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The occurrence of *Phellinus torulosus* in Apulia and Basilicata (Southern Italy): identification of isolates by morphologic, microscopic, and molecular means

Grazia Campanile and Nicola Luisi

Dipartimento di Biologia e Patologia Vegetale, Università degli Studi,
Via G. Amendola 165/A, 70126 Bari, Italy

Summary. Basidiomes of *Phellinus torulosus* were found in 9 oak woods in Apulia and Basilicata (Southern Italy); from these basidiomes 138 isolates of *P. torulosus* were obtained and identified by their morphologic, microscopic, and molecular characteristics. Based on the type of aerial mycelium (fluffy, cottony or powdery) and its growth, 9 morphotypes were identified. The morphology of the cultures was not correlated with the microscopic character of the 9 morphotypes. Molecular analysis, such as intergenic transcribed spacers-restriction fragment length polymorphism (ITS-RFLP) and sequencing of the ITS region, confirmed the results obtained with microscopy analysis.

Key words: *Phellinus torulosus,* white rot, ITS region, RFLP, sequence analysis.

Introduction

The wood of old trees is frequently subject to rot from biotic agents such as bacteria, fungi and insects. The most important degrading microorganisms are the wood-rotting fungi, generally referred to as decay fungi, which include the basidiomycetes (brown and white rots) and the ascomycetes (soft rot) (Solveig *et al.,* 2002). Wood decay is of considerable importance, both economically, due to the loss of ligneous material it causes, and ecologically, due to adverse changes in wood composition (Fischer and Wagner, 1999). Until recently, decay fungi in wood were detected by cultivating the fungi on selective media and by biochemical, chemical, and immunological analyses (Stalpers, 1978; Johannesson and Stenlid, 1999). However, these methods are time-consuming, laborious and complex, and even so, most are not sensitive enough to detect decay in the early stages (Fischer, 2000; Annesi *et al*., 2003).

Molecular approaches, such as polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), are potential means for early detection of decay fungi (Bruns *et al*., 1991; Schulze *et al*., 1997; Chillali *et al*., 1998; Edel, 1998; Mazzaglia *et al*., 2001). Such approaches are based on the enzymatic amplification of suitable DNA regions, usually located within the nuclear encoded ribosomal DNA (rDNA). The amplified fragments span the entire region of the intergenic transcribed spacers (ITS), as well as the 5.8S rRNA gene, which are then digested with restriction endonucleases, resulting in specific restriction phenotypes (Fischer and Wagner, 1999; Fischer, 2000).

Corresponding author: N. Luisi
Fax. +39 080 5442906
E-mail: luisin@agr.uniba.it

Phellinus torulosus (Pers.) Bourd. *et* Galz. is a white rot fungus on wood that also decays heartwood in roots and lower stems and invades and kills cortical tissue (Kotlaba, 1975; Panconesi *et al.,* 1994). Its range of distribution is limited to areas with higher temperatures (Gilbertson and Burdsall, 1972). *P. torulosus* infects a very wide range of hosts, which it frequently kills, although the time required for tree death varies. In Europe, *P. torulosus* occurs both as a parasite and as a saprobe on many broadleaf trees (Fischer and Bresinsky, 1992) but only rarely on conifers. In the United States of America, however, *P. torulosus* seems to be restricted exclusively to conifers.

The basidiomes produced by this fungus are perennial, pileate, and sessile and grow to considerable size (15–30 cm in width and 1–5 cm or more in thickness). They are found at the stem base of a number of tree species, both dead and living (Kotlaba, 1975; Gilbertson and Ryvarden, 1987; Panconesi *et al.,* 1994). Recently, based on the phylogenetic analysis of sequence data of a region of the large subunit of the nuclear-encoded ribosomal DNA (nLSU rDNA), Wagner and Fischer (2001) suggested a subdivision of the genera *Phellinus s. lat*. and *Inonotus s. lat*. into five subunits each. The following genera are accepted: *Phellinus s. str. Porodaedalea, Fomitiporia, Fuscoporia, Phellinidium, Phylloporia,* and *Inonotus s. str. Inocutis, Mensularia*, *Pseudoinonotus* and *Inonotopsis*. *P. torulosus* has been re-combined as *Fuscoporia torulosa* (Pers.) T. Wagner and M. Fischer. In this work we used the traditional nomenclature: "*P. torulosus* Bourd. *et* Galz." because the work of Wagner and Fischer (2001) is essentially restricted to European poroid *Hymenochaetales*. More detailed studies on non-European taxa are desirable.

