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ORIGINAL ARTICLE

A phylogenetic analysis of *Antrodia s.l.* based on nrDNA ITS sequences, with emphasis on rhizomorphic European species

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Abstract The rhizomorphic European species of *Antrodia*, belonging to the traditionally called *Antrodia radiculosa* group, are investigated. On the basis of morphological and molecular analysis, the genus *Fibroporia* is supported. Specimens from Central Europe previously ascribed to *Antrodia radiculosa* constitute a species of their own, and are herein described as *Fibroporia bohemica*. Moreover, a new combination, *Fibroporia citrina*, is proposed.

Keywords *Antrodia* · *Fibroporia* · Wood-inhabiting fungi · Polypores · ITS · Molecular phylogeny

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Introduction

The genus *Antrodia* P. Karst. in a wide sense includes species with a dimitic hyphal system, clamped generative hyphae and causing a brown rot (Ryvarden 1991; Ryvarden and Gilbertson 1993). The genus thus defined is heterogeneous, and it has long been regarded as a polyphyletic group due to variations in macro- and micromorphological characters such as the structure of basidiomata, basidiospores morphology, an iodine reaction of hyphae and sexuality (Lombard 1990; Ryvarden 1991; Ryvarden and Gilbertson 1993; Kim et al. 2001). About 50 species have been described (Kirk et al. 2001; Dai and Niemelä 2002), of which around 30 are known to be present in Europe.

Among the polyporoid fungi, *Antrodiella* Ryvarden & I. Johans. and *Diplomitoporus* Domanski have been traditionally regarded as the closely related genera to *Antrodia*, sharing similar basidiomata and dimitic hyphal system with clamped generative hyphae, but differing in the type of rot, brown in *Antrodia* and white rot in *Antrodiella* and *Diplomitoporus*. Wood rot type seems to be of phylogenetic importance and *Antrodia* has been shown more closely related to other genera with brown rot such as *Fomitopsis* P. Karst., *Daedalea* Pers., *Gloeophyllum* P. Karst., and *Oligoporus* Bref. than to other polypores with white rot such as *Antrodiella, Diplomitoporus, Junghuhnia* Corda, and *Ceriporiopsis* Domanski (Kim et al. 2003).

Parmasto (1968) described the genus *Fibroporia* to accommodate species with fimbriate to rhizomorphic margin and ellipsoid basidiospores with *Polyporus vaillantii* DC. as the generic type species, proposing the following combinations: *Fibroporia vaillantii* (DC.) Parmasto, *F. gossypium* (Speg.) Parmasto, *F. destructor* (Fr.) Parmasto, *F. radiculosa* (Peck) Parmasto, and *F. overholtsii* (Pilát.) Parmasto. Ryvarden (1991) regards the rhizomorphs as an adaptive

character not enough to justify a generic separation and synonymised *Fibroporia* to *Antrodia*. Molecular studies by Kim et al. (2001), however, support the monophyletic group of *F. vaillantii* and *F. gossypium*. In addition, *Fibroporia* is distinguished from *Antrodia* by the generative hyphae that do not possess irregularly thickened walls, distinct to slightly thick-walled basidiospores, and tetrapolar sexuality (usually homothallic or heterothallic bipolar in *Antrodia*) (Rajchenberg 2006).

Neolentiporus Rajchenb. is phylogenetically closely related to *Antrodia s.str.* as was recently showed by nuc-LSU rDNA sequence data (Yu et al. 2010). *Amyloporia* Bondartsev & Singer is separated from *Antrodia s.str.* by the skeletal hyphae that either are amyloid or swell in KOH solution, and by the heterocytic nuclear behaviour (normal in *Antrodia*). The phylogenetic relationships of *Amyloporia* are still not fully resolved (Yu et al. 2010).

In the present study, rhizomorphic *Antrodia* species (*Antrodia radiculosa* complex) from Europe have been morphologically and phylogenetically analyzed and compared with American and East Asian specimens of *Antrodia radiculosa* as well with other non-rhizomorphic *Antrodia* species.

