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Grapevine wood decay and lignicolous basidiomycetes

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Summary. Morphological, anatomical and molecular features of the white rot basidiomycetes on grapevine, *Fomitiporia punctata* and *Phellinus igniarius*, were examined. Species specific restriction phenotypes were detected for an enzymatically amplified region of nuclear-encoded ribosomal DNA (including ITS-1, 5.8S, ITS-2). The procedure described allows reliable identification of mycelia not assignable to a particular fruiting body. A sequence analysis of the ribosomal ITS region was performed with twelve strains of *F. punctata* and two strains of *P. igniarius*. The data obtained indicate a subdivision of *F. punctata* into three subgroups, assignable to different geographic regions. Italian isolates from *Vitis vinifera* were found to be closely related to North American isolates from *Salix hindsiana* and *S. lucida* and to be distinct from isolates from Central and Northern Europe.

Key words: esca, *Fomitiporia punctata*, ITS region, *Phellinus igniarius*, RFLP, sequence analysis.

Introduction

A considerable number of lignicolous basidiomycetes are ranked among the most harmful pathogens on wood. The total number of taxa of basidiomycetes living on wood is unknown, but probably numbers of several thousand are good estimates for North America and Europe (Eriksson *et al.*, 1973-88; Gilbertson and Ryvarden, 1986-87; Breitenbach and Kränzlin, 1986-95; Ryvarden and Gilbertson, 1993-94). Only very few taxa have been reported as occurring on grapevine, *Vitis vinifera* L. Such taxa are:

Armillaria mellea (Vahl. : Fr.) Kumm., *Flammulina velutipes* (M.A. Curt. : Fr.) P. Karst., *Megacollybia platyphylla* (Pers. : Fr.) Kotl. & Pouz. (all Kreisel, 1961), *Inonotus hispidus* (Bull. : Fr.) P. Karst. (Ryvarden and Gilbertson, 1993), *Phellinus igniarius* (L.) Quél. (see Chiarappa, 1997), *Fomitiporia punctata* (P. Karst.) Murrill [= *Phellinus punctatus* (Fr.) Pilát; see Larignon and Dubos, 1997], and *Stereum hirsutum* (Willd. : Fr.) Pers. (see Mugnai *et al.*, 1996).

All these species cause a white rot of heartwood and/or splintwood; except for *M. platyphylla* and *S. hirsutum* they mostly live as parasites. Corresponding to the vast majority of lignicolous basidiomycetes, propagation is by airborne basidiospores, spread by mycelial growth occurs in *A. mellea* only. The basidiomycetes most often associated with esca symptoms are *P. igniarius* and *F. punctata* (Chiarappa, 1959, 1997; Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Cortesi *et al.*, 2000), and the present study focuses on these species. On the basis of collections from different parts of Europe and North America, the following problems should be addressed.

i) For a long time *F. punctata* on grapevine was misidentified as *P. igniarius*. Only recently has *F. punctata* been demonstrated as the white rot ba-

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sidiomycete associated with esca (Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Cortesi *et al.*, 2000). Referring to the morphology and anatomy of fruiting bodies, a checklist of distinct characters should be provided for both species.

ii) If fruiting bodies are not available, the identification rests on other features, for example the mycelium that can be isolated from infected wood. Thus, an accurate correlation between fruiting bodies and cultured mycelia is required, and this should be achieved by an analysis of restriction fragment length polymorphisms (RFLP) of an amplified fragment of the nuclear-encoded rRNA genes, i.e., the ribosomal ITS region (ITS-1, 5.8S, ITS-2).

iii) Esca disease is a widespread phenomenon and has been reported, for instance, from the Mediterranean region and North America (Chiarappa, 1959; Mugnai *et al.*, 1999). Isolates of *F. punctata* of different geographic origin and collected from different hosts should be checked for the existence of genetic variability. The relationships between isolates were inferred from sequence data of the ribosomal ITS region.

Materials and methods

Fungal strains and culture conditions

Fomitiporia punctata: 85-74, Germany, on *Salix caprea* L., 4 July 1985; 89-826b, Estonia, on *Sorbus aucuparia* L., 26 August 1989; Dai 2727, Finland, on *Sorbus aucuparia*, 5 October 1997; 87-511, Germany, on *Rhamnus cathartica* L., 11 May 1987; CA 3, Italy, on *Vitis vinifera*, August 1997; VD 24, Italy, on *Vitis vinifera*, August 1997; VD 44, Italy, on *Vitis vinifera*, August 1997; PVFiAs3, Italy, on *Vitis vinifera* L., 1998; 91-42a, USA, on *Salix hindsiana* Benth., 2 April 1991; 91-42b, USA, on *Salix hindsiana*, 2 April 1991; 91-42c, USA, on *Salix hindsiana*, 2 April 1991; 91-42d, USA, on *Salix lucida* (Benth.) Murray, 2 April 1991.