The aim of this study was to evaluate the frequency of occurrence of *P. torulosus* in 13 oak woods in Apulia and Basilicata, to study the morphological features of the fungus cultured *in vitro*, and to identify the fungal isolates collected using microscopic and molecular techniques such as ITS-RFLP and sequencing of the ITS region.

Materials and methods

Sampling strategy

The occurrence of *P. torulosus* was estimated by sampling 13 mixed oak woods in Apulia and Basilicata, the latter including Monte Vulture in autumn 2000. The Apulia region was subdivided into 5 areas: Salento, South-East Murge (SEM), North-West Murge (NWM), Lower Apennine Dauno, and Gargano (Fig. 1; Table 1). In each of the woods, 2 causal and orthogonal transepts were laid out, along each of which about 150 trees or stumps of trees were examined for basidiomes. Basidiomes were collected from trees at least 25–35 m apart.

Microscopic and morphologic analyses

Phellinus torulosus was isolated from the basidiomes by placing small pieces on a selective medium for basidiomycetes (Kuhlman and Hendrix, 1962). After 6 days of incubation at 22±3°C in the dark, hyphal tips were transferred to 2% malt extract agar (MEA). This procedure was repeated as often as necessary to obtain pure isolates (Intini, 1987; Motta *et al*., 1996). One hundred and thirty eight isolates were identified as *P. torulosus* by comparing with the characteristics of this fungus as described in the literature (Stalpers 1978; Bernicchia, 1990).

For morphological studies of *P. torulosus*, Petri dishes containing MEA were inoculated with a

Fig. 1. Map of Apulia and Basilicata, Southern Italy, showing the localities (left column, 1–13) and areas of North-West Murge (NWM), South-East Murge (SEM), Salento, Gargano, Lower Appennine Dauno and Monte Vulture where basidiomes of *Phellinus torulosus* were sampled.

Table 1. Frequency of occurrence (%) of host species with *Phellinus torulosus* basidiomes in the different localities and oak woods sampled.

Oak wood	Host species	Frequency (%)
Apulia		
Salento		
- Scorrano, "Pecorara"	*Quercus ilex*, *Arbutus unedo*	11
- Frigole, "Cervalura"	*Q. ilex*, *Viburnum tinus*	12
South-East Murge		
- Martina Franca, "San Paolo"	*Q. trojana*, *Crataegus monogyna*	17
- Mottola, "Terzi"	*Q. trojana*	5
North-West Murge		
- Cassano Murge, "Mesola"	*Q. coccifera*	2
- Corato, "Pedale"	*Q. pubescens*, stumps of *Quercus* sp.	17
- Andria, "Tenuta Cocevola"	*Q. pubescens*, *Pistacia lentiscus*	10
- Ruvo di Puglia, "Scoparello"	*Pinus halepensis*, *Cupressus sempervirens*, *C. monogyna*, *A. unedo*, *P. lentiscus*, stumps of *Quercus* sp.	21
Lower Appennine Dauno		
- Biccari, "Valle in Vincoli"	-	0
- Bovino, "Acquara"	-	0
Gargano		
- Vico del Gargano, "Foresta Umbra"	-	0
- Monte Sant'Angelo, "Quarto"	-	0
Basilicata		
Monte Vulture		
- Atella, "San Martino"	Stumps of *Quercus* sp., *Pistacia terebintus, Cornus mas*, *C. monogyna*	5
Total		100

piece of mycelium and incubated in diffused light at 22±3°C and examined at 5-day intervals. The isolates were scored for type and growth of aerial mycelium, radial growth, and colony colour.

