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Variations among Italian isolates of the *Heterobasidion annosum* P Group detected using the M13 minisatellite

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Summary. The genetic variability of 22 Italian isolates of *Heterobasidion annosum* belonging to intersterility group (ISG) P was studied by using the M13 minisatellite, as marker, in PCR experiments. Phylogenetic dendrogram constructed with the UPGMA method clearly differentiated the P group isolates from F and S ones, used for comparison. Within the tested population, P isolates related by the same geographical origin tend to form separated clusters. A stronger differentiation was observed in this Italian P population than in other European P populations previously studied with the same methods. The observed stronger genetic differentiation may be related to a more marked differentiation in host populations, considering that Italy was a refugee area during glaciation and a differentiation centre after. Also the specific morphology of Italy may not allow the genetic flow between the pathogen populations of different areas. Further investigations are needed to relate the genetic differentiation with isolates virulence.

Key words: *Heterobasidion annosum*, intersterility groups, M13 minisatellite, genetic differentiation.

Introduction

Heterobasidion annosum (Fr.) Bref., a basidiomycete causing root and butt rot, is one of the most harmful pathogens in conifer forests of the Northern hemisphere (Hodges, 1969; Korhonen and Stenlid, 1998). Three intersterility groups (ISGs) of this fungus are known: F, P and S, distinguished by host range, ecological, morphological, genetic and biochemical differences (Korhonen, 1978; Johansson, 1988; Mugnai and Capretti, 1989; Karlsson and Stenlid, 1991; Stenlid *et al.,* 1994; Korhonen *et al.,* 1998; Comparini *et al.*, 2000). Based on these differences, Niemelä and Korhonen (1998) recently proposed raising the three groups to the rank of species, where groups P, S and F would become respectively *H. annosum* (Fr.) Bref. *sensu stricto*, preferentially colonising the genus *Pinus*, *H. parviporum* Niem. and Korh., most common on *Picea* spp., and *H. abietinum* Niem. and Korh., mostly on the genus *Abies*.

In Italy *H. annosum* causes serious losses in wood production, especially in pure artificial conifer stands. In natural forests the fungus exists in an ecological equilibrium which makes it a weakness parasite, related to ageing process. All three ISGs are found in Italy, P and F groups in various parts of the country, from the Alps in the north to Calabria in the south, and S group only in the Alps (Barzanti and Capretti, 1997; Capretti, 1998; Ko-

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rhonen *et al.,* 1998). As regards the host trees, S group colonises almost exclusively *Picea abies,* F group much prefers *Abies alba* while P group, the most polyphagous, has been reported not only on pine (*Pinus pinea, P. pinaster, P. cembra, P. mugo, P. murrayana, P. nigra* and *P. sylvestris*) but occasionally also on other conifers (*Abies alba, Larix decidua, Picea abies, Pseudotsuga menziesii)* and on some broadleafs (*Fagus sylvatica, Fraxinus ornus, Sorbus aucuparia, Quercus pubescens* and *Q. ilex)* (Capretti and Moriondo, 1983; Capretti, 1986, 1998; Capretti *et al.,* 1990; Nicolotti *et al.,* 1999).

The occurrence of this pathogenic fungus on numerous hosts in countries bordering the Mediterranean sea suggested the desirability of determining whether the ISG P differed in relation to the tree species it attacks and the various soils and climates in which it is found. These differences were explored with the analysis of amplification profiles by "Direct Amplification of the Minisatellite DNA–PCR" (DAMD-PCR), using as a primer the minisatellite M13 already used in similar studies with *H. annosum* (Karlson, 1994; Stenlid *et al.,* 1994).

Materials and methods

Fungal isolates.

