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Comparison of RAPD and AFLP marker analysis as a means to study the genetic structure of *Botrytis cinerea* populations

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Abstract

To assess the genetic relationships of *Botrytis cinerea* populations in Almería (Spain), 44 isolates of *B. cinerea*, collected from six commercial greenhouses (subpopulations), were analysed by Random Amplified Polymorphic DNA (RAPD) and amplified-fragment length polymorphism (AFLP). Polymorphisms were more frequently detected per primer with AFLP than with RAPD (16 compared to 4). However, RAPD detected polymorphisms more frequently per loci than AFLP (56% compared to 32%). The analysis of population structure revealed that the genetic diversity within subpopulations (H_S) accounted for 96% of the total genetic diversity (H_T) , while genetic diversity among subpopulations represented only 4% of the total diversity, independently of whether they were analysed with RAPD or AFLP markers. The relative magnitude of gene differentiation between subpopulations (G_{ST}) and the estimate of the number of migrants per generation (N_m) averaged similar values when estimated with RAPD or AFLP markers (0.039 and 0.036, or 12.32 and 13.39, respectively). The results obtained in dendrograms were in accordance with the gene diversity analysis. However, the diversity of *B. cinerea* was higher when analysed by RAPD than with AFLP. In these cases, the isolates could not be grouped by greenhouse or fungicide resistance (except those sensitive to carbendazim and resistant to procymidone). Both the RAPD and AFLP technologies are suitable for studies of genetic structure of *B. cinerea* populations, although RAPD generated more polymorphisms per *loci* than AFLP, and provided a better explanation of the genetic relationships between isolates.

Introduction

Botrytis cinerea, the anamorph of *Botryotinia fuckeliana*, is a haploid, filamentous, heterothallic fungus that causes grey mould on many economically important crops. Growers protect their crops by applying fungicides, but strains resistant to these fungicides frequently develop (Katan et al., 1989; Raposo et al., 1996; Wang et al., 1986). In the case of *B. cinerea*, a major cause of the difficulties in managing plant disease arises from the limited understanding of the genetic structure of *B. cinerea* populations. The complexity and variability of this fungus make it difficult to control. Despite the importance of this pathogen and the availability of appropriate molecular genetic tools, there have been few studies on the genetic structure of *B. cinerea* populations (Alfonso et al., 2000; Giraud et al., 1997; 1999; Kerssies et al., 1997; Thomson and Latorre, 1999; Van der Vlugt-Bergmans et al., 1993; Yourman et al., 2000).

Most of the molecular genetic studies on *B. cinerea* populations have been made with Random Amplified Polymorphic DNA (RAPD) markers (Alfonso et al., 2000; Kerssies et al., 1997; Thomson and Latorre, 1999; Van der Vlugt-Bergmans et al., 1993; Yourman et al., 2000). One of the newest and most promising method is Amplified-Fragment Length polymorphism (AFLP) analysis (Vos et al., 1995). As in the case of

RAPD markers, AFLP markers are dominant and particularly well suited for genetic studies of haploid fungi such as *B. cinerea,* because there is no loss of genetic information caused by the dominant inheritance.

In our work, the genetic relationships among *B. cinerea* isolates were investigated by RAPDs and by AFLPs markers in order to compare the techniques for analysing the fungus genome. The present project explored the hypothesis that genetic parameters determining the use of RAPD and AFLP will be essentially identical, thus vindicating the use of either in studies of *B. cinerea* variability.

Materials and methods

Collection and culture of B. cinerea

Forty-four isolates of *B. cinerea* were collected at the end the grey mould epidemic, from six commercial tomato greenhouses located in the western region of Almería (Spain). Fifteen isolates were collected from greenhouse 1 (denominated 1-1 to 1-15), six isolates from greenhouse 2 (2-1 to 2-6), six isolates from greenhouse 3 (3-1 to 3-6); six isolates from greenhouse 4 (4-1 to 4-6), five isolates from greenhouse 5 (5-1 to 5-6), and six isolates from greenhouse 6 (6-1 to 6-6). Air-borne spores were collected on 9 cm Petri dishes containing selective medium for *B. cinerea* (Kerssies, 1990). These traps were placed in greenhouses for at least 4 h and incubated at 20–25 °C until colonies of *B. cinerea* grew (15-20 days). The growing mycelium was then transferred to potato-dextrose agar (PDA) slants. The collection was stored at −80 °C in 20% glycerol until use. Isolates were grown on PDA at 25 °C in the dark for mycelium production.