DNA isolation

Fungal colonies were grown on cellophane sheets placed on MEA for 6 days in diffuse light at 22±3°C. Mycelium was collected, transferred to Eppendorf® tubes, and ground under liquid nitrogen. DNA extraction and purification was carried out according to Murray and Thompson (1980). The concentration and purity of the extracted DNA was determined by spectrophotometric measurement at 260 and 280 nm.

RFLP analysis

The ITS region of the 138 isolates was amplified with primers ITS1 and ITS4B, as described by White *et al*. (1990). The nucleotide sequence of ITS1 was 5'-TCCGTAGGTGAACCTGCGG-3' and of ITS4B 5'-CAGGAGACTTGTACACGGTCCAG-3' (Medical Genenco, Florence, Italy). PCR was performed in 25-μl volumes, each containing 50 ng of genomic DNA; 1.5 U of *Taq*-polymerase (Promega, Madison, WI, USA); 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100; 10 pmol μl^{-1} of primer (Medical Genenco), 200 μM of each dNTP (Promega) and 1.5 mM of $MgCl_2$. Reactions were carried out in a thermal cycler (Bio-Rad laboratories, Hercules, CA, USA) programmed as follows: 3 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, 3 s at 72°C; and a conclusive extension phase of 5 min at 72°C.

The ITS-PCR products were digested with *Cfo*I, *EcoR*I, *Msp*I, *Hinf*I, *Hind*III, *Alu*I and *Taq*I (Promega), according to manufacturer's instructions. The amplification products and restriction fragments

were separated on 2% agarose gels which were stained with ethidium bromide and photographed under UV light using the Gel Doc 2000 system (Bio-Rad Laboratories). The molecular size was estimated using the 100 bp DNA Ladder (New England Bio-Labs, Beverly, CA, USA). Each reaction was repeated three times to check reproducibility of the bands.

Sequencing of the ITS regions

The ITS of isolates P7, P59, P90, P125, P137, P142, P134, P115, P77 and P11 was sequenced by MWG-Biotech (Florence, Italy). The sequences were aligned using the ClustalX program compared with the GenBank database using BLAST (Basic Local Alignment Search) analysis. For each fungal isolate the entire procedure of DNA extraction, amplification and sequencing was repeated twice.

Results

Frequency of *P. torulosus* basidiomes

The frequency of occurrence of *P. torulosus* basidiomes in the various woods is summarized in Table 1. *P. torulosus* basidiomes were found in 9 of the 13 woods. No basidiomes were found in the Gargano area or in the Lower Appennine Dauno area even though here the likelihood of infection was high since this fungus produces basidiomes during the last stage of infection. The greatest occurrence of basidiomes was at Scoparello (21%), San Paolo and Pedale (17%). The main host species were oaks: *Quercus pubescens*, *Q. ilex*, *Q. trojana*, and oak stumps. Basidiomes were also frequently found on *Crataegus monogyna*, *Arbutus unedo*, *Viburnum tinus*, *Pistacia lentiscus* and *Pinus halepensis,* and more rarely at the base of *Cupressus sempervirens, Cornus mas,* and *Pistacia terebintus* (Table 2). More than 200 fruiting bodies were collected from the 13 oak woods, yielding 138 isolates of *P. torulosus* (Table 3).

Microscopic and morphologic analysis of *P. torulosus* isolates

Microscopic analysis of vegetative mycelium revealed 2 types of hyphae: skeletal and generative. The skeletal hyphae were yellow to brownish yellow with thick walls ranging from 2.5 to 5 μm; the hyphae were rarely branched, pigmented (Fig. 2A), with terminal swellings (Fig. 2B) encrusted,

Table 2. Number and frequency (%) of *Phellinus torulosus* basidiomes collected from each host species.

