Molecular Characterization of Apulian Fig (*Ficus carica* L.) Germplasm Collection Using Fluorescence-Based AFLP Markers

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Abstract

Accurate germplasm characterization and elucidation of the genetic relationships among the accessions maintained in a plant collection serve as essential links between the conservation and appropriate utilization of plant genetic resources. The present study was undertaken to assess polymorphism and relationships among 24 Apulian fig accessions using fluorescence-based AFLP (amplified fragment length polymorphism) markers. Five selective primer pairs resulted in 553 amplification products of which 535 were polymorphic among the analysed genotypes. A high degree of polymorphism was revealed by these primer combinations that ranged from 91.6 to 100%. The genetic relationships among the studied figs were estimated using the Dice similarity index that was calculated between each pair of genotypes. The pairwise genetic similarities ranged from 0.30 to 0.88 with a mean value of 0.60, thus showing a good degree of inter-cultivar genetic diversity at the DNA level. Intra-cultivar diversity was also investigated in the two cultivars San Giovanni (0.78) and Dottato (0.84), for which three and two different accessions were analysed, respectively. Dendrogram constructed using UPGMA cluster analysis showed that cv. Potentino separated from the other 23 genotypes at a genetic similarity value of 0.35 pointing out a relatively high genetic divergence of this cultivar from the others. The remaining 23 genotypes formed two principal clusters diverging at a genetic similarity value of 0.59. The results of this study will help in the formulation of appropriate strategies for conservation and cultivar improvement in Apulian figs, for which limited knowledge of the genetic diversity is available.

INTRODUCTION

Fig (*Ficus carica* L.; 2*n*=2*x*=26) is characterized by a various and wide genetic patrimony which can be ascribed to its ancient origin (Zohary and Hopf, 1988). Fig trees used to be widely cultivated in Italy especially in Apulia region. At the beginning of the last century, 94 cultivar names were re-counted in the peninsula of Salento, South of the Apulia region. Unfortunately, over the last 50 years, the fig monoculture fell from 40,000 to 2,000 hectares in Italy, and from 22,800 to 500 hectares in the Apulia region. Nowadays, genetic relationships among different fig cultivars in the Salento area remain ambiguous and the nomenclature is hampered by the occurrence of different names used for the same cultivar (synonymy) in different regions or the presence of genetically distinct cultivars with similar morphological characteristics uniformly named (homonymy).

Plant identification and estimation of their relationships and diversity are traditionally established on the basis of morphological, physiological and agronomical characteristics (Mars, 2001). Although morphological descriptors are still considered a basic tool for the identification and classification of fig germplasm (Giraldo et al., 2008; Küden et al., 2008), the use of molecular markers is becoming widely accepted for cultivar characterization, as well as for the assessment of genetic relatedness among

cultivars and clones.

Several studies, based on RAPD (random amplified polymorphic DNA) markers, revealed the genetic relatedness among fig genotypes belonging to different fig collections (Cabrita et al., 2001; Papadopoulou et al., 2002). The use of inter-simple sequence repeats (ISSRs) allowed to estimate the genetic diversity among Tunisian figs (Salhi-Hannachi et al., 2004) and SSR (simple sequence repeats) markers were widely applied for studying genetic diversity and for cultivar characterization of figs (Khadari et al., 2001; Saddoud et al., 2005). Phylogenetic relationships among fig cultivars were also estimated by ribosomal DNA analysis (Weiblen, 2000). Moreover, AFLPs (amplified fragment length polymorphism) were used for cultivar identification purposes (Cabrita et al., 2001) and in establishing genetic relationships among cultivars (Resta et al., 2003). AFLP markers (Vos et al., 1995) are a reliable method of genetic fingerprinting and have been successfully used for characterization and evaluation of genetic relationship in many other crops (Russell et al., 1997; Pejic et al., 1998).

Accurate germplasm characterization and elucidation of the genetic relationships among the accessions maintained in a plant collection serve as essential links between the conservation and appropriate utilization of plant genetic resources (Rodriguez et al., 1999; Papadoupulo et al., 2002). Thus, the objectives of the present study were to fingerprint and to assess polymorphism and genetic relationships among a number (21) of South Italian fig cultivars using AFLP markers.