Material and methods

Macro- and microscopic examinations

Specimens were studied in Melzer's reagent (IKI) and KOH (3%) under a Leitz Dialux 22 EB microscope. Measurements (where basidiospores apiculus is excluded) and drawings were made in KOH (3%) reagent.

Phylogenetic analysis

Genomic DNA of strains was extracted from basidiomata of the different analyzed species. The extraction method of Lohdi et al. (1994) with CTAB was adapted. Primers ITS4 and ITS5 (White et al. 1990) were used to amplify part of the nuclear ribosomal DNA (rDNA). PCR was carried out in a final volume of 25 µl containing: 5 µl of diluted sample, 0.75 units of GoTaq Flexi DNA polymerase (Promega, Madison, Wi, USA), 5X Green GoTaq Flexi Buffer, 0.2 mM dNTP, 3 mM $MgCl_2$ (Promega) and 2.5 pmol of each primer.

Amplifications were performed in a thermal cycler (T3 Thermocycler, Biometra) using an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 56°C and elongation for 1 min at 72°C. This was concluded with a final extension for 10 min at 72°C. PCR products were analyzed by electrophoresis in 1% agarose gel in TBE 1X (54 g Tris Base, 27.5 g boric acid, 20 ml EDTA pH 8.0), stained with ethidium bromide (0.4 µg ml-1) and photographed under UV. The molecular weight of the amplified DNA was estimated by comparison with a "100 bp DNA ladder" (Promega).

The amplified products were purified (Wizard SV Gel and PCR Clean-Up System, Promega) and ligated into the pGEM-T easy vector (Promega), which was then used to transform competent cells of JM109 *Escherichia coli* (Promega), and recombinant plasmid DNA from transformed cells was purified (Wizard Plus SV Minipreps DNA Purification System, Promega). Colonies containing the insert were screened by PCR with primers designed on the polylinker of the vector. Sequencing work was done by a commercial company (MWG - Germany).

Sequences were aligned using the multiple-sequence alignment program Clustal W (Higgins and Sharp 1989) from the Megalign package (DNAStar, USA). Phylogenetic analysis were performed by the maximum parsimony method using the heuristic search algorithm of PAUP* program version 4.0b10 (Swofford 1998), and the neighbor-joining method of MEGA 4 (Tamura et al. 2007). Maximum parsimony trees were obtained with heuristic research with 1000 random taxa stepwise addition sequences, tree-bisection-reconnection (TBR) branch swapping algorithm was performed. Robustness of clades was assessed by the bootstrap method using 1000 heuristic search replicate. Trees were edited in MEGA 4 (Tamura et al. 2007). The sequences of *Antrodia juniperina* (AY 966454), *Antrodia malicola* (AY 966449), *Antrodia sinuosa* (AY 966450), *Antrodia sitchensis* (AY 966451), *Antrodia variiformis* (AY 966453), *Antrodiella semisupina* (AF126905), *Auricularia auricula-judae* (EU520237), *Daedalea quercina* (FJ403214), *Diplomitoporus flavescens* (FN907908), *Exidia recisa* (AF291276), *Fibroporia radiculosa* (FJ644284), *Fibroporia vaillantii* (AM286436, AJ421008), *Fomitopsis pinicola* (EF530947), *Gloeophyllum abietinum* (GQ354271), *Gloeophyllum sepiarium* (AY089732), *Laetiporus sulphureus* (AY089742), *Oligoporus lacteus* (FJ627254), and *Piptoporus betulinus* (EU661888) are from the National Centre for Biotechnology Information (NCBI) GenBank. New sequences of *Antrodia sinuosa* (GU991578), *Fibroporia bohemica* (GU991575, GU991577, HM590887, HM590888, HM590889, HM590890), *Fibroporia citrina* (GU991573, HM542005, HM542006), *Fibroporia gossypium* (GU991576, HM590880, HM590881), *Fibroporia radiculosa* (GU991574, HM590882, HM590883), *Fibroporia vaillantii* (HM590884, HM590885, HM590886), *Poria saxonica* (GU991572) were deposited in GenBank (see Table 1). *Auricularia auricula-judae, Exidia recisa, Piptoporus betulinus*, and *Oligoporus lacteus* were used as outgroups.