Thirty isolates were examined: 22 from ISG P, 5 from ISG F and 3 from ISG S. The ISG was determined by pairing each isolate, in Petri dishes, with tester isolates, homokaryotic cultures of known ISG, as described in Korhonen (1978). Out of 22 P isolates 6 were from two location in Tuscany and from two different hosts (*P. nigra*, 3 isolates; and *P. pinea*, 3 isolates). Ten isolates were from two locations in Calabria collected from different host (*P. nigra,* 2 isolates; *P. pinea*, 1 isolate; *P. menziesii,* 7 isolates). The additional 6 isolates were from different hosts and localities on northern Italy (*P. sylvestris*, 2 isolates; *P. nigra*, 2 isolates; *P. pinaster,* 2 isolates). A complete list of isolates, with their host trees and their geographic origin, is given in Table 1.

Isolates were grown in potato dextrose broth (PDA, Difco Laboratories, Detroit, MC, USA) and after three weeks of incubation in static culture at room temperature, mycelium was collected by vacuum filtration onto filter paper and lyophilised.

DNA extraction.

For DNA extraction the mycelium was ground in a mortar with quartz sand and liquid nitrogen according to the protocol reported by Goggioli *et al.* (1998). Approximately 20 mg of mycelium were homogenised with 600 μl of extraction buffer (700 mM NaCl, 50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% β-mercaptoethanol, 1% cetyl-trimethl-ammonium bromide). The sample was incubated at 65°C for 30 minutes, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged for 5 minutes. The resulting supernatant was twice recovered and mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and centrifuged. The upper aqueous phase was precipitated using 75 μl of ammonium acetate (75 mM) and 600 μl of cold absolute ethanol. The nucleic acid precipitate was recovered by centrifugation, washed with 500 μl of 70% ethanol, dried and suspended again in 50 μl of TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA). The DNA concentration of each sample was estimated by comparing band intensities with known standards of lambda DNA on agarose gel.

Amplification of DNA.

Amplifications were performed in volumes of 25 μl containing: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.2 mM of each dNTPs (Polymed, Italy), 2.5 U Taq polymerase (Polymed, Italy); 200 μM of the core sequence of M13 minisatellite DNA (Innovagen, Sweden) (Stenlid *et al.,* 1994) and 20 ng of fungal DNA as template.

The amplifications were carried out in a thermal cycler (Programmable Thermal Cycler Delphi 1000™, Oracle Biosystems™, MJ Research Inc., Watertown, MA, USA) programmed for a pre-denaturation step at 93°C for 3 min, followed by 45 cycles at 93°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 4 min.

Five microliters of each amplification product were loaded onto a 1.4% agarose gel (Ultrapure DNA grade agarose, Bio-Rad, Hercules, CA, USA) with TBE buffer (40 mM Tris-borate, 1 mM EDTA, pH 8).

Electrophoresis was carried out at 100 V for 1.5 h. Gels were visualised through UV fluorescence and photographed after ethidium bromide staining (0.5 μg/μl).

The molecular size of the amplification products was estimated by comparison with a marker (1 kb

Table 1. List of isolates of *Heterobasidion annosum* used in the investigation grouped according their geographical origin.