MATERIALS AND METHODS

The present study was carried out on a collection of fig cultivars established in the "Hortus Lupiaense" Botanical Garden, at the Department of Biological and Environmental Science and Technology, University of Lecce, Italy. The present collection includes a sizeable number of the denominations known in the Salento area. Name, definition and characteristics of the 24 fig genotypes analysed are reported in Table 1. Young leaves of the studied fig accessions were sampled for DNA isolation performed by the CTAB method (Busconi et al., 2003). The isolated DNA was diluted to 100 ng μl^{-1}.

The AFLP procedure was carried out as described by Vos et al. (1995), with minor modifications using the AFLP™ Plant Mapping kit for Regular Plant Genome (Applied Biosystems, California, USA) and the capillary electrophoresis system ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). The primer combinations used for the selective amplifications are shown in Table 2. The *Eco*RI-primers used for selective amplifications were either labeled with the fluorescent dye FAM or NED (Applied Biosystems, AFLP™ Selective Amplification Start-Up Module). Dye-labeled AFLP fragments were analysed using GeneScan analysis software and displayed as peaks in electropherograms. Tabular data, including the peak size, peak height, peak area and data point, were also generated. Polymorphic and reproducible peaks were scored as present (1) or absent (0) among the analysed genotypes and entered into a data matrix. The outcome matrix was processed to estimate the genetic similarity (GS) of Dice (1945) for all pairs of the fig accessions.

A dendrogram was constructed based on the similarity matrix data applying the un-weighted pair-group method with arithmetic average (UPGMA) cluster analysis. Cophenetic matrix for the dendrogram was generated and compared to the similarity matrix using the Mantel matrix (Mantel, 1967) correspondence test. All statistical analyses were conducted by using the NTSYS-PC software, version 2.11a (Exeter Publishing, Setauket, NY) (Rohlf, 2002). The data were also subjected to Bootstrap Analysis in order to estimate the reliability of the clustering pattern. Bootstrap Analysis, using 2000 iterations, was computed using the WinBoot software (Yap and Nelson, 1996). Finally, polymorphic information content (PIC) and marker index (MI) were calculated across assay units. Each AFLP primer combination was assumed an assay unit. PIC value was calculated applying the formula of Roldán-Ruiz et al. (2000). The marker index was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell et al., 1996).

RESULTS

Fifteen primer combinations containing three selective nucleotides were pre-screened for their ability to provide a high level of polymorphism in two of the fig accessions examined later in this study. Five selected combinations that produced the highest number of amplified fragments were used to screen the 24 fig genotypes. Only reliable fragments between 100 and 450 bp in length were employed for subsequent analysis. Amplified markers less than 100 bp and more than 450 bp in length were excluded in order to consider that the size calling method used for the analysis (GeneScan^{TM} analysis software, Applied Biosystems) cannot assign the size of fragments smaller than 100 bp and larger than 450 bp unambiguously.

The number of amplification products and the percent polymorphism per primer combination as well as the PIC and MI values are shown in Table 2. Based on 553 useful AFLP markers, Dice similarity index was determined between each pair of genotypes. Intra-cultivar diversity was found in the two cultivars San Giovanni and Dottato, for which three and two different accessions were analysed, respectively. Intra-cultivar similarity was 0.78 for 'San Giovanni' and 0.84 for 'Dottato'.

The result of cluster analysis was shown on the dendrogram in Figure 1 depicting the pattern of relationships between the studied genotypes. The robustness of the branches was evaluated statistically by 2000 cycles of bootstrapping. The cophenetic matrix computed from the tree matrix and compared with the original similarity data showed significant correlation of 0.95, revealing a good fit of the cluster analysis performed.

DISCUSSION

The present study allowed the discrimination between all the analysed cultivars and between clones of the same fig cultivar. The analysis of AFLP profiles found in our set of fig cultivars showed a good genetic variability among germplasm of Salento region, making it a valuable source for incorporation into potential breeding programs for the region. In particular, five primer pairs produced a relatively high number (535) of polymorphic bands among the 24 fig genotypes (Table 2), which indicated a good level of genetic diversity within the fig germplasm cultivated in a restricted geographic area of Apulia region (Salento). The percentage of AFLP polymorphic bands (97%) obtained in the current study corresponds to a high polymorphic rate when compared to other studies using other molecular markers such as RAPD (77.4%; Sadder and Ateyyeh, 2006) and I-SSR (90.4%; Salhi-Hannachi et al., 2004).