Fungicide resistance

Phenotypic characterisation of field isolates was carried out by qualitative tests for fungicide resistance using discriminatory doses (Raposo et al., 1996). Plugs of 6 mm diameter of actively growing mycelium were transferred to each plate containing PDA alone or supplemented with 1 μg ml^{-1} carbendazim or 5 μg ml^{-1} procymidone. Plates were kept at 20 °C in the dark for 3 days. Isolates were rated as resistant if they grew on fungicide-amended media and classified as sensitive when they failed to grow. All the tests were repeated at least twice.

DNA isolation

Isolates of *B. cinerea* isolates were grown on PDA Petri dishes which were incubated at 25 °C under fluorescent light, and at 21 °C in the dark, with a 16-h photoperiod. After 7 days incubation, conidia were transferred to Petri dishes with Gamborg's B5 minimal medium (Sigma-Aldrich Quimica S.A., Av. Valdelaparra 53, 28100 Alcobendas, Madrid, SPAIN) supplemented with a low glucose concentration (0.2%). Plates were then incubated for 20 h in the dark in stationary culture at 20 °C. Germinating conidia were collected, freeze-dried and stored at −20 °C. DNA was isolated, as described by Drenth et al. (1993) with slight modifications. A polysaccharide precipitation with 1 M NaCl was made before the DNA precipitation with isopropanol.

RAPD analysis

Eleven random decamer primers purchased from Operon Technologies Inc., Alameda CA, USA (OPB-01, OPB-03, OPB-04, OPB-05, OPB-08, OPB-10, OPB-13, OPB-15, OPB-18, OPB-20, OPD-02) that were previously tested in Alfonso et al. (2000), were used. The PCR–RAPD method for *B. cinerea* was based on that described by Alfonso et al. (2000). Amplification reactions were performed in volumes of 25 μl containing 2.5 μl of Amersham 10× PCR reaction buffer, 2.3 mM $MgCl_2$, 1.25 U Taq polymerase (Perkin-Elmer, Norwalk CT, USA), 100 μM dNTP, 26 ng of primer and from 0.4 to 1.5 ng of DNA template. The reaction mixtures were overlaid with 25 μl of mineral oil. PCR reactions were performed in a Perkin-Elmer Cetus DNA Thermal Cycler (GeneAmp PCR System 9700) using the following conditions: 94 °C for 6 min initially to denature the DNA, followed by 40 cycles of 1 min each at 94 °C, 2 min at 38 °C and 2 min at 72 °C. These cycles were followed by a final extension of 5 min at 72 °C. PCR amplification products were size-separated by electrophoresis on 1.5% agarose gels in TBE buffer (3.5 h at 5 V cm^{-1}). A 123 bp ladder (Pharmacia-Biotech, Uppsala, Sweden) was used as a molecular size standard for RAPD markers. The gel was stained with ethidium bromide and photographed under UV light with Polaroid 667 film.

AFLP analysis

AFLP markers were developed following the protocol described by Vos et al. (1995). DNA digestion was

carried out using the restriction enzymes *Eco*RI and *Mse*I. The selective amplification was determined by three primer pairs (AA, AC and AT). Aliquots (2 µl) of PCR products were mixed with 12 µl of formamide and 0.5 µl of a red DNA size standard (GENESCAN-500 ROX). Samples were denatured prior to separation by capillary gel electrophoresis in an ABI Prism 310 DNA Genetic analyser (PE Biosystems) and analysed by using Genescan Analysis software 3.1 (PE Biosystems).

Data analysis

Polymorphisms and monomorphisms were identified by the name of the primer and the size of the DNA amplification product. The presence or absence of each band RAPD or peak AFLP were scored as 1 and 0, respectively, and all fragments were given equal weights. Scores were compiled as two individual matrixes of isolates and markers. All the processes were repeated at least two times to ensure reproducibility.

Analysis of genetic diversity and structure of population

Gene diversity in the total population (H_T) was divided into the gene diversities within (H_S) and between (D_{ST}) subpopulations, $H_T = H_S + D_{ST}$ (Nei, 1987). We used the correction for small samples and haploidy for *B. cinerea* recommended by Brown (1996).

$$\hat{H}_T = 1 - \sum \overline{x_i^2} + \hat{H}_S / n_S$$

$$\hat{H}_S = n \left(1 - \sum \overline{x_i^2}\right) \Big/ (n - 1)$$

where x_i is the frequency of the ith allele at a locus (the frequency of the marker) and n is the harmonic mean of the sample sizes (n_i) from each of the S subpopulations. Both polymorphic and monomorphic markers were included, as recommended by Nei (1987).