Table 1 Specimens included in this study. In bold the new accession Genbank number referred to the sequences obtained from this study

Species	Collector number or strain	Locality	GenBank accession n°
Antrodia juniperina	H.S. Wu & Y.S. Yu, strain FP97452T	?	AY966454
Antrodia malicola	H.S. Wu & Y.S. Yu, strain MJL1167SP	?	AY966449
Antrodia sinuosa	H.S. Wu & Y.S. Yu, strain RLG1182R	?	AY966450
Antrodia sinuosa	HUBO 8400	Italy, Sardinia	**GU991578**
Antrodia sitchensis	H.S. Wu & Y.S. Yu, strain HHB12513	China	AY966451
Antrodia variiformis	H.S. Wu & Y.S. Yu, strain FP90100SP	?	AY966453
Antrodiella semisupina	Coll. P. Renvall 3497 (KUO)	Finland, Lammi	AF126905
Auricularia auricula-judae	Coll. Z. Yu, isolate NW484	China ?	EU520237
Daedalea quercina	Coll H.H. Burdsall, Jr. 8735	U.S.A.	FJ403214
Diplomitoporus flavescens	H. Gunter s.n. lab no O. Miettinen X84	Germany	FN907908
Exidia recisa	M. Weiss 315	Germany	AF291276
Fibroporia bohemica	Coll. P. Vampola 12/12/2000	Czech Republic, Bohemia	**HM590888**
Fibroporia bohemica	Coll. P. Vampola 06/09/2002	Czech Republic, Bohemia	**HM590887**
Fibroporia bohemica	Coll. P. Vampola 3/04	Czech Republic, Bohemia	**HM590889**
Fibroporia bohemica	Coll. P. Vampola 327/91, HUBO 8479	Czech Republic, Bohemia	**GU991575**
Fibroporia bohemica	BRNM 612444, HUBO 8480	Italy, Trentino Alto-Adige	**GU991577**
Fibroporia bohemica	PRM 859138, holotypus	Czech Republic, Bohemia	**HM590890**
Fibroporia citrina	Coll. HUBO G. Trichies 7237	France	**HM542006**
Fibroporia citrina	HUBO 7887	Italy, Emilia-Romagna	**HM542005**
Fibroporia citrina	HUBO 7715	Italy, Emilia-Romagna	**GU991573**
Fibroporia gossypium	HUBO 7724	Italy, Sardinia	**HM590880**
Fibroporia gossypium	HUBO 7952	Italy, Veneto	**HM590881**
Fibroporia gossypium	HUBO 7725	Italy, Sardinia	**GU991576**
Fibroporia radiculosa	Coll. HUBO Y.C. Dai 3577	China	**HM590883**
Fibroporia radiculosa	Coll. B.K. Cui 2797	China	FJ644284
Fibroporia radiculosa	Coll. HUBO Y.C. Dai 6473	China	**HM590882**
Fibroporia radiculosa	Coll. Rogers 89668, HUBO 8478	U.S.A.	**GU991574**
Fibroporia vaillantii	HUBO 8160	Italy, Sardinia	**HM590885**
Fibroporia vaillantii	HUBO 8158	Italy, Sardinia	**HM590886**
Fibroporia vaillantii	Coll. K. Grimm 1991	Germany, Karlsruhe	AJ421008
Fibroporia vaillantii	O. Schmidt & U. Moreth, isolate 240	Germany, Karlsruhe	AM286436
Fibroporia vaillantii	Herb. H. Ostrow 2120	Germany, Bavaria	**HM590884**
Fomitopsis pinicola	Coll. S. Brar. UBC F16252	Canada	EF530947
Gloeophyllum abietinum	Baars et al., strain DSM 1210	?	GQ354271
Gloeophyllum sepiarium	Isolate Fp-105551-sp	?	AY089732
Laetiporus sulphureus	S.H. Kim et al. Da-11(Tb)	?	AY089742
Oligoporus lacteus	Coll. B.K. Cui 5724	China	FJ627254
Piptoporus betulinus	D. Cui, M. Zhao & H. Yang, strain 009	China	EU661888
Poria saxonica	HUBO 8481, MTB 5434, typus	Germany, Bavaria	**GU991572**