To compare the efficiency of the primer combinations utilised, other variability indices were calculated, such as PI and MI. Combination *Eco*RI-ACA/*Mse*I-CAA totalized the maximum PIC (0.29) and MI (41.47). Moreover, the average PIC value of 0.25 (Table 2) across all scored AFLP bands agreed well with the results obtained in AFLP-based genetic diversity studies on several species such as maize (Lüberstedt et al., 2000), ryegrass (Roldán-Ruiz et al., 2000), wheat (Bohn et al., 1999), and soybean (Powell et al., 1996).

Cluster analysis of the AFLP results reported on the dendrogram in Figure 1 shows that the genotype Potentino was clearly separated by the other accessions, as confirmed by the low similarities with all other cultivars, probably due to its different geographic origin (Basilicata region). The high boostrap value (100) that supported this branch of the dendrogram confirmed the hypothesis that Potentino was characterized by a unique genetic background. Another cluster supported by 100 boostraps was the group B, enclosing cultivars Ottata Rossa, Dottato and Rigato, resulting in a high similarity at the molecular level among them and with different molecular characteristics from the others. Based on morphological characteristics of Rigato and Dottato cultivars, they appear so similar so that it is a common belief among Apulian growers that 'Rigato' could derive from a bud mutation of 'Dottato'.

Information regarding the geographic origin of the different genotypes is often helpful but, as seen in our study, it seems that there is no clear correlation between the estimated relationships and the geographical origins of these genotypes with the exception

of the two accessions Fracazzano Bianco and Fracazzano Rosso which were collected from the same location, Novoli, and presenting a high genetic similarity value (0.88) supported by 100 boostraps. We can hypothesize that these cultivars should have a local origin and should derive from a common ancestor. In fact, they showed very similar phenotypic characteristics. The lack of relationship between the spatial and genetic proximity of the most cultivars analysed might be explained by the fact, that these fig genotypes are 'naturalized' because they have been established, adapted and persisted in areas distant from their initial origin. Concerning the study of genetic polymorphism among different accessions of the same cultivar, we can observe that all entries selected from the same cultivar were grouped together with high boostrap values (i.e. A31, A41, and A39 for 'San Giovanni'; A40, and A22 for 'Dottato'). Finally, the phenotypic characteristics (Table 1) had suggested the occurrence of a possible case of synonymy between Culummo Fasanese and San Giovanni cultivars and between Marangiana and Ottata Rossa cultivars; however this synonymy was not supported by molecular analysis as a distinct clustering was recorded for the former genotypes (Fig. 1).

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Tables

Table 1. Accession number, cultivar name and representative phenological traits of 24 figs sampled in different sites of the Peninsula of Salento (Apulia region, Italy) and grown in the "Hortus Lupiaense" Botanical Garden (University of Lecce, Italy).

Accession number	Cultivar name	Collecting site	Crop setting fruit	Full maturity*	
				Breba	Main crop
A4	Calimera	Cerfignano	Two-crops	Late	Mid-season
A6	Culummo Fasanese	Latiano	Two-crops	Mid-season	Early
A7	Culummo Nero	Marittima	Two-crops	Late	Mid-season
A9	Dei Greci	Marittima	Two-crops	Late	Mid-season
A10	Dell'Abate	Salice	One-crop	-	Late
A11	Della 'Mota	Novoli	One-crop	-	Mid-season
A13	Fracazzano Bianco	Novoli	Two-crops	Late	Mid-season
A14	Fracazzano Rosso	Novoli	Two-crops	Late	Mid-season
A15	Gniura	Latiano	One-crop	-	Mid-season
A16	Lancina	Borgagne	One-crop	-	Mid-season
A18	Marangiana	Marittima	One-crop	-	Early
A19	Marinese	Martano	Two-crops	Late	Mid-season
A22	Dottato	Marittima	Two-crops	Late	Early
A24	Pasulita	Marittima	One-crop	-	Early
A26	Potentino	Novoli	Two-crops	Late	Mid-season
A29	Rigato	Marittima	One-crop	-	Mid-season
A31	San Giovanni	Salice	Two-crops	Mid-season	Early
A32	Stiddhianese	Martano	One-crop	-	Mid-season
A38	Ottata Rossa	Novoli	One-crop	-	Early
A39	San Giovanni	Marittima	Two-crops	Mid-season	Early
A40	Dottato	Cerfignano	Two-crops	Late	Early
A41	San Giovanni	Cerfignano	Two-crops	Mid-season	Early
A42	Varnea Nera	Cerfignano	One-crop	-	Late
A43	Della Monaca	Latiano	Two-crops	Very late	Mid-season