Phellinus igniarius: 85-625, Germany, on *Salix caprea*, 25 June 1985; TN 5758, Finland, *Salix* sp., 25 May 1994.

All cultures were grown on 2% ME medium (2% malt extract, 2% agar, 0.05% yeast extract, in distilled water) at 23°C and 65% humidity in the dark.

DNA isolation

DNA was isolated from fresh and lyophilized mycelium. Isolation was essentially as described by Lee and Taylor (1990). About 50 mg were taken for lyophilized mycelium, about 100 mg for fresh mycelium.

Polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify a portion of the nuclear encoded 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 (90 s), 53°C annealing step (45 s), 72°C primer extension (90 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 (Diagnostic s.p.a. Roche molecular, Mannheim, Germany) was used as a standard.

For RFLPs, the amplified products were extracted with one volume of phenol:chloroform and centrifuged at 10,000 *g* for 15 min. Eighty µl of the aqueous portion were removed, and the DNA was precipitated by the addition of 8 µl NaAc (pH 8.0) and 190 µl of 100% EtOH (>1 h, -20°C). Precipitates were collected by centrifugation (10,000 *g*, 15 min), washed with 750 µl of 70% EtOH, air-dried and resuspended in an appropriate amount of TE buffer.

For sequencing, the amplified products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was suspended in 50 µl Tris-HCl buffer (10 mM, pH 8.0).

Restriction analysis

Usually 10 µl of each DNA solution were digested in 20 µl volumes. The restriction endonucleases *Alu*I, *Csp6*I, *Hae*III, *Hinf*I, *Mbo*I, and *Taq*I (MBI Fermentas, New England Biolabs, Beverly, California, USA) were used according to the manufacturer's instructions. The restriction products were separated on 2.6-3.3% agarose gels; molecular weight marker VI was used as a standard. Results were recorded by photographing gels over a UV transilluminator. The size of the fragments was estimated by comparison with the molecular weight marker.

Sequencing

Fragments were sequenced with the AmpliTaq DNA Polymerase FS Dye Terminator Cycle Sequencing kit (Perkin Elmer, Foster City, USA), using 2 µl of premix, 1 µl of the primers (8 pmol of ITS1 and an equal amount of ITS4), and 3.5 µl of the PCR products. The reactions were set up in 11 µl volumes, overlaid with one 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 the 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 neighbour-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 1,000 replications using the programs Seqboot and Consense.

Results

***Fomitiporia punctata* and *Phellinus igniarius*: fruiting bodies and mycelia**

The availability of fruiting bodies usually allows a reliable identification of *F. punctata* and *P. igniarius* (Table 1). The two taxa are readily distinguished by the shape of the fruiting bodies (Figs 1 and 2), the number of pores per mm, the occurrence of setae in the hymenium, and the size and colour reactions of the basidiospores.

In other respects, the two taxa hardly differ (Table 2; also see Stalpers, 1978). Basically, lignicolous

Table 1. *Fomitiporia punctata* and *Phellinus igniarius*: morphology and anatomy of fruiting bodies.

Fungal characters	*Fomitiporia punctata*	*Phellinus igniarius*
Fruiting body	resupinate	pileate (effused-reflexed)
Pores	6-8/mm	5-6/mm
Setae	none	12-20 x 4.5-6 µm
Basidiospores	6.5-8.5 x 5.5-7 µm	5-6.5 x 4.5-6 µm
	strongly dextrinoid	non-dextrinoid
	strongly cyanophilous	non-cyanophilous

Table 2. *Fomitiporia punctata* and *Phellinus igniarius*: mycelial characters.

Fungal characters	*Fomitiporia punctata*	*Phellinus igniarius*
Mycelial growth (20-25°C)	2.5-5.5 cm in 2 weeks	2-5 cm in 2 weeks
Germination of spores	< 1% in 4 weeks	>20% in 4 weeks
Reproduction	homothallic (intermingling)	heterothallic unifactorial (mating type factors)
Heterokaryon x heterokaryon	demarcation line	demarcation line
Nuclear behavior	oligonucleate	oligonucleate

basidiomycetes are easy to cultivate on ME medium; the optimum growth of *F. punctata* and *P. igniarius* is between 20 and 25°C. Appearance of cultivated mycelia is variable for both taxa (Fischer, 1987; for additional mycelial characters, see Stalpers, 1978). Basidiospores of *F. punctata* are difficult to germinate; on ME medium a pH between 5.0 and 5.5 as well as a certain period of stratification may facilitate the germination process. In contrast, spores of *P. igniarius* easily germinate. *Fomitiporia punctata* is a homothallic species, and sibling single spore isolates intermingle in pairing tests. In the heterothallic species *P. igniarius* such mating reactions are regulated by a two-allelic mating type factor. In both taxa, pairings between genetically different heterokaryons consistently result in the formation of a dark line of demarcation. The nuclear behaviour is identical as well, and both homokaryotic and heterokaryotic mycelia are oligonucleate, i.e., between two and eight nuclei can be observed per hyphal segment (Fischer, 1996).

