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Preliminary notes on the population structure of *Heterobasidion abietinum* from *Abies pinsapo* in Spain

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Summary. A molecular study using M13 minisatellite amplification was carried out to ascertain the origin of the fungal isolates of *Heterobasidion abietinum* collected from *Abies pinsapo* in Andalucia (Spain). Isolates were compared with those from *A. alba* in neighboring countries. Analysis of molecular variance (AMOVA) detected significant differences between *H. abietinum* isolates from *A. pinsapo* and isolates from *A. alba* in France and Italy. Variance within the population from *A. pinsapo* was lower. The genetic differentiation of the fungus reflects differences in the distribution of the host species such as *A. pinsapo* and *A. alba*.

Key words: DAMD-PCR, minisatellite, Spanish fir.

Introduction

Heterobasidion annosum (Fr.) Bref. sensu lato is a fungus that causes root rot in several conifer species (Woodward *et al.*, 1998; Asiegbu *et al.*, 2005). In Europe this species was initially separated into three intersterility groups on the basis of the host tree (Capretti *et al.*, 1990; Korhonen *et al.*, 1998). Later the taxonomy was revised and each intersterility group was designed as a different species: *H. parviporum*, primarily infecting *Picea abies*, *H. annosum* sensu stricto (s.s.), prevalent on *Pinus* spp., and *H. abietinum*, which mainly attacks *Abies alba* (Niemelä and Korhonen, 1998).

In Spain, *Heterobasidion* although it occurs, is not a common pathogen of forest trees. To date, it has been reported in the Spanish Pyrénées, where it infects declining Silver fir (*A. alba* Mill.) (Oliva and Colinas, 2007), and in Andalucia, in the south of the Iberian peninsula, where it causes root disease on Spanish fir (*A. pinsapo* Boiss.) (Sanchez *et al.,* 2005). The fungus on *A. pinsapo* was recently identified as *H. abietinum* (Sanchez *et al.*, 2007) on the basis of paring tests (Mitchelson and Korhonen, 1998).

Since the *A. pinsapo* population is endemic in the Spanish peninsula, the aim of the present work was to analyse the population structure of *H. abietinum* recently collected from Spanish fir and to evaluate whether this population differed from neighboring Italian and French populations. For this purpose a molecular approach was chosen based on repetitive DNA with tandem repeats of a core or consensus sequences (minisatellites) already used to study genetic variations in many other organisms, including fungi pathogenic on forest trees (Stenlid *et al.*, 1994). This type of analysis includes the direct amplification of minisatellite-region DNA by the polymerase chain reaction (DAMD-PCR) using the M13 core sequence (Petta *et al.*, 2001; Zamponi *et al.*, 2007).

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Materials and methods

Fungal isolates

Seventeen isolates of *H. abietinum* from *A. pinsapo,* recently collected from different regions of southern Spain (Sanchez *et al.*, 2007), were used for genetic characterization. Thirteen *H. abietinum* isolates from *A. alba* collected from France and Italy were used for comparison. Three *H. annosum* s.s., and two *H. parviporum* strains were also tested as outgroup species. A complete list of the isolates, with their hosts and geographic origin, is shown in Table 1.

DNA extraction

Fungal cultures were grown on 300PT cellophane discs (Celsa, Varese, Italy) on 1.5% malt agar (Difco, Detroit, MI, USA) in 90-mm Petri dishes, and incubated at 20°C in the dark. After 10 days, mycelium was scraped off and ground using a mortar and pestle in liquid nitrogen and quartz sand. Genomic DNA was extracted using the DNeasy Plant Minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration of the DNA extracted was measured by a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

DAMD - PCR amplification

Fungal DNA was amplified in a PCR thermal cycle (Programmable Thermal Cycler Delphi 1000™, Oracle Biosystem™, MJ Research Inc., Watertown, MA, USA).

The PCR reaction was performed in a 25 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM each of dATP, dCTP, dGTP and dTTP (Fermentas GMBH, St. Leon-Rot, Germany); 2.5 U *Taq*-polymerase (Fermentas GMBH); 1 μM of the core sequence of M13 minisatellite DNA (5'-GAGGGTGGCGGTTCT-3') (MWG-Biotech AG, Ebersberg, Germany), and 25 ng of extracted DNA. Two samples of negative control with no template DNA were included to ensure that the reagents were not contaminated with extraneous DNA.

The PCR conditions were as follows: 3 min predenaturation at 94°C; 45 cycles of denaturation at 93°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min.

The PCR amplification products were separated by electrophoresis (90V for 100 min) in 1.6% agarose gel (Invitrogen, Milano, Italy) in 1× Tris-boric acid-EDTA (TBE) and stained with 0.4% ethidium bromide. The DNA size marker used for electrophoresis was the O'GeneRuler™ DNA Ladder Mix (Fermentas GMBH) banding from 100 to 10,000 bp.