*The full maturity was recorded when 50% of the fruits matured, according to IPGRI and CIHEAM (2003). Breba full maturity: Mid-season (1-15 June); Late (16-30 June); Very late (>1 July). Main crop full maturity: Mid-season (11-31 August); Early (1-10 August); Late (1-30 September).

Table 2. Polymorphic information content (PIC) and marker index (MI) per amplified fragment length polymorphism primer combination in 24 genotypes of fig.

Primer combination	Total no. of bands	No. of polymorphic bands	Polymorphic bands (%)	PIC	MI
*Eco*RI-AAG/*Mse*I-CTA	114	112	98.3	0.22	24.64
*Eco*RI-ACA/*Mse*I-CAA	143	143	100.0	0.29	41.47
*Eco*RI-ACA/*Mse*I-CAT	81	79	97.5	0.21	16.59
*Eco*RI-ACA/*Mse*I-CTA	107	98	91.6	0.26	25.48
*Eco*RI-ACC/*Mse*I-CAA	108	103	95.4	0.25	25.75
Total	553	535	-	-	-
Average	111	107	96.6	0.25	26.79

Figures

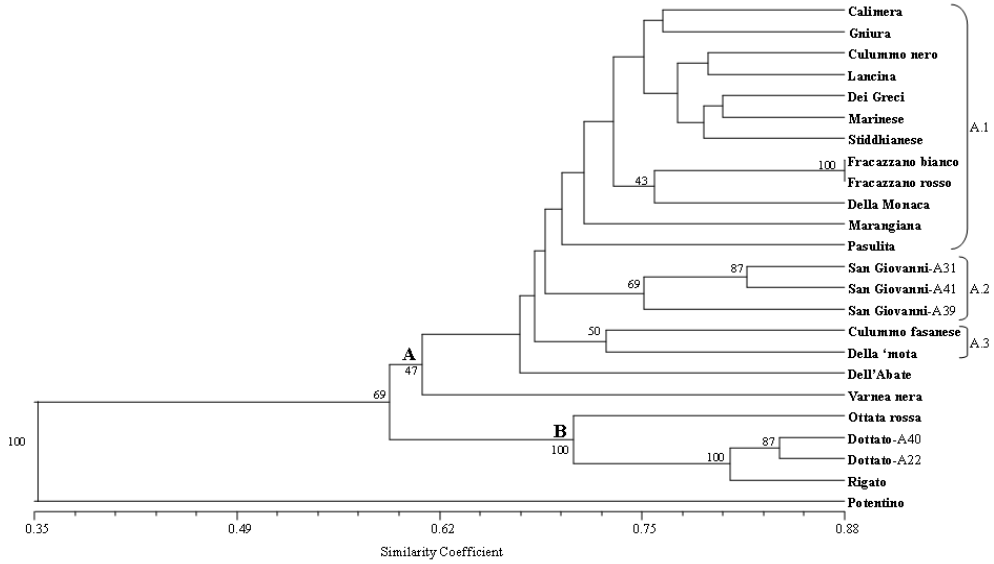

Fig. 1. Association among 24 genotypes of fig revealed by UPGMA cluster analysis of Dice's genetic similarity coefficients calculated from AFLP data of five primer combinations. Numbers at the nodes indicate the bootstrap values, in percentages, of the consensus tree obtained (branches lacking the value received <40% bootstrap support).