Host species	Basidiomes	
	Number	Frequency (%)
Quercus pubescens	40	16
Q. ilex	40	16
Q. trojana	38	15
Stumps of *Quercus* sp.	36	15
Crataegus monogyna	28	12
Arbutus unedo	14	6
Pistacia lentiscus	10	4
Viburnum tinus	10	4
Pinus halepensis	11	4
Cupressus sempervirens	8	3
Q. coccifera	8	3
Cornus mas	2	1
Pistacia terebintus	2	1
Total	247	100

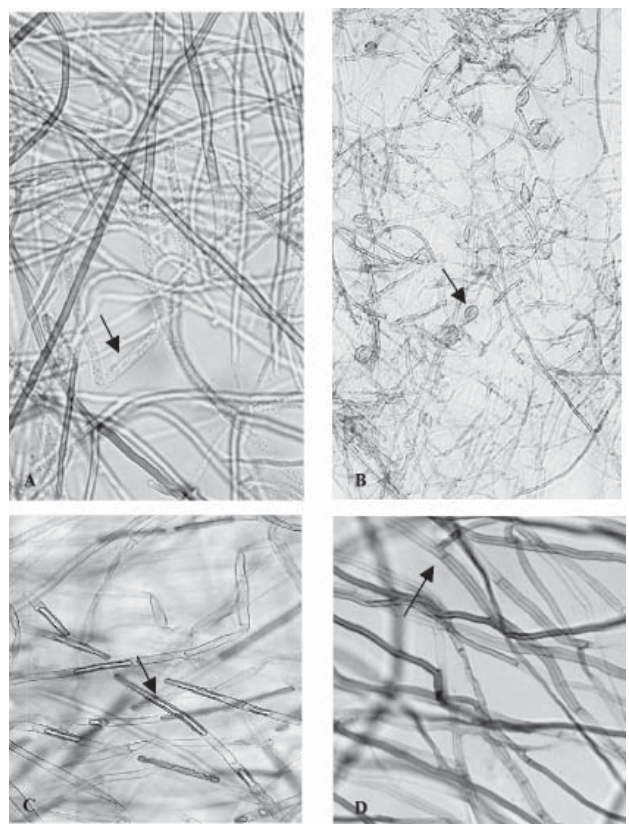

Fig. 2. Skeletal hyphae (40×): A, pigmentate; B, with swellings; C, encrostate; D, generative hyphae with septa (40×).

(Fig. 2C), and lacking in clamps. The generative hyphae were septate, branched and with thin, 2 to 3 μm walls (Fig. 2D). Basidiospores obtained from fruiting cultures were ovoid (4–6×3–4 μm). Isolates of *P. torulosus* showed high variability in culture, especially in diameter growth, and type of the aerial mycelium. After several transfers in culture the isolates maintained their phenotypic traits. Based on the type of mycelium (fluffy, cottony or powdery) and its growth (moderate, abundant or very abundant), 9 morphotypes were identified to which the following letters were assigned: A, B, C, D, E, F, G, H and I (Fig. 3; Table 4). Morphotypes A, B, and C presented fluffy aerial mycelium with moderate, abundant, and very abundant mycelium, respectively; these 3 morphotypes occurred at a frequency of 9, 24, and 10%, respectively. Morphotypes D, E, F and G presented cottony aerial mycelium but with varying types of growth: moderate in D, abundant in E; while in F the mycelium grew only at the centre of the colony, and in G it grew in the peripheral area. Morphotype H had fluffy mycelium in the centre and cottony mycelium in the peripheral area. The frequencies of occurrence of the latter morphotype were 11% for D, 9% for E, 12% for F, 7% for G and 7% for H. Morphotype I, characterized by powdery aerial mycelium, had an 11% frequency. All colonies except those from morphotypes A, D and F, had the same average diameter (85 mm) after incubation for 30 days at 22±2°C in diffuse light. Colony colour was dark brown in morphotypes A, C, D, G, and I and creamy in morphotypes B, E, F, and H.

Restriction analysis and sequencing of the ITS region

Using primers ITS1 and ITS4B, each isolate of *P. torulosus* yielded an amplification product of approximately 700 bp (Fig. 4). Only fragments larger than 100 bp were scored because smaller fragments were difficult to score clearly and tended to be obscured by the prominent "primer dimer" band produced during amplification. For all enzymes tested, restriction sites and phenotypes

Table 3. Isolates of *Phellinus torulosus* (P) used in this study.