Data analysis

The DAMD-PCR electrophoretic profiles were utilized to construct a dendrogram after cluster analysis. The presence (1) or absence (0) of each amplification product was scored for each isolate; only clear and reproducible markers were considered. The similarity level between each pair of isolates was determined by Jacquard's coefficient. The dendrogram was produced by cluster analysis of the similarity coefficients using UPGMA (unweighted pair-group method using arithmetic averages). These calculations were performed with the programs SIMQUAL and SAHN of the software NTSYS-pc version 2.1 (Exter software Co., New York, NY, USA).

The analysis of molecular variance (AMOVA) was calculated based on the DAMD markers. Total variation was divided into two components, within populations and between populations. This test was calculated using Arlequin Software (ver 3.01) (Excoffier *et al.*, 2005).

Results and discussion

In a number of fungal species DAMD-PCR was used not only to classify species but also to differentiate populations (Zhou *et al.*, 2001; Santini *et al.*, 2005). This kind of analysis was also useful on *Heterobasidion* spp., where it discriminated both the old intersterility groups (Karlsson, 1994; Stenlid *et al.*, 1994) and the fungal populations (Petta *et al.*, 2001; Dai *et al.*, 2003; Zamponi *et al.*, 2007).

The electrophoretic profiles of DAMD-PCR showed a total of 20 bands with molecular weights ranging from 400 to 2600 bp. Only one marker was common to all isolates tested, while the remaining 19 were polymorphic. This is consistent with the results of a previous study (Zamponi *et al.*, 2007).

A dendrogram generated by UPGMA, based on DAMD analysis showed that the *Heterobasidion* isolates used in this study formed three clusters corresponding to *H. annosum s.s.*, *H. parviporum* and *H. abietinum*. This last included all the Spa-

Table 1. *Heterobasidion* fungal isolates used in the direct amplification of minisatellite region DNA polymerase chain reaction by M13 minisatellite.

Fungal species	Strain	Code	Host	Geographic origin
H. abietinum	AP1	Sp1	*Abies pinsapo*	La Nava, Spain
H. abietinum	AP7	Sp2	*Abies pinsapo*	La Chaparrera, Spain
H. abietinum	AP8	Sp3	*Abies pinsapo*	La Chaparrera, Spain
H. abietinum	AP57	Sp4	*Abies pinsapo*	Merendero, Spain
H. abietinum	AP58	Sp5	*Abies pinsapo*	Merendero, Spain
H. abietinum	AP76	Sp6	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	AP77	Sp7	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	AP86	Sp8	*Abies pinsapo*	Era de los Gamones, Spain
H. abietinum	AP89	Sp9	*Abies pinsapo*	Alhucemal, Spain
H. abietinum	AP91	Sp10	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	AP92	Sp11	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	AP93	Sp12	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	AP96	Sp13	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	AP109	Sp14	*Abies pinsapo*	Alhucemal, Spain
H. abietinum	AP110	Sp15	*Abies pinsapo*	Alhucemal, Spain
H. abietinum	AP116	Sp16	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	AP117	Sp17	*Abies pinsapo*	Pozo de la Nieve, Spain
H. abietinum	03164/1[b]	It1	*Abies alba*	Vigo di Ton (TN)[a], Italy
H. abietinum	03165/1[b]	It2	*Abies alba*	Vigo di Ton (TN), Italy
H. abietinum	910910.10	It3	*Abies alba*	Vallombrosa (FI)[a], Italy
H. abietinum	960528.1/1/2	It4	*Abies alba*	Vallombrosa (FI), Italy
H. abietinum	921218.3/2	It5	*Abies alba*	Foresta Umbra (BA)[a], Italy
H. abietinum	920701.2/4/5	It6	*Abies alba*	M. Vulture (PZ)[a], Italy
H. abietinum	920701.2/4/3	It7	*Abies alba*	M. Vulture (PZ), Italy
H. abietinum	971120.6/2	It8	*Abies alba*	Mongiana (VV)[a], Italy
H. abietinum	971120.2/2	It9	*Abies alba*	Mongiana (VV), Italy
H. abietinum	970907.8/3	Fr1	*Abies alba*	Pyrenee, France
H. abietinum	970907.5/3	Fr2	*Abies alba*	Pyrenee, France
H. abietinum	970907.2/1/1	Fr3	*Abies alba*	Pyrenee, France
H. abietinum	970907.4/2/1	Fr4	*Abies alba*	Pyrenee, France
H. parviporum	03245/1[b]	Hp1	*Picea abies*	Val di Sella (TN), Italy
H. parviporum	98036/3[b]	Hp2	*Picea abies*	Hameenlinna, Finland
H. annosum s.s	042611/1.1.1	Ha1	*Pseudotsuga menziesii*	S. Brigida (FI), Italy
H. annosum s.s.	03016/1[b]	Ha2	*Pinus sylvestris*	Layliainen, Finland
H. annosum s.s.	96006/1[b]	Ha3	*Pinus pinea*	San Rossore (PI)[a], Italy

[a] Italian Provinces: FI, Firenze; PI, Pisa; TN, Trento; PZ, Potenza; PA, Palermo; VV,Vibo Valentia.