Isolate	Code	ISG[a]	Host species	Geographical origin
3-t	920601 3/1/7	P	*Pinus nigra*	Monte Amiata, Tuscany, Central Italy
4-t	920601 3/1/6	P	*Pinus nigra*	Monte Amiata, Tuscany, Central Italy
5-t	920601 3/1/1	P	*Pinus nigra*	Monte Amiata, Tuscany, Central Italy
8-t	920213 1.1 05	P	*Pinus pinea*	Tirrenia, Tuscany, Central Italy
9-t	920213 1.1 02	P	*Pinus pinea*	Tirrenia, Tuscany, Central Italy
10-t	911116 1.1	P	*Pinus pinea*	Tirrenia Tuscany, Central Italy
13-c	921006 1.2	P	*Pseudotsuga menziesii*	Mercurella, Calabria, South Italy
14-c	921006 1.4	P	*Pseudotsuga menziesii*	Mercurella, Calabria, South Italy
15-c	921006 1.7	P	*Pseudotsuga menziesii*	Mercurella, Calabria, South Italy
16-c	921006 1.10	P	*Pseudotsuga menziesii*	Mercurella, Calabria, South Italy
17-c	921006 1.1/1	P	*Pseudotsuga menziesii*	Mercurella, Calabria, South Italy
18-c	921006 2.5 ve	P	*Pinus nigra*	Mercurella, Calabria, South Italy
19-c	921006 2.10 ve	P	*Pinus pinea*	Mercurella, Calabria, South Italy
25-L	881028 2.2 ve	P	*Pinus sylvestris*	Bressanone, Alto Adige, North Italy
26-L	881028 2.8	P	*Pinus sylvestris*	Bressanone, Alto Adige, North Italy
30-L	890505 1.1	P	*Pinus nigra*	Centocroci, Liguria, North Italy
31-L	890505 1.2	P	*Pinus nigra*	Centocroci, Liguria, North Italy
34-L	910115 1 ve	P	*Pinus pinaster*	Marina di Ravenna, Emilia Romagna, North Italy
35-L	910115 2 ve	P	*Pinus pinaster*	Marina di Ravenna, Emilia Romagna, North Italy
55-c	931030 4.13	P	*Pseudotsuga menziesii*	Gambarie, Calabria, South Italy
56-c	931030 4.23	P	*Pinus nigra*	Gambarie, Calabria, South Italy
57-c	931030 4.28	P	*Pseudotsuga menziesii*	Gambarie, Calabria, South Italy
58-c	931030 4.1	F	*Pinus nigra*	Gambarie, Calabria, South Italy
59-c	931030 4.6	F	*Abies alba*	Gambarie, Calabria, South Italy
60-c	931030 4.8	F	*Abies alba*	Gambarie, Calabria, South Italy
64-t	920401 2 ve	F	*Pseudotsuga menziesii*	Vellano, Romita, Tuscany, Central Italy
65-t	920401 3 ve	F	*Pseudotsuga menziesii*	Vellano, Romita, Tuscany, Central Italy
75-v	931007 3.3 ve	S	*Picea abies*	Valgrande, Veneto, North Italy
76-v	931007 3.2 ve	S	*Picea abies*	Valgrande, Veneto, North Italy
77-v	930519 7.4	S	*Picea abies*	Valgrande, Veneto, North Italy

[a] ISG, Intersterility group.

Plus DNA Ladder, GIBCO BRL, Life Technologies, Gaithersburg, MD, USA).

Statistical data analysis.

Photographs of the amplification products were used to construct a binary matrix of absence/presence (1/0) of the bands. Genetic similarity coefficient (CSxy) was estimated by using the formula of Nei and Li (1979):

$$CSxy = 2Nxy/ (Nx + Ny)$$

where Nxy is the number of bands that the two isolates have in common, Nx the total number of bands of isolate x and Ny the total number of bands of isolate y. Only bands that were clear and easily distinguishable were evaluated. The coefficient of genetic similarity (CS) for each ISG was calculated as the arithmetic mean CS (CSM) of that ISG in accordance with Gilbert *et al.* (1990) and Stenlid *et al.* (1994), considering isolates from the same region to belong to the same population even if they were collected at different times and locations.

Phylogenetic dendrograms were constructed with unweighted pair-group method averaging (UPGMA) using the SIMQUAL programme of the NTSYS-pc v. 1.60 package.

Statistical analysis of genetic diversity (AMOVA) within *H. annosum* groups was performed

using the Arlequin software version 1.1 (Schneider *et al.,* 1992).

Results

The electrophoretic profiles of the amplification products yielded a total of 29 bands with molecular weights ranging from 200 to 1,650 bp, some of which specific to each ISG.

The isolates of ISG P used in this study were characterised by a number of bands between 15 and 24, compared with 11-13 bands for ISG F and S.

ISG P exhibited considerable variation: none of the isolates of this ISG showed identical banding patterns, whereas 2 isolates (64[-t] an 65[-t]) from ISG F, collected from 2 trees in the same stand, did (Fig. 1, Table 1 and Table 2).

ISG P isolates exhibited 8 monomorphic bands (most of them with a molecular weight around 650 bp), ISG F isolates 8, and ISG S isolates 11.