Host	Isolates	Origin
Quercus pubescens	P69, P72, P74, P75, P76, P77, P78, P81, P82, P83, P84, P87, P88, P90	North-West Murge
Q. ilex	P57, P92, P94, P95, P98, P99, P100, P101, P102, P108	Salento
Q. trojana	P6, P8, P12, P16, P19, P22, P24, P25, P27, P28, P32, P38, P39, P40, P42, P43, P49	South-East Murge
Stumps of *Quercus* sp.	P31, P50, P51, P52, P53, P54, P55, P56, P104, P109, P110, P122, P124, P125 P126, P137, P131, P134, P142	North-West Murge Monte Vulture
Crataegus monogyna	P1, P3, P4, P5, P7, P9, P11, P15, P26, P144 P29, P30, P34, P37, P65, P66, P71, P80, P93 P41, P46, P59, P61	South-East Murge North-West Murge Monte Vulture
Arbutus unedo	P86, P91, P96, P97, P111, P113, P114 P103, P115, P118, P135	North-East Murge Salento
Pistacia lentiscus	P89, P136, P138, P139	North-East Murge
Viburnum tinus	P117, P119, P120, P121, P123, P129, P140	Salento
Pinus halepensis	P35, P36, P112, P116, P127, P128, P130, P132, P133, P141, P143	North-East Murge
Cupressus sempervirens	P58, P60, P62, P63, P64, P67, P68, P70, P73	North-East Murge
Q. coccifera	P2, P10, P13, P14, P17, P18, P20, P21	South-East Murge
Cornus mas	P44, P47, P48	Monte Vulture
Pistacia terebintus	P23, P33	Monte Vulture