Results

Morphological analysis

All the species under observation: *Fibroporia radiculosa* from the United States (coll. Rogers 89668) and China (coll. YC 3577, YC6473), *Poria saxonica* (coll. typus MTB 5434, Herb. H. Ostrow 2120), *"Fibroporia radiculosa"* from Czech Republic (Bohemia) (coll. Vampola: 336/92, 327/91, 3/04, VI/09/2002, 12/VIII/2000), *"Fibroporia radiculosa"* from Italy (coll. BRNM 612444), *Fibroporia vaillantii* (coll. HUBO 8158, 8160, MTB 5434), *Fibroporia gossypium* (coll. HUBO 7952, 7724, 7725), *Antrodia citrina* (coll. HUBO 7715, 7887) and a close related

specimen of *Antrodia* (cf. *citrina*) from France (coll. G. Trichies 7237).

Fibroporia radiculosa from the United States and China and *"Fibroporia radiculosa"* from Europe belong to the *Fibroporia radiculosa* group, presenting a yellow colour of the hymenophore. *Antrodia citrina* and *Antrodia* sp. (cf. *citrina*) from France have a cream to pale yellow colour of fertile surface, while *F. vaillantii* and *F. gossypium* have a whitish colour.

Antrodia citrina shows a cream to pale yellow or lemon olivaceous basidiomata with conspicuous bright yellow rhizomorphs. Basidiospores are broadly ellipsoid to subglobose 4–5 × 3–3.5(3.8) μm (Fig. 1b), differing clearly both from the larger ellipsoid basidiospores of *Fibroporia radiculosa* from North America and those of the Central European taxon. For a complete description see Bernicchia (2005).

Fibroporia radiculosa shows a yellowish to bright orange pore surface with yellowish rhizomorphs. It has been impossible to keep a look of the type of *F. radiculosa*, because the specimen was a mass of powdery material. Basidiospores in *F. radiculosa* from North America are broadly ellipsoid, measuring 6–8 × 3–4 μm (Fig. 1c). As stated by Ryvarden and Gilbertson (1993), specimens of *Poria saxonica* Dörfelt are morphological similar to *F. radiculosa*, and this type of species, showing a cream orange colour of the pore surface (probably darkened by age), differs neither macroscopically nor microscopically from *F. radiculosa* from North America, showing similar bur shorter basidiospores (Fig. 1d).

Specimens from Czech Republic and Italy, previously identified as *Fibroporia radiculosa* (Vampola 1992) or *Antrodia radiculosa* (Ryvarden and Gilbertson 1993; Bernicchia 2005), differ from American specimens of *F. radiculosa* in having smaller basidiospores: (4.8–)5–5.5 × (2.6–)2.8–3(–3.2) μm of an ellipsoid to subcylindrical shape.

Phylogenetic analysis

The complete alignment of the ITS region was 679 bp long, but 30 extreme 5' positions and 59 extreme 3' positions were excluded from all analyses, because they were incomplete for some taxa. The final alignment was 590 including 215 constant characters, 68 parsimony-uninformative variable characters and 307 parsimony-informative characters.

Analyses of the nrDNA ITS dataset were grouped into two main groups: "*Fibroporia"* group and "*Antrodia"* group (Fig. 2). The *Fibroporia* group comprises a well-supported group with a 95% (100% in NJ, data not shown) bootstrap support value and includes *F. gossypium, F. bohemica, F. radiculosa, Antrodia citrina, and Poria saxonica*, as well as the generic type species, *F. vaillantii.* The new species reported here appeared in the well-supported *Fibroporia* clade and differed from other *Fibroporia* species. *Antrodia citrina* is phylogenetically recovered among the *Fibroporia* group.