The dendrogram (Fig.1) shows the various clusters that identified and separated the three ISGs. The isolates belonging to the P group were separated significantly (*P*>0.05) from those belonging to the F and S groups, which were themselves closer together. The total CSMs were 0.828±0.075 for ISG P isolates, compared with 0.881±0.076 for ISG F and 0.943±0.023 for ISG S .

In the ISG P a number of clusters comprising individuals with the same geographic origin were identified. It was noted that isolates from Tuscany (3-t, 4-t, 5-t, and 8-t, 9-t, 10-t) fell into different clusters than samples from Calabria (13-c, 14-c, 15-c, 16-c, 17-c,18-c, 19-c, and 55-c,56-c, 57-c) or from northern Italy (25-L, 26-L, and 30-L, 31-L and 34-L, 35-L) (Fig. 1, Table 1). These differences were statistically significant (*P*>0.01).

Percent variation between isolates from different ISG P clusters was 57.92% while that of isolates from within the same cluster was 42.08%. Although significant, variation between clusters was not very high, corroborating that all isolates belonged to the same ISG.

The amplification products from ISG F, even if of few isolates, also presented different clusters for Tuscany and Calabria (Fig. 1, Table 1).

Isolates did not differ depending on the host tree: individual clusters contained isolates from both pine and Douglas fir.

Table 2. Presence-absence band score matrix of *Heterobasidion annosum* isolates tested with the M13 primer.

Isolate	ISG[a]	Scored bands
3-t	P	11001101011101111110011111111
4-t	P	11001001011101111110011110111
5-t	P	11001101011101111110011101111
8-t	P	11011100111101111110001111011 1
9-t	P	11011100110101111110001111011 1
10-t	P	11011100111001111110001111011 1
13-c	P	11010110010101011100001111001
14-c	P	11010110010001111100001111001
15-c	P	11010110001101111100001111001
16-c	P	11010110011101111100001111001
17-c	P	11010110010001110100001111001
18-c	P	11010110010101111100001111001
19-c	P	11010110011001111100001111001
25-L	P	11111110011101111101111111011
26-L	P	11111110011101111100101111011
30-L	P	11111110010101111101001101011
31-L	P	11111110011101111101101101011
34-L	P	11101110110101111100011101011
35-L	P	11111110110101111100011101011
55-c	P	11010110010001111100001110011
56-c	P	11010110000101111100001110011
57-c	P	11010110010101111100001110011
58-c	F	10000011000011001110000110011
59-c	F	10000011000101001110000110011
60-c	F	10000011000111001110000110011
64-t	F	10000001000011100110000110011
65-t	F	10000001000011100110000110011
75-v	S	10000011001100110010000110011
76-v	S	10010001001100110010000110011
77-v	S	10000001001100110010000110011

[a] ISG, Intersterility group.

Discussion

The minisatellite M13 used as a primer confirmed its capability to detect both isolate-specific and group-specific differences of *H. annosum.* (Stenlid *et al.,* 1994). It was also noted, in agreement with Karlsson (1993), that based on UPGMA clustering of M13 minisatellite bands, strains with the same geographical origin tended to form clusters. This tendency was found with both *H. annosum* (Karlsson, 1993) and other pathogenic fungi on forest and ornamental trees such as *Gremmeniella abietina* (Hellgren, 1995), *Nectria fuckeliana* (Vasiliauskas and Stenlid, 1997) and *Ceratocystis*