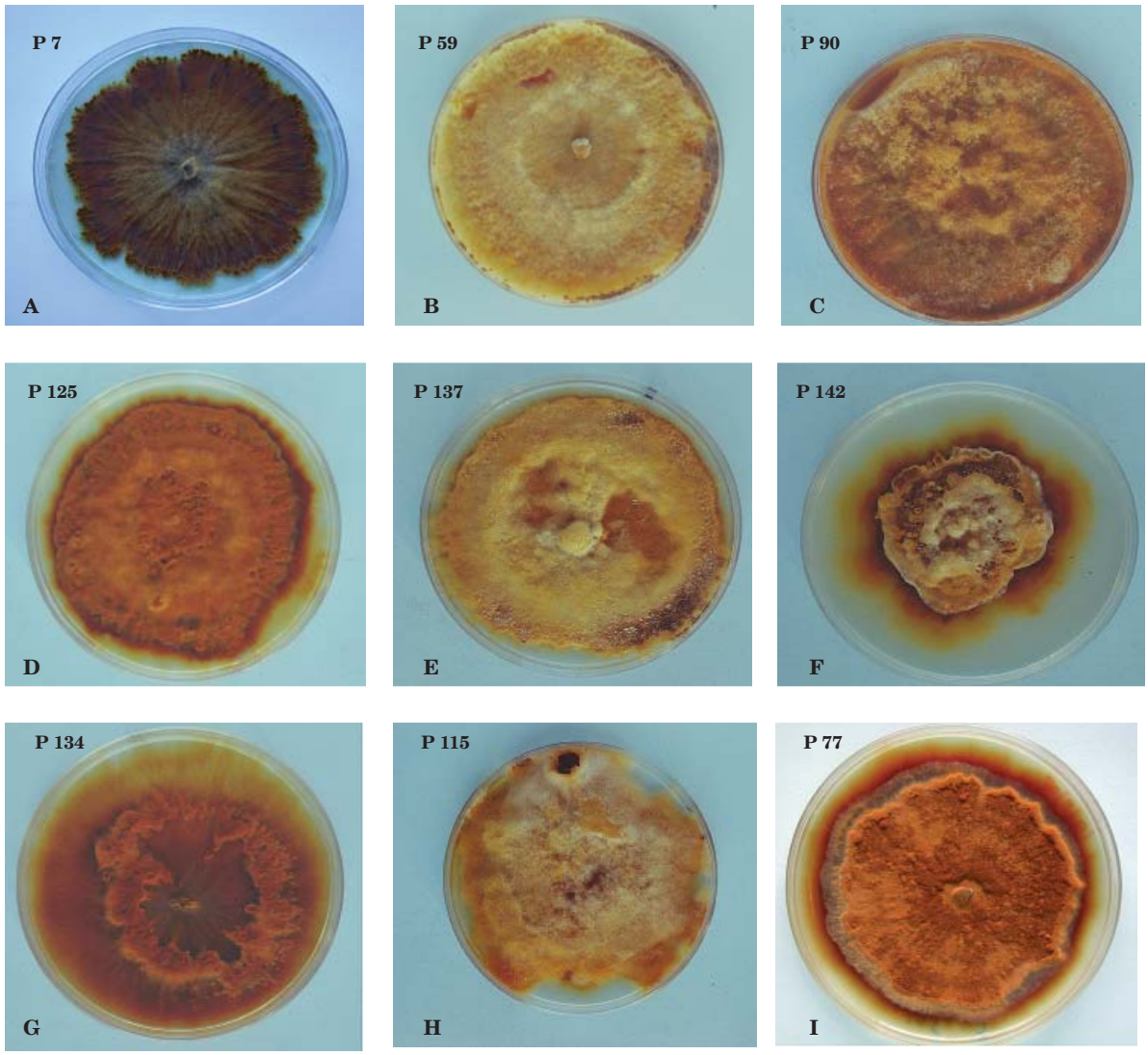

Fig. 3. Morphotypes A, B, C, D, E, F, G, H and I identified among colonies of *Phellinus torulosus* (P). The isolate code of each colony is shown in the upper left-hand corner of each picture.

within the ITS region of *P. torulosus* isolates were similar; no intraspecific variation was observed. Table 4 shows the RFLP fragments after digestion with restriction enzymes. Representative banding patterns from two different enzymes, *Taq*I and *Cfo*I, are illustrated in Figure 5. The total length of the alignment was 656 nucleotides. Alignment of ITS sequences did not reveal any intraspecific polymorphisms. BLAST analysis excluded the presence of identical sequences among available gene bank databases. Higher levels of sequence homology were found with *Phellinus tremulae*, (accession number AF200240), *P. nigrolimitatus* (accession number AJ289627) and *P. laevigatus*, (accession number AF058765). In the future, the ITS sequences could be used to design pairs of specific primers for the detection of *P. torulosus*.

Table 4. Morphotypes identified among *Phellinus torulosus* isolates.

Isolate	Morphotype[a]
P98, P53, P54, P109, P91, P111, P103, P117, P60, P102, P110, P60	A
P69, P75, P76, P87, P57, P92, P99, P100, P8, P22, P16, P24, P27, P28, P50, P51, P104, P7, P9, P15, P29, P30, P86, P96, P97, P113, P10, P17, P119, P128, P129, P132, P23	B
P83, P84, P42, P43, P32, P71, P59, P114, P136, P73, P2, P44, P40, P59	C
P81, P94, P95, P31, P124, P134, P11, P65, P66, P93, P118, P47, P33, P63, P64, P89	D
P72, P88, P52, P125, P138, P120, P140, P35, P36, P58, P101, P108	E
P55, P3, P4, P5, P46, P121, P123, P127, P133, P141, P70, P13, P18, P130, P131, P137, P139	F
P6, P38, P39, P142, P1, P26, P34, P74, P112, P116	G
P82, P90, P19, P61, P115, P20, P21, P14, P62	H
P77, P78, P12, P25, P49, P144, P41, P135, P143, P67, P68, P48, P37, P56, P80	I

[a] A, B and C, fluffy aerial mycelium with moderate, abundant, and very abundant mycelium, respectively. D, E, F and G, cottony aerial mycelium but with different growth habit: D, moderate; E, abundant; F, restricted only to the centre of the colony; G, restricted to the peripheral area. H, fluffy mycelium in the centre and cottony mycelium in the peripheral area. I, powdery aerial mycelium.