The *Antrodia* group comprises a series of species belonging to genus *Antrodia*: *A. juniperina*, *A. malicola*, *A. sinuosa*, *A. sitchensis*, *A. variiformis*, as well as *Daedalea* and *Fomitopsis*. Wu et al. (2004) also recovered *Fomitopsis pinicola* in the *Antrodia* group.

Discussion

Fibroporia vaillantii, the type of species of *Fibroporia*, forms with the other rhizomorphic species a well differentiated and supported clade, named *Fibroporia* group, separated from that of *Antrodia s.str.* species (Fig. 2), which confirms the generic delimitation proposed by Parmasto (1968) and suggested also by other molecular studies (Kim et al. 2001).

The type of *Poria saxonica*, identified as *Antrodia radiculosa* by Ryvarden and Gilbertson (1993), and included in the present molecular study, clusters with other specimens of *Fibroporia vaillantii*, and therefore, we can regard *P. saxonica* as a heterotypic synonym of *F. vaillantii*. Some rhizomorphic specimens of *Fibroporia* (HUBO 8160 – Sardinia, Italy; Ostrow 2120 – Bavaria, Germany) clustered also with specimens of *F. vaillantii*.

Antrodia citrina Bernicchia & Ryvarden, was recently described as a new species from Italy (Bernicchia 2005), with the following latin diagnosis: "*Antrodia radiculosa* (Peck) Gilbn. & Ryvarden affinis, sed sporis ellipsoideis, 4–5 × 3.2–3.5(3.8) μm". *Antrodia citrina*, with cream to yellowish lemon basidiomata and bright yellow rhizomorphs, belongs in the complex of yellowish rhizomorphic species, and it is separated from *F. radiculosa* both in basidiospore and pore size. Based on the results suggested by the present molecular study, we propose the following combination: ***Fibroporia citrina*** (Bernicchia & Ryvarden) Bernicchia & Ryvarden **comb**. **nov**. Mycobank MB 516775 –basionym: *Antrodia citrina* Bernicchia & Ryvarden, *Polyporaceae s.l.* Fungi Europaei 10: 98, 2005. A specimen collected from France (G. Trichies 7237), very similar morphologically to *Antrodia citrina*, is confirmed by molecular data to belong to the same species, even if we could appreciate some small differences accepted as an intraspecific variability. Therefore, the distribution of *Antrodia citrina*, up until recently limited to Italy, has now expanded to France.

Fibroporia radiculosa was described as being from the United States, and it is a widely distributed species in North America (Gilbertson and Ryvarden 1986). Vampola (1992) and Ryvarden and Gilbertson (1993) synonymised with *F. radiculosa* some specimens from Central Europe with