Restriction fragment length polymorphisms

The size of the amplified PCR product, defined by the primer combination ITS1 and ITS4, was found to be approximately 780 base pairs for both taxa. After application of restriction enzymes, in several cases the sum of the fragment sizes was not identical with the size of the undigested PCR product, probably on account of undetected double bands. For all enzymes tested, *F. punctata* restriction phenotypes were different from *P. igniarius*. Most obvious results were obtained after application of *Alu*I and *Csp*6I. For *Alu*I two fragments of about 580 and 200 base pairs were detected for *F. punctata*, three fragments, with sizes of about 280, 190 and 70 base pairs, for *P. igniarius*. For *Csp*6I, the fragments were about 500, 140 and 80 base pairs for *F. punctata* and there was no restriction site for *P. igniarius*.

Sequencing

The total length of the alignment was 772 nucleotides. The aligned region comprises a small portion of the flanking 18S and 28S rRNA genes, the complete ITS-1 and ITS-2 region, and the complete 5.8S rRNA gene.

The neighbour-joining method produced a phylogenetic tree exhibiting a certain genetic divergence among the isolates of *F. punctata*, which was correlated with different geographic regions (Fig. 1). Sequence variability was mostly due to insertions and/or deletions within the ITS-1 and ITS-2 region. Three subgroups were formed, corresponding to central and northern Europe (four strains from three host genera), Italy (four strains from grapevine), and North America (four strains from *Salix*). The Italian material appeared as a well supported sister group to the North American isolates (bootstrap value 95%) and was separated from the remaining European material. *Phellinus igniarius* was clearly divergent from *F. punctata* (bootstrap value 100%).

Discussion

Fomitiporia punctata is generally considered a harmful pathogen in the Mediterranean vine-growing regions (France, Greece, Italy, Portugal, and Spain); in other parts of Europe, however, the species does not play an important role as a parasite (Jahn, 1963 and 1967; Ryvarden, 1978).

The character states listed in Table 1 allow a reliable identification of fruiting bodies of *F. punctata* and *P. igniarius*. For unknown reasons, fruiting bodies of *F. punctata* have only rarely been observed within vineyards. Outside of vineyards, the species seems to be quite common in the Mediterranean area, for instance on *Olea europaea* (Plank, 1980). Basically, *F. punctata* exhibits a wide distribution and is found on many different hardwood genera (Jahn, 1967; Breitenbach and Kränzlin, 1986; Erkkilä and Niemelä, 1986; Gilbertson and Ryvarden, 1987; Ryvarden and Gilbertson, 1994).

The identification of cultures of *F. punctata* rests mainly on molecular data. In the present study, RFLP data of the enzymatically amplified ribosomal ITS region produced the information desired. After application of several restriction enzymes, for example *Alu*I and *Csp*6I, unequivocal phenotypes were obtained for *F. punctata* and *P. igniarius*. Under favourable conditions, the typing of collections can be performed within a few weeks, most of which is required for the cultivation of mycelia. In any comparative study of restriction phenotypes, special attention should be paid to the possible existence of intraspecific variation. Previous reports on the rRNA fragment chosen for this study have dem-

Fig. 1. Fruiting body of *Fomitiporia punctata* on sorb.

Fig. 2. Fruiting body of *Phellinus igniarius* on willow.

onstrated little or no variation between strains of a given species (Hibbett and Vilgalys, 1991; Fischer and Wagner, 1999).

The sequence data show the examined strains of *F. punctata* to be divided into three subgroups (Fig. 3). These subgroups correlate well with the geographic origin of the strains. The tree topology points to a close relationship between the Italian isolates, collected from grapevine, and the North American isolates, collected from *Salix* (Fig. 1). Strongly supported by a bootstrap value of 100%, the isolates of Central and Northern Europe are positioned as a separated clade. A close relationship between Italian and North American isolates is supported by unpublished observations on the lignin decomposing enzyme laccase: preliminary results demonstrate a high level of extracellular laccase produced by these strains, whilst distinctly less enzyme was found for the remaining European strains.