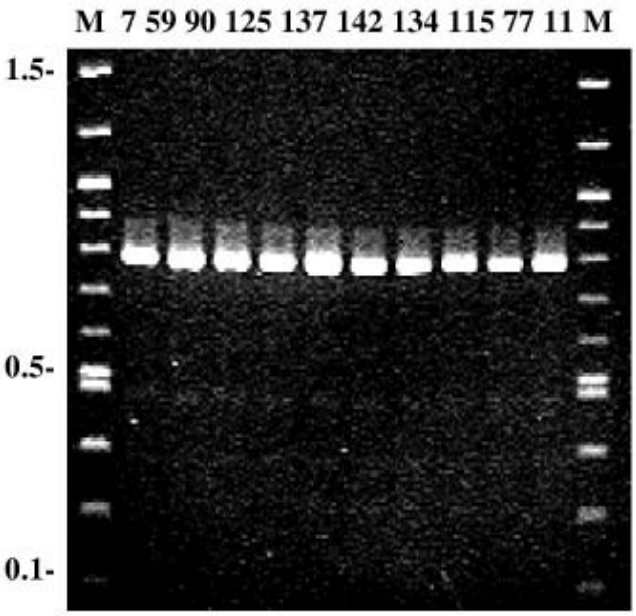

Fig. 4. ITS amplification products of different *Phellinus torulosus* isolates using the primer pair ITS1 and ITS4B. In the migration lanes the following are shown from left to right: M, 100 base pairs marker ladder and code of the *P. torulosus* isolates tested.

Table 5. Restriction fragment analysis of the rDNA spacer digested with different enzymes in *Phellinus torulosus* isolates.

Enzyme	Fragment size (bp)		
*Hind*I	360	200	140
*Taq*I	300	260	170
*Alu*I	400	300	
*EcoR*I	400	300	
*Cfo*I	390	310	
*Hind*II	700		
*Msp*I	700		
*Dra*I	700		

Discussion

The occurrence of *P. torulosus* in oak woods located in the bioclimatic mid- and upper Mediterranean plains of Apulia confirmed the existing data on the spread of this fungus (Kotlaba, 1975). *P. torulosus* is a pathogen and saprobe on many broadleaf trees in Italy (Panconesi *et al.,* 1994; Luisi *et al.,* 1998). Basidiomes of this fungus occur with varying frequency both at the base of living trees and on the stumps of dead trees in the oak woods

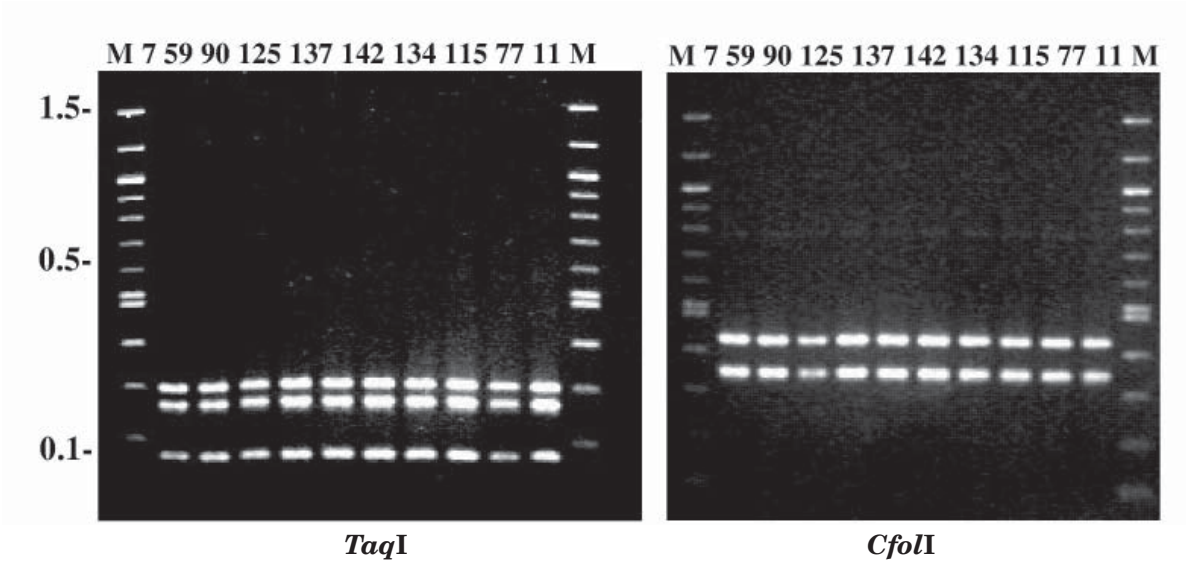

Fig. 5. Restriction profiles obtained with the enzymes *Taq*I, *Cfo*I by amplification of the ITS region of rDNA of *Phellinus torulosus* isolates. In the migration lanes the following are shown from left to right: M, 100 base pairs marker ladder and code of the *P. torulosus* isolates tested.