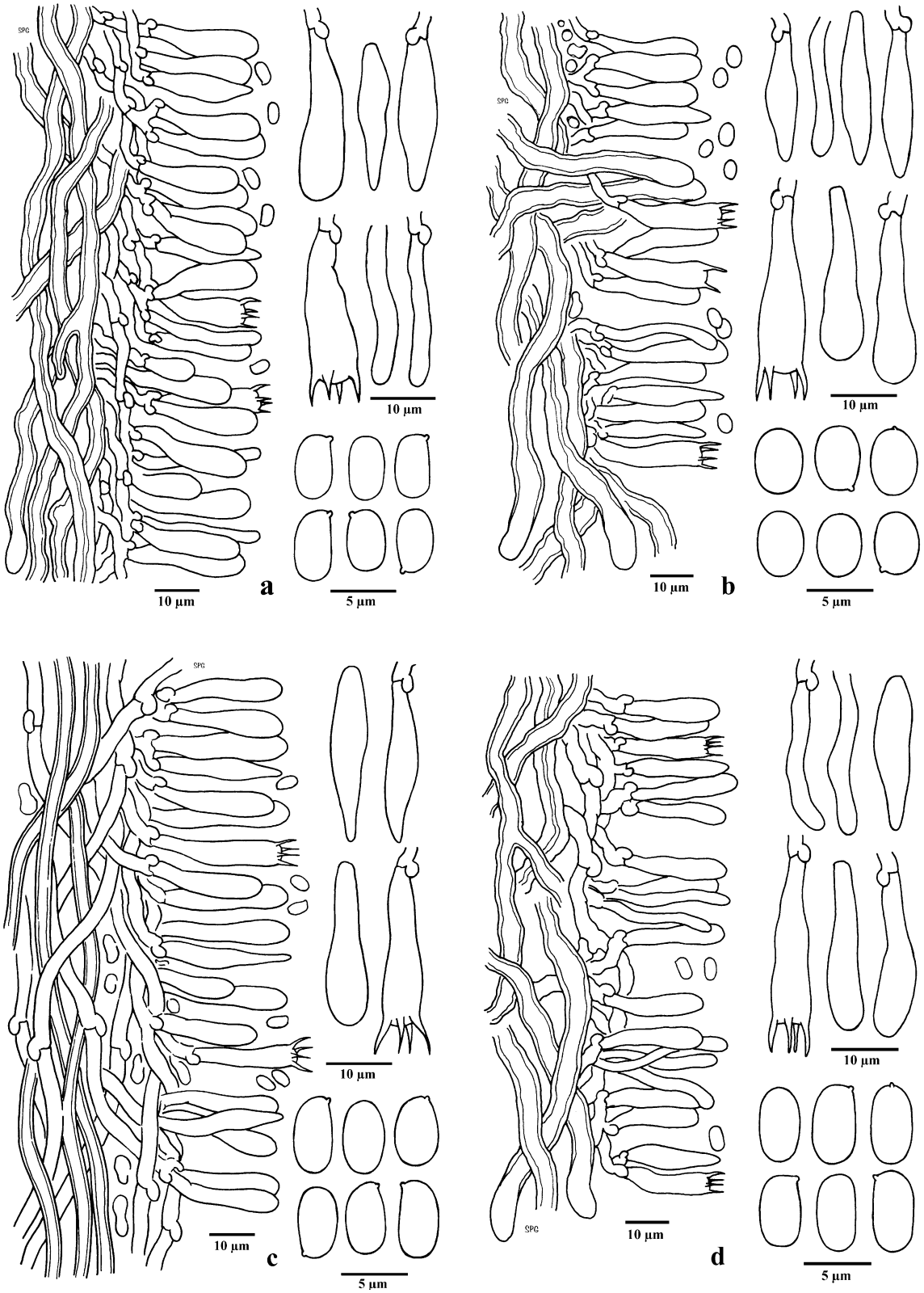

Fig. 1 Hymenial elements showing dimitic hyphal system, basidia, cystidiols and basidiospores. **a** *Fibroporia bohemica* sp. nov., coll. HUBO 7723. **b** *Fibroporia citrina*, coll. HUBO 7887. **c** *Fibroporia radiculosa*, coll. HUBO 8478. **d** type of *Poria saxonica* (= *F. vaillantii*), coll. HUBO 8481

