; tubuli juvenes luteoli, deinde rubiginosi, ad 1.5 cm longi, sero cum contexto fusi; systema hypharum dimiticum; omnia septa fibulis egentia; hyphae skeletales luteobrunneae, 2.5-3.5  $\mu\text{m}$  latae; setae frequentes in hymenio, plerumque subulatae, raro ventricosae, (15-)-20-45(-50)  $\times$  (4-)-5-11  $\mu\text{m}$ . Sporae ellipsoideae ad subglobosae, hyalinae, tenui vel modice crassitunicatae, modice cyanophilicae, inamyloideae, (4.5-)-5-6(-7)  $\times$  (3.5-)-4-5(-5.5)  $\mu\text{m}$ .*

Fruiting-bodies perennial, effused-reflexed to pileate; pileal surface reddish brown to black, concentrically sulcate, irregularly cracked and becoming encrusted with age; pore surface yellowish brown to rust brown, pores circular to more or less daedaleoid, (1-)-2-3 per mm; context rust brown, woody hard, ca. 5 mm thick; tubes yellowish when young, becoming rust brown with age, up to 1.5 cm long, old tubes merging into context; hyphal system dimitic; all septa without clamp connections, skeletal hyphae yellowish brown, 2.5-3.5  $\mu\text{m}$  wide; setae frequent in the hymenium, mostly subulate, rarely ventricose, (15-)-20-45(-50)  $\times$  (4-)-5-11  $\mu\text{m}$ ; spores ellipsoid to subglobose, hyaline, thin- to slightly thick-walled, slightly cyanophilous, inamyloid,  $(4.5-)-5-6(-7) \times (3.5-)-4-5(-5.5) \mu\text{m}$ .

Holotype: Finland, South-Häme, Juupajoki, Hyytiälä, on living tree of *Larix sibirica*, 20.VIII.1995 T. Niemelä 5877 (REG).

#### Discussion

The data presented in this study demonstrate that besides *P. pini* and *P. chrysoloma* another taxon of *Porodaedalea* exists in Europe. The Finnish strain TN 5877, originating from *Larix sibirica*, was sharply delimited by pairing tests and molecular sequences of the ribosomal ITS region (Table 1; Fig. 1). With respect to characters of the fruiting-body, the differentiation remains more questionable. In both collections of *P. niemelaei*, hymenial setae were similar to those of *P. chrysoloma*. Basidiospores were larger than those in *P. pini* and *P. chrysoloma*, but, although belonging to one single individual, some varia-

Table 1. *Porodaedalea* in Europe, North America, and Asia: Pairings of single spore testers.

	<i>P. pini</i>	<i>P. chrysoloma</i>	TN 5877	<i>P. piceina</i>	N-VI	As-II	As-III
<i>P. pini</i> (Europe)	50 <sup>a</sup>	–	–	(–)	(–)	–	–
<i>P. chrysoloma</i> (Europe)		50	–	–	–	–	–
TN 5877 (Europe)			50	–	–	–	–
<i>P. piceina</i> (Canada)				50	(–)	–	–
N-VI (U.S.A.)					50	–	–
As-II (Russia)						50	–
As-III (China)							50

<sup>a</sup> bifactorial pattern of sexuality in intra-strain pairings = 50; intersterility complete = –; intersterility incomplete = (–).

tion of the spore size was noted among the specimens. Additional collections of *P. niemelaei* are needed to elucidate this phenomenon. In general, spores and setae of *P. pini* and *P. chrysoloma* as observed in this study were smaller than those reported by Cerný (1985), Ryvarden and Gilbertson (1994), and, in part, Jülich (1984). However, the data provided in the literature are problematical since they may have been based on different taxa.

The geographic distribution of *P. niemelaei* in Europe remains unclear. Possibly the taxon is restricted to autochthonous stands of *Larix* in Northern and Central Europe. Reports of *Porodaedalea* on *Larix* are very rare; such findings have been mentioned by Jahn (1976), Cerný (1985), and Breitenbach and Kränzlin (1986). Very recently, I have reexamined a putative locality of *P. cf. pini* on *Larix decidua* near Innsbruck, Austria, but without any success.

No strict correlation between the molecular data on one hand and geographic or host criteria on the other hand is evident in the phylogenetic tree (Fig. 1). The North American taxa, *P. piceina* and N-VI, originate from different host genera, but appear as very close sister groups. In these taxa, divergence of ribosomal sequences is preceded by the establishment of far-reaching reproductive isolation (Table 1; Fischer 1994). The

European species, *P. pini* and *P. chrysoloma*, came out as clearly separated genetically. In other respects these taxa are well-defined as well. Collections of *P. pini* and *P. chrysoloma* usually can be distinguished by their substrate, the shape of fruiting-bodies and the dimensions of spores and setae. Consequently, *P. pini* and *P. chrysoloma* may be considered as phylogenetically old taxa. The strains from *Larix*, albeit of different geographic origin, are unified in one clade. Fruiting-bodies of the Chinese collection from *Larix olgensis* have distinctly smaller spores than *P. niemelaei*. Nevertheless, these taxa exhibit similar ITS sequences. This is another case, where reproductive isolation and – possibly – anatomical differentiation precede a distinct diversity of molecular data.

As has been indicated before (Niemelä 1985; Fischer 1994, 1996), *P. piceina* represents a separate species, next related to the North American intersterility group N-VI. While fruiting-bodies of *P. piceina* occur saproparasitically on *Picea glauca*, *P. mariana* and, less often, on *Larix laricina* (Niemelä 1985), strains belonging to N-VI all originate from living trees of *Pinus muricata* (Fischer 1994). Additional taxa, so far undescribed, exist in Asia. Dai (1999) cites four species of *Porodaedalea* (as *Phellinus* subg. *Porodaedalea*) as occurring in East Asia: *Phellinus*

*himalayensis*, *P. cf. laricis* (Jaczewski in Pilát) Pilát, *P. cf. pini*, and *P. yamanoi*. With the data at hand, the exact relationships of strains P-86 (China, *Larix olgensis*, As-II) and 151400 (Russia, *Larix dahurica*, As-III) to these taxa remain uncertain. The spores of P-86,  $3.5\text{--}4 \times 2.5\text{--}3.5 \mu\text{m}$ , are distinctly smaller than any dimensions reported by Dai (1999). Probably the closest relative is *Phellinus cf. laricis*, which was reported from species of *Larix* including *Larix olgensis* and other hosts; however, spores are bigger in this taxon,  $4.1\text{--}5.2 \times 3.5\text{--}4.5 \mu\text{m}$  (Dai 1999).

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