The putative connection between Italian and North American strains might be a result of the transfer of (infected) North American rootstocks to Europe as a consequence of the epidemic of Phylloxera, which devastated large parts of European vine-growing regions during the last century. If this hypothesis is correct, North American strains from grapevine should be genetically more or less identical with those from *Salix*. With the data at hand, another question remains open: no sequence data

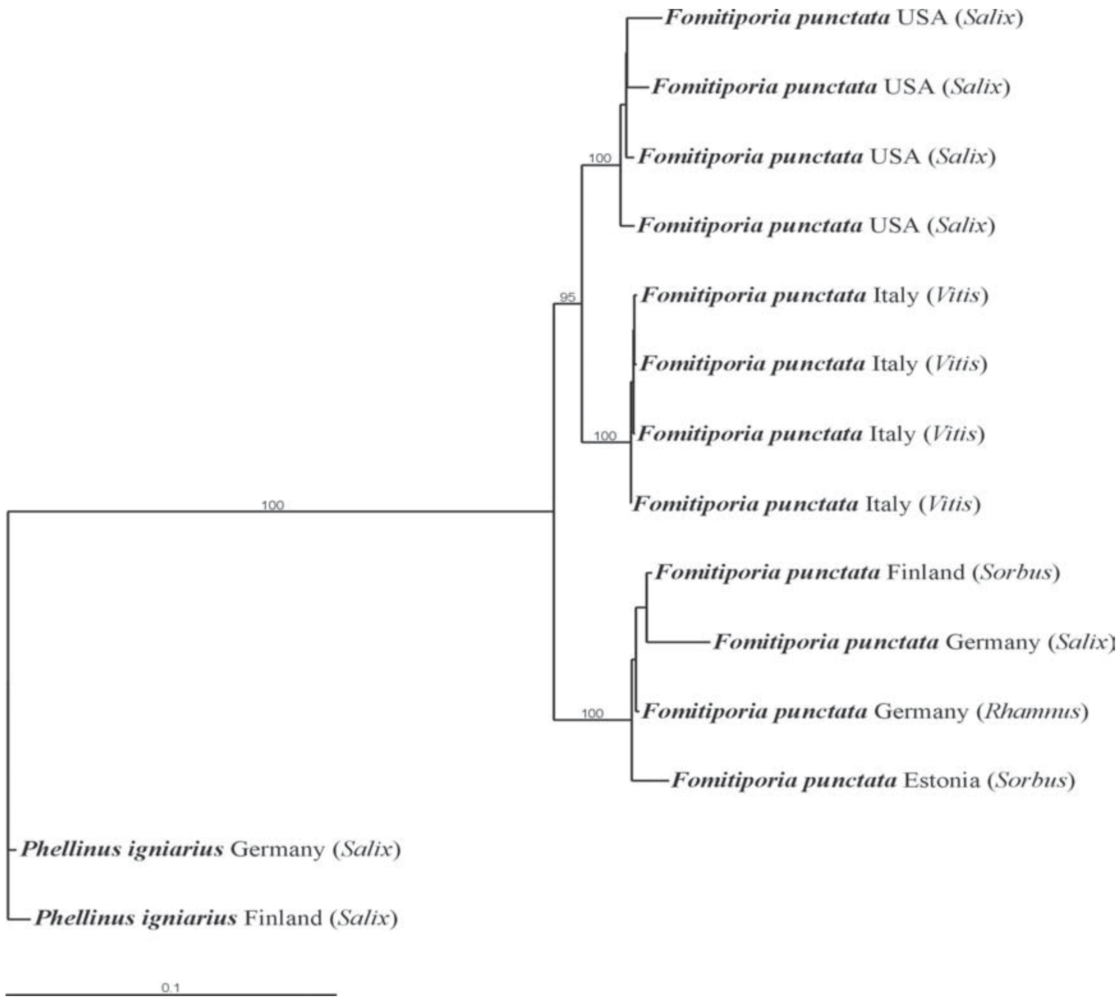

Fig. 3. Relationships between isolates of *Fomitiporia punctata* and *Phellinus igniarius* inferred from the nuclear ITS region (ITS-1, 5.8S, ITS-2) using the neighbour-joining method. 1,000 bootstrap replications.

are available for Mediterranean isolates of *F. punctata* from non-grapevine hosts. Genetically, such isolates may either belong to the Italian strains tested in this study or to the Central and Northern European strains. In the first case, infection of grapevine could be induced by any fruiting body occurring outside of vineyards; in the second case, infection processes are more likely to be initiated by fruiting bodies already existing within vineyards. These problems are currently under investigation with a larger number of strains.

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