[b] Fungal isolates kindly provided by Kari Korhonen (Finnish Forest Research Institute, Vantaa, Finland).

nish isolates collected from *A. pinsapo* as well as 9 isolates from Italy and 4 isolates from Pyrénées (France) collected from *A. alba* (Fig. 1).

The fact that the isolates from *A. pinsapo* were included in the last cluster validates their correct identification as *H. abietinum* previously made by Sanchez *et al.* (2007) and confirms the differences among the *H. abietinum* population recently reported by Zamponi *et al.* (2007), who found that isolates fell into separate clusters depending on their geographical origin. AMOVA detected no significant differences among the isolates from *A. pinsapo* (6.08%), but found significant differences ($P<0.005$) when comparing isolates from *A. pinsapo* with isolates from Italy (14.79%) and France Pyrénées (36.91%). Variation within populations was 74.15%, and total variance was 21.40.

In this note the variability of *H. abietinum* populations was extended to the isolates from *A. pinsapo* collected in Andalucia. This suggests that the relationship between the fungus and the host species, which is considered an interesting endemism (Arista, 1995) in southern Spain, has existed for a long time.

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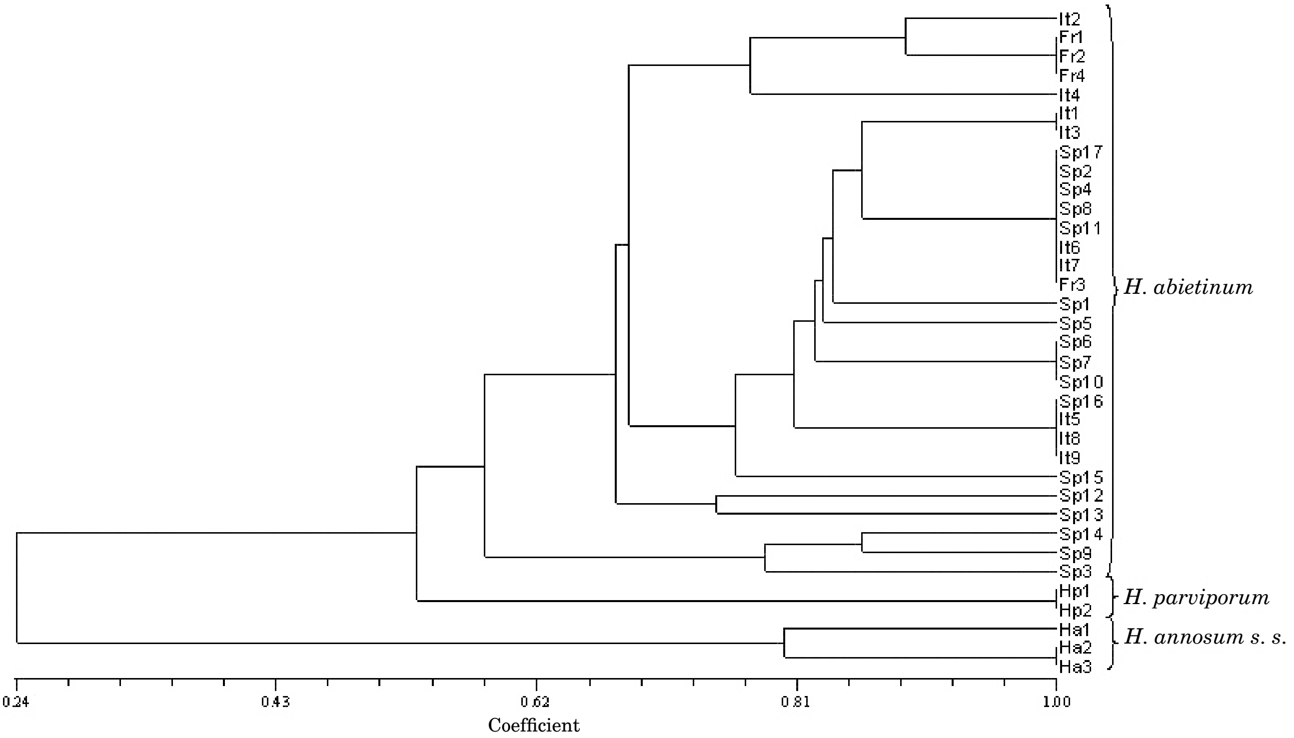

Fig. 1. Dendrogram generated by UPGMA, based on the direct amplification of minisatellite region DNA polymerase chain reaction. The three main clusters of *Heterobasidion* are shown.

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