Genetic differentiation relative to the total population was calculated by the coefficient of gene differentiation (Nei, 1987): $G_{ST} = D_{ST}/H_T.G_{ST}$ can take values between 0.0 (no differentiation between subpopulations) and 1.0 (complete identity within each subpopulation and complete differentiation between subpopulations).

The amount of gene flow between populations, N_m, where N is the effective population size and m is the fraction of individuals in a population that are immigrants, was estimated by using the following formula (Boeger et al., 1993).

$$N_m = 0.5[(1/G_{ST}) - 1]$$

If $N_m < 1$, then local populations tend to differentiate; if $N_m \geq 1$, then there will be little differentiation among populations and migration is more important than genetic drift (Wright, 1951).

Cluster analysis

The genetic similarity of isolates was assessed, based on RAPD and AFLP markers, by the similarity coefficient (SC) of Dice (1945). The resulting matrixes of matching coefficients were constructed and analysed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) version 1.8 (Rohlf, 1993). The distance matrices of Dice's SC were computed using the SIMQUAL program and dendrograms were generated by the unweighted pair-group arithmetic average method (UPGMA) using the SAHN clustering program.

Results

On the basis of their response to carbendazim and procymidone, isolates fell into the following phenotypic classes (Figure 1): resistant to carbendazim and procymidone (64%), sensitive to carbendazim and procymidone (11%), resistant to carbendazim and sensitive to procymidone (11%), and sensitive to carbendazim and resistant to procymidone (14%).

Eleven 10-mer oligonucleotides produced a total of 79 RAPD reproducible markers (35 monomorphic and 44 polymorphic). In the case of the AFLP analysis, the three primers produced 152 markers (105 monomorphic and 47 polymorphic).

The analysis of population structure revealed that genetic diversity within subpopulations accounted for 96% of the total genetic diversity, while genetic diversity between subpopulations represented only 4% of the total diversity, independently of being analysed with RAPD or AFLP markers. Both by using RAPD and AFLP markers, similar values of gene differentiation between subpopulations were obtained (0.039 and 0.036, respectively; see Table 1). However, values of total gene diversity and genetic diversity within subpopulations were twice as high when estimated with RAPD than with AFLP markers (Table 1). The