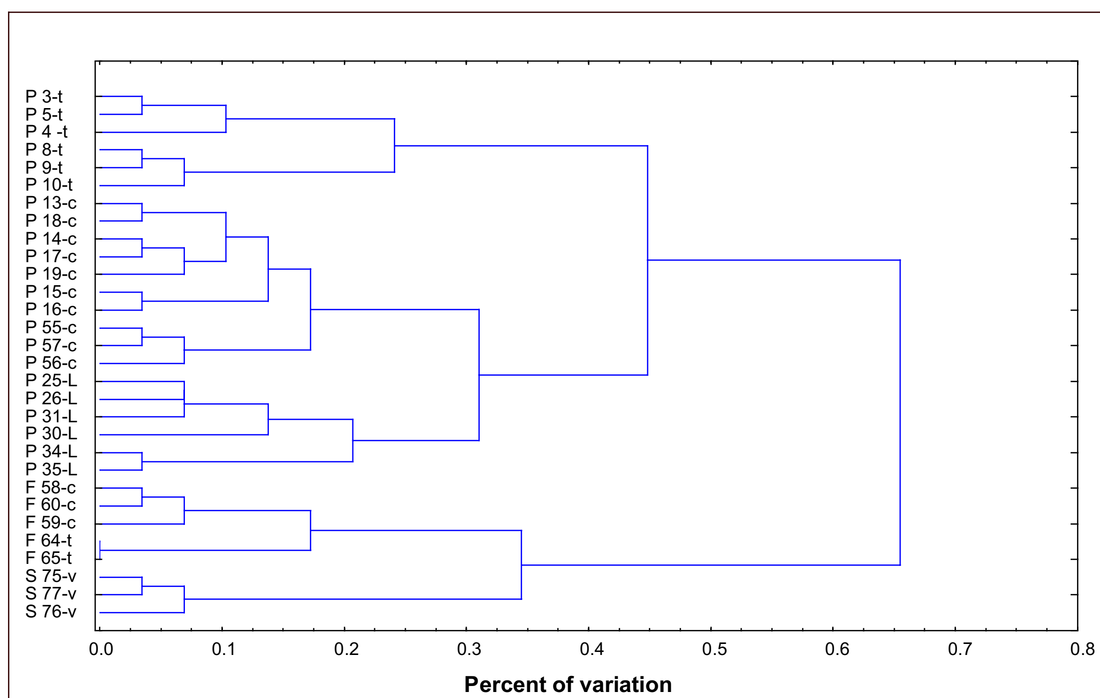

Fig. 1. Dendrogram based on the similarity index matrix of 30 Italian *Heterobasidion annosum* isolates, after PCR by using the M13 minisatellite as primer. List of isolates see Table 1.

fimbriata f.sp. *platani* (Santini and Capretti, 2000).

The CSMs revealed a shorter genetic distance between S and F groups than with P group, confirming previous studies (Stenlid *et al.,* 1994; La Porta *et al.,* 1997; Goggioli *et al.,* 1998).

The present study also showed a certain genetic variation within P group, indicating that isolates from the Mediterranean region have a different behaviour from those studied by Stenlid *et al.* (1994) in northern Europe. These researchers found that P group populations were genetically more stable than S group. They related the lower genetic stability to a greater gene flow in ISG S, or alternatively to a 'bottleneck' through which this group might have been forced in the course of its evolution.

By contrast, the findings of the present study agree with more recent data reported by Vanio and Hantula (1999) who examined electrophoretic patterns of P group isolates mostly from central and northern Europe with the Random Amplified Minisatellite (RAMS) technique and found a greater variability than that found by Stenlid *et al.* (1992).

The variability of ISG P populations in Italy was probably due to the mountainous nature of the Italian peninsula which reduced the normal genetic gene flow between the local populations, leading to differences that could be detected with molecular markers, and indeed the Italian isolates of P group studied here fell into clusters that were clearly related to their geographic origin.

In this connection, it should be borne in mind that during the last ice age Italy was an important area of refugee for many host species, including the genus *Pinus*. This was the first genus of conifers to become dominant in the Mediterranean region, where it became distributed in a large but fragmented area, and the same development later occurred also with Norway spruce and later still with Silver fir (*A. alba*) (Bernetti, 1998). The greater number of native host trees may have had a not negligible role in the genetic diversification of greater genetic variation found in *H. annosum.* These considerations suggest that differences within the *H. annosum* population can be explored by relating the evolutionary history of the pathogen to that of the trees it infects (La Porta *et al.,* 1997; Goggioli *et al.,* 1998).

Further work needs to be undertaken to determine whether the differences found in this study are also related to differences in isolate virulence.

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