studied. In this study it appeared that *P. torulosus* entered the trees through wounds on the stumps or on large exposed roots. Attacks of *P. torulosus* in the areas studied were attributable to various stress factors that have affected the woods over the past 10 years: intense and lengthy periods of drought, low fertility, tree senescence, and improper silvicultural practices (Giordano, 1993; Vannini *et al.,* 1993; Luisi *et al.,* 1995; Luisi and Lerario, 2000). The greatest concentration of *P. torulosus* basidiomes was found in aged and abandoned oak coppices because the aging phase of the trees had not been preceded by the required operations of thinning and conversion to high forest. These woods require appropriate management based on periodical cutting and low-intensity pasturing to minimize the risk of further disrupting their precarious equilibrium (Sicoli *et al.,* 1993, 1998). *P. torulosus* should be kept under close watch since it could become a serious risk to broadleaf trees in the Mediterranean-type ecosystem (Panconesi *et al.,* 1994).

Phellinus torulosus cultures *in vitro* revealed a high degree of morphologic variability. Isolates were classified into 9 morphotypes which occurred at different frequencies. Morphologic variations among *P. torulosus* isolates have been reported by other authors. Fischer and Bresinsky (1992) found 2 types of mycelia, a bleaching type (B-type) and a staining type (S-type), in *P. torulosus* isolates from Europe and the Canary Islands. The B-type was characterised by well-developed aerial hyphae, rapid growth (approximately 2–2.5 cm wk^{-1}), and weak pigmentation of the medium. The S-type was characterised by appressed hyphae with sparse aerial development, slow growth (approximately 0.8–1.1 cm wk^{-1}), and deep reddish-brown pigmentation of the medium. In a study on biological and epidemical aspects of *P. torulosus* in Southern Italy, Luisi *et al*. (1998) observed considerable variations in colony morphology, colour, and aerial mycelium growth. A recent study by Campanile *et al.* (2004) also found genetic and morphologic variability of *P. torulosus* isolates in some oak woods of Southern Italy; there were no correlations between random amplified polymorphic DNA (RAPD)-PCR banding and morphotype. The isolates showed the same microscopic traits, and no differences in morphology were found confirming the findings of other authors (Wronski *et al.,* 1997; Cherrab *et al.*, 2000) for *Verticillium dahlie* and *Cryphonectria parasitica*, respectively, although these fungi are not ecologically similar to *P. torulosus*.

The RFLP analysis showed similarity among *P. torulosus* isolates; no polymorphisms were observed. These results confirmed microscopic obser-

vation suggesting a low genetic variability in this DNA region. This result is in agreement with current findings that ITS is good for identification at the species level (Lee and Taylor, 1992). Similar results with RFLP have been reported for other taxa of *Phellinus*. Thirteen taxa of the *Phellinus pini* group from Europe (*P. pini*, *P. chrysoloma*), North America (*P. piceinus*), Morocco (*P. vorax*) and Asia were characterized by restriction fragment analysis of rDNA (Fischer, 1996).

Alignment of ITS sequences did not reveal any intraspecific polymorphisms. The data obtained with sequence analysis of the ribosomal ITS region showed the same sequence for the isolates studied.

In conclusion, *P. torulosus* is a widely spread fungus found in the oak woods of Apulia and Basilicata and on numerous host species at different frequencies. The results of this study confirm that RFLP-PCR is an efficient and reliable method for the detection and identification of *P. torulosus* from basidiomes. For the proper management of forests and urban trees, we hope to develop efficient methods of extraction of fungal DNA from wood to detect and identify *P. torulosus* and other fungi in the absence of basidiomes by comparing RFLP patterns.

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