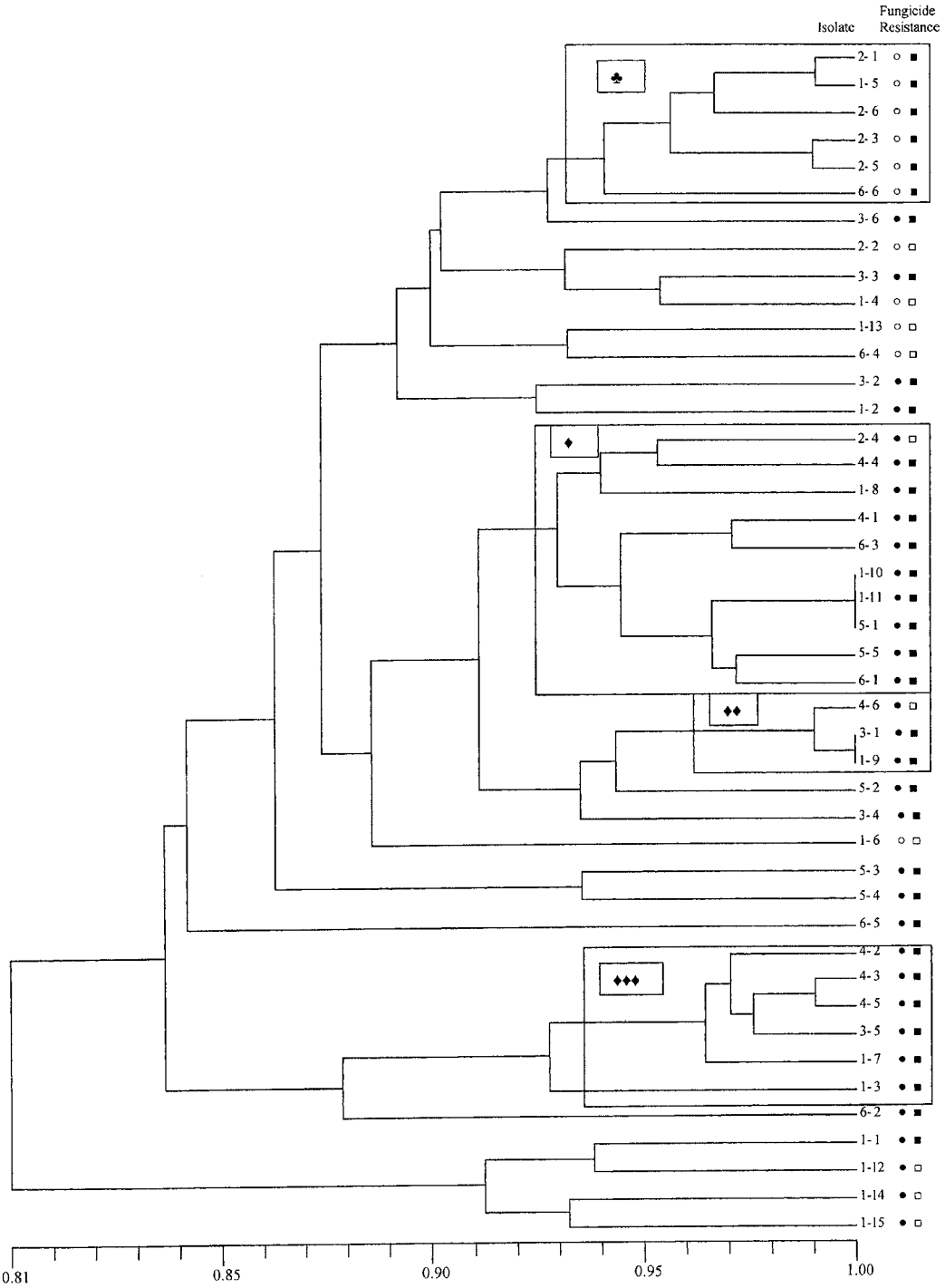

Figure 1. The UPGMA dendrogram showing the genetic similarity among 44 isolates of *Botrytis cinerea* sampled in different greenhouses of Almería (Spain) and analysed by RAPD. Isolates have different resistance phenotypes to carbendazim (●: resistant, and ○: sensitive) and procymidone (■: resistant, and □: sensitive).

Table 1. Genetic diversity among subpopulations of *Botrytis cinerea* in greenhouses in Almería estimated by RAPD and AFLP markers

	Marker analysis	
	RAPD	AFLP
H_S^a	0.4582	0.2163
H_T^b	0.4724	0.2252
G_{ST}^c	0.036	0.039
N_m^d	13.3	12.3
n^e	44	44

[a]Gene diversity within subpopulations. [b]Total gene diversity. [c]Gene differentiation between subpopulations relative to the total population. [d]Estimation of the number of migrants per generation. [e]Number of individuals.

estimated number of migrants per generation (N_m) was similar for both methodologies (12.32 and 13.39, for RAPD or AFLP markers, respectively).

Dendrograms generated with RAPD or AFLP markers grouped isolates in different clusters (Figures 1 and 2). Some clusters are reproducible and are identified by both marker methodologies (e.g. clusters ♦, ♦♦, ♦♦♦ in Figures 1 and 2) whereas other clusters are inconsistent and dependent on the marker type used (e.g. cluster ♠, Figure 2). More distinct differentiation between isolates was obtained when using RAPD markers, despite the fact that approximately the same number of polymorphic markers were used in RAPD (44) and in AFLP (47) analysis. For instance, only two clusters of isolates were obtained in the dendrogram generated with RAPDs at 100% similarity between isolates, while six in the case of AFLPs. At 97.5% similarity between isolates 37 clusters were obtained with RAPDs compared to 16 with AFLPs; and at 93% similarity, 15 clusters were obtained with RAPD and only one with AFLPs (Figures 1 and 2).

Isolates from the same and from different greenhouses were grouped randomly in dendrograms generated with RAPDs or with AFLPs (Figures 1 and 2). Isolates belonging to phenotype sensitive to carbendazim and resistant to procymidone were grouped in a cluster in both the dendrograms at approximately 95% similarity (cluster ♣, Figures 1 and 2). Isolates of this phenotype were from 3 greenhouses (1, 2 and 6). Isolates of the other three fungicide-resistance phenotypes did not cluster together.