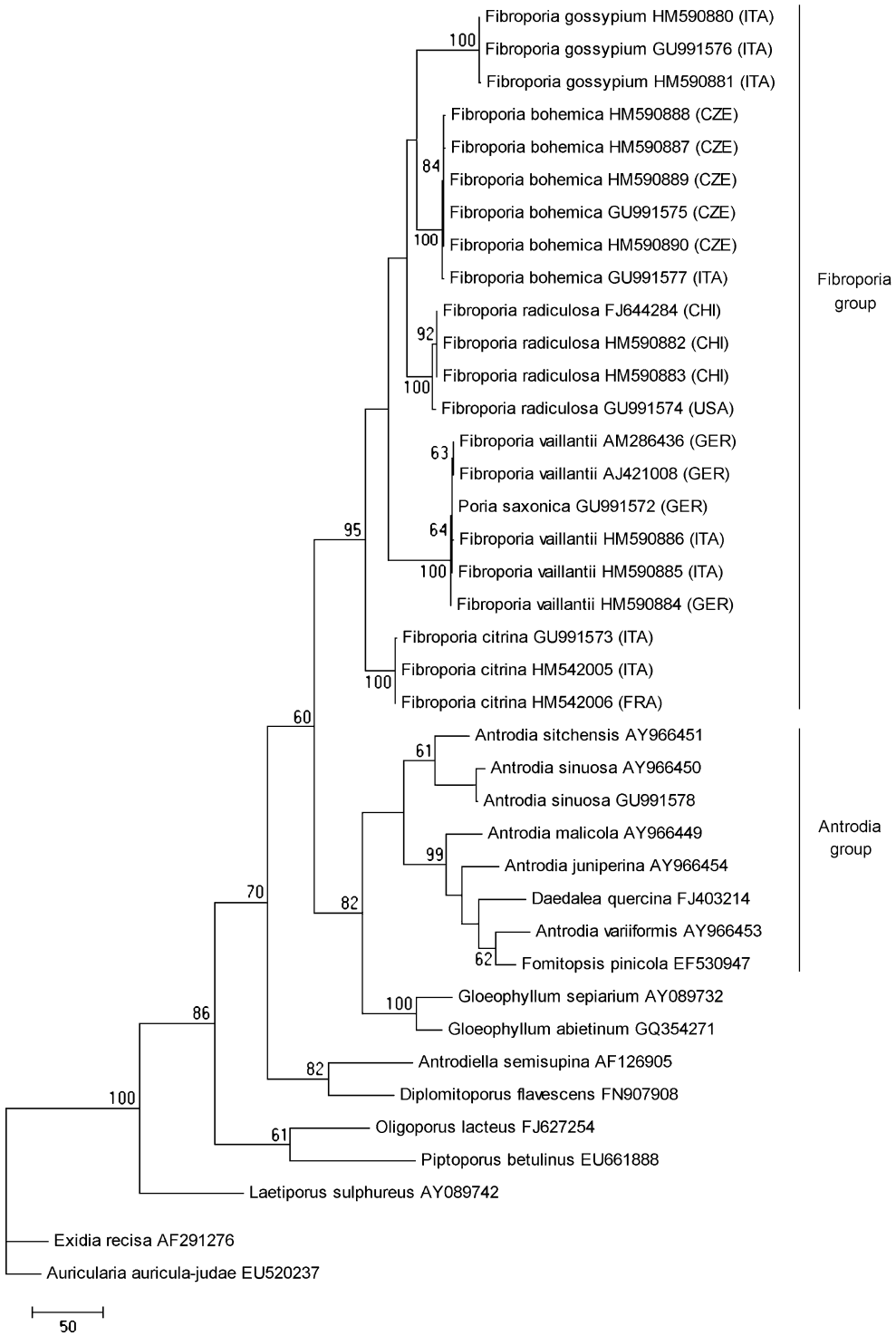

Fig. 2 Maximum parsimony tree (tree length = 1324, CI = 0,5325, RI = 0,7242) conducted in PAUP* based on aligned internal transcribed spacer (ITS) sequences and 5.8S rRNA sequences. Bootstrap percentages higher than 65% based on 1,000 replications are shown next to the branches. The bar indicates the number of expected substitutions per position. In brackets: (CHI) China; (CZE) Czech Republic; (FRA) France; (GER) Germany; (ITA) Italy; (USA) United States of America

similar basidiomata and somewhat similar basidiospores. Effectively, macroscopical characters of European specimens are almost coincident. They differ from *F. radiculosa* mainly in microscopical characters because of shorter and ellipsoid to subcylindrical basidiospores (broadly ellipsoid and wider in North American specimens, 6–8 × 3–4 μm), which was initially taken as an intraspecific variability. The results of the molecular analysis show that specimens from Central Europe (Czech Republic and Italy) form a well-differentiated and supported clade, different from that of the North American and East Asiatic specimens of *F. radiculosa*. It seems obvious that European specimens, previously ascribed to *F. radiculosa*, constitute a species of its own; despite the fact that they are macro- and microscopically closely related, we propose it as a new species as follows:

Fibroporia bohemica Bernicchia, Vampola & Prodi **sp. nov**.