Discussion

Polymorphisms obtained with AFLP and RAPD markers have different underlying causes at the molecular level and thus may provide differing information in the analysis of genetic relationships. Operationally, it is useful to compare the absolute levels of polymorphism shown by these two classes of markers, although it must be taken into consideration that as long as the changes occur randomly throughout the genome, you can expect their resolving power to be similar (Noli et al., 1997). In our study, four polymorphisms were generated per primer in the case of RAPD and 16 in the case of AFLP, indicating that in *B. cinerea* populations polymorphisms are more frequently detected (per primer) with AFLP than with RAPD. However, RAPD detected polymorphism more frequently per locus than AFLP: 56% of the RAPD were polymorphic compared to 32% of the AFLP loci. In plants, AFLP analysis has been found to be more informative than RAPD analysis (Powell et al., 1996; Russell et al., 1997), but fewer studies have been done with plant pathogens (Majer et al., 1996; 1998) and none with *B. cinerea.* Majer et al. (1996) reported a high efficiency in the AFLP technique for detecting polymorphisms, even in species where little variation could be previously found by other molecular analysis. Twenty one primer combinations were used on four isolates of *Pyrenopeziza brassicae*, detecting a total of 162 polymorphisms (mean = 4.1 polymorphisms per primer combination per pair of isolates); and with 4 primer combinations used on eight isolates of *Cladosporium fulvum*, a total of 32 polymorphisms were detected (mean = 3.3 polymorphisms per primer combination per pair of isolates) (Majer et al., 1998).

Diversity of *B. cinerea* appeared higher when analysed by RAPD than by AFLP (Table 1) due to the use of a higher proportion of polymorphic markers (56% compared to 32%). Estimations of gene differentiation between subpopulations (G_{ST}) (Table 1) were similar for both the markers since this parameter is the proportion of the total variation that is different between subpopulations.

The number of isolates used in this study (44) as well as the number of polymorphic marker fragments (44 for RAPD and 47 for AFLP) is low and not sufficient for making any extremely conclusive groupings in dendrograms. However, the results obtained in dendrograms were in accordance with those obtained in the genetic diversity analysis. In dendrograms constructed with

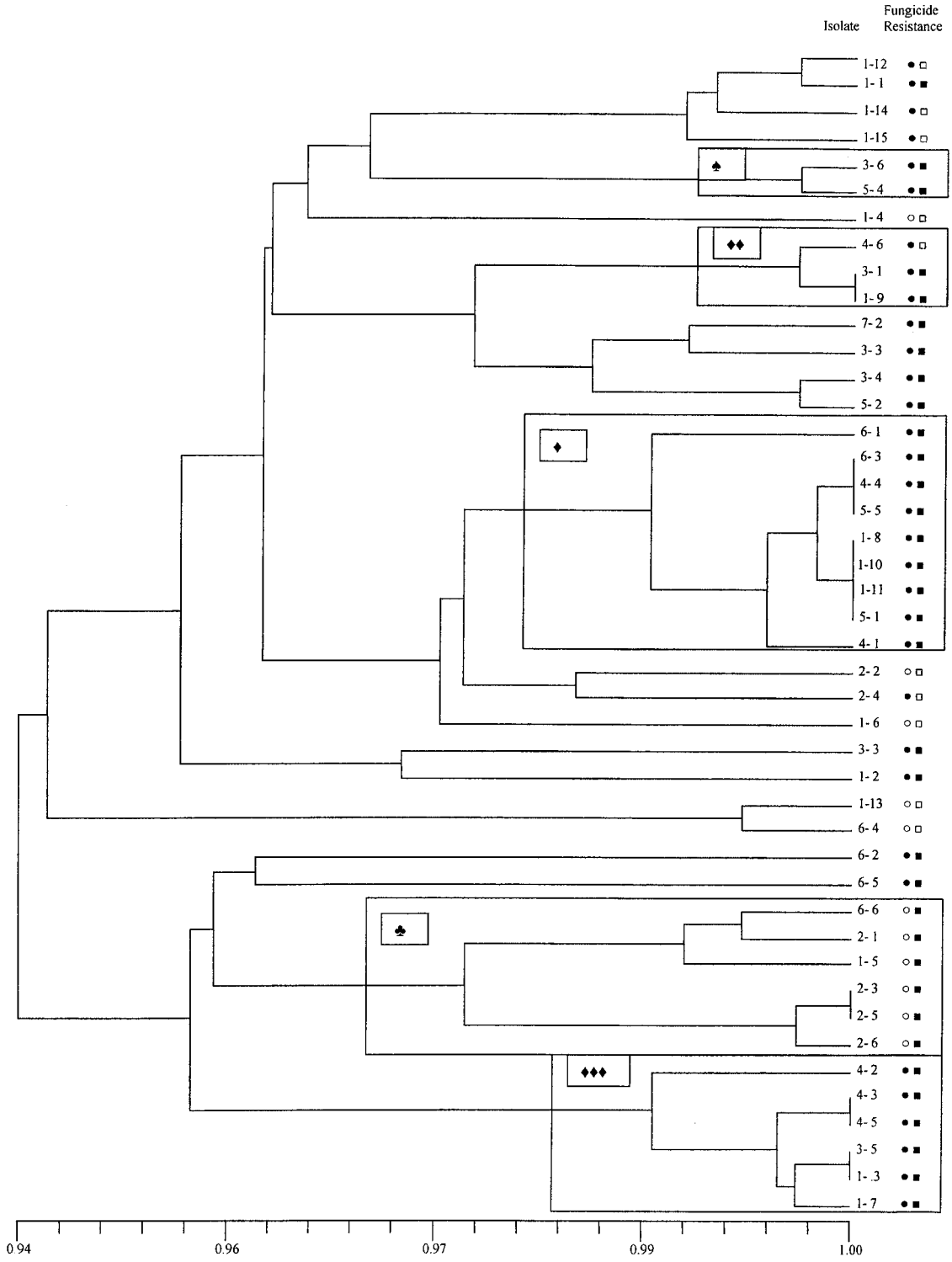

Figure 2. The UPGMA dendrogram showing the genetic similarity among 44 isolates of *Botrytis cinerea* sampled in different greenhouses of Almería (Spain) and analyzed by AFLP. Isolates have different resistance phenotypes to carbendazim (●: resistant, and ○: sensitive) and procymidone (■: resistant, and □: sensitive).

both markers, the isolates were not grouped by greenhouse, as occurred in other *B. cinerea* population studies. Kerssies et al. (1997), carried out a study of isolates from gerbera using RAPD markers. No aggregation of isolates was obtained in relation to pathogenicity, inside–outside greenhouses or with regard to the month of the year. Likewise, Van der Vlugt-Bergmans et al. (1993) did not detect genetic clustering by host, year of isolation, geographic origin or mating type. Giraud et al. (1997) did not detect differentiation between isolates from different organs, different sampling dates, grape varieties or geographic origin, although some degree of host specialization was obtained in French populations of *B. cinerea* (Giraud et al., 1999).

Clustering of isolates in relation to fungicide resistance or sensitivity did not occur with the exception of the phenotype sensitive to carbendazim and resistant to procymidone. All the six isolates having this phenotype (from 3 different greenhouses) clustered together in both dendrograms. Yourman et al. (2000) detected clustering in relation to benzimidazole or dicarboximide sensitivity phenotype in populations of *B. cinerea* from South Carolina (USA).

Results obtained by both methods of comparing populations of *B. cinerea* validate previous results obtained by Alfonso et al. (2000) using RAPD markers in relation to population structure of *B. cinerea* in Almería. Values of G_{ST} and N_m were similar according to RAPD or AFLP estimations. These values indicate that most of the genetic diversity in *B. cinerea* populations is present within subpopulations (greenhouses). The estimate of the number of migrants between greenhouses (always higher than 1) indicates the importance of migration in preventing genetic differentiation. Migration of isolates may occur between greenhouses (Alfonso et al., 2000). However, the RAPD data demonstrated that only two haplotypes contained more than one member, thus indicating that haplotypes as such did not migrate readily. Haplotypes might undergo genetic recombination after migration. Strong evidence, from molecular marker studies by Giraud et al. (1997), indicated that sexual recombination occurs frequently in *B. cinerea* populations in French vineyards. Both the mating types *MAT1-1* and *MAT1-2* are present in populations of *B. cinerea* from Almería, which enables sexual reproduction to occur (Delcan and Melgarejo, 2002). Other sources of genetic recombination such as heterokaryosis have been proposed for *B. cinerea*, but its occurrence has not been clearly demonstrated (Beever and Parkes, 1993; Delcán and Melgarejo, 2002; Weeds et al., 1998). However, other explanations may apply. Large recombining *B. cinerea* populations may exist outside greenhouses, acting as the source of the pathogens, which then migrate regularly into the greenhouses. This large population is diverse and widely distributed, and greenhouses merely subsample the large population.

These results suggested that both RAPD and AFLP technologies are suitable for studying the genetic structure of *B. cinerea* populations, although RAPD generated more polymorphisms per *loci* than AFLP and explained better the genetic relationships between isolates.

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