Mycobank MB 516774

Basidiomata annua, resupinata, effusa, membranacea et a substrato separabilis, subalba deinde flava. Subiculum album et tubuli flavi. Pori rotundi vel rotundi-angulati, 2-4 per mm, dissepimentis fimbriatis. Systema hypharum dimiticum: hyphae generativae hyalinae, ramosae, fibulatae, tenuitunicatae, 2–4.5 μm latae. Hyphae skeletales 3–6 μm latae, crassitunicatae, solidae, flexuosae, rariter ramosae, leviter amyloideae. Cystidia desunt. Cystidiola hyalina, cylindrata vel hyphoidea, 20 × 2.5–3 μm. Basidia clavata, hyalina, 4-sterigmatibus, 17–27(–30) × 6–7.5 μm. Basidiosporae hyalinae, leves, subcylindratae vel ellipsoideae, (4.8–)5–5.5 μm longae et (2.6–)2.8–3(–3.2) μm latae, inamyloideae, indextrinoideae, acyanophileae. Ad lignum arborum coniferarum.

Etymology: *bohemica*, from Bohemia, the western part of the Czech Republic.

Holotypus: Czech Republic, Bohemia-Moravian Uplands, Zbilidy (distr. Jihlava), the forest "Pansky les", 13 Km WNW of Jihlava, s.m. 650 m, *Picea abies* – on stump, 27.VIII.2008, leg. et det. P. Vampola (n.29/08), PRM 859138. Isotypus: HUBO 7723.

Habitat: in coniferous forests, on stumps of *Picea abies*, rarely of *Pinus sylvestris*.

Basidiomata annual, non-layered and resupinate, irregularly growing, later quite large, pelliculose, separable, covering up to tens of cm^2 the surface of the substrate, at first smooth and whitish to slightly pinkish, sometimes sulphur-yellow on vertical surface. The tubes are built only later, thick-walled when fresh, sulphur-yellow (similar to those of *Laetiporus sulphureus* (Bull.) Murrill), contrasting with white subiculum on a cross-section, up to 6 mm long, sometimes cascaded. Edges of the tubes finely fimbriate, creating apex-like protrusions on the dissepiments. Pores round to round-angular, 2-4 per mm. Rhizomorphs numerous, cream-coloured, 0.5–2(5) mm thick, penetrating deep into the substrate, representing a remarkable character. They are most abundant in fully developed fruitbodies growing on very decayed wood; in young fruitbodies, or on less decayed wood they are almost unnoticeable. Taste mild to slightly acid when fresh, odour strong and unpleasant, reminding one of a mixture of *Fomitopsis pinicola* (Sw.) P. Karst. and *Gelatoporia pannocincta* (Romell) Niemelä.

Hyphal system dimitic: generative hyphae thin-walled, richly branched, with clamps at septa, 2–4.5 μm in diameter. Skeletal hyphae 3–6 μm in diameter, thick-walled, unbranched or with only few branches. Hyphal walls negative or very slightly amyloid in Melzer's reagent, while are swelling inside using KOH solution, filling up the whole volume of the hypha. Cystidia absent. Cystidiols among the basidia numerous, hyphoid, cylindrical with obtuse apex, about 20 × 2.5–3 μm. Basidia clavate, 4-sterigmate, sometimes slightly constricted in the upper part, basally clamped, 17–27(–30) × 6–7.5 μm. Basidiospores hyaline, smooth, ellipsoid to subcylindrical, (4.8–)5–5.5 × (2.6–)2.8–3(–3.2) μm, inamyloid, indextrinoid, acyanophilous. (Fig. 1a).

Additional specimens examined: as *"Antrodia radiculosa"*, Czech Republic, Bohemia, Zbilidy, 16. August 1991, on *Picea abies*, P. Vampola 6558 (O), and from the same locality and substratum: coll. P. Vampola 336/92, 327/91, 3/04, 6/09/2002, 12/12/2000, coll. BRNM 612444 from Italy.

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