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SUMMARY - The more than 100 almond cultivars grown in Apulia (South Italy) could be a source of genetic variation adapted to the Mediterranean environment, provided that a proper classification, identifying similar characteristics and synonymous cultivars, is made. DNA-based assays could integrate traditional classifications based on morphology. In the comparison of 17 Apulian almond cultivars, by random amplification of their DNAs (RAPD) with 60 primers, 418 bands were detected, of which 241 were polymorphic (57.7%). Primers distinguishing the majority of the tested cultivars were found. Cultivar relationships were estimated through cluster analysis (UPGMA) based on RAPD data. High similarity between two pairs of cultivars was revealed, and was in agreement with previous observations. Only tentatively, two additional aggregations were suggested. RAPDs seemed to be effective classification tools for germplasm conservation and breeding.

Key words: Almond, *Amygdalus*, RAPD, genetic diversity, DNA polymorphism.

RESUME - *"RAPD chez des cultivars d'amandier (*Amygdalus communis *L.) dans les Pouilles". Plus de 100 cultivars d'amandier plantés dans les Pouilles (Italie du Sud) pourraient constituer une source de variation génétique adaptée au milieu méditerranéen, à condition qu'on réalise une classification appropriée, identifiant des caractéristiques similaires et des cultivars synonymes. Des essais reposant sur l'emploi du DNA pourraient complémenter les classifications traditionnelles fondées sur la morphologie. En comparant 17 cultivars d'amandier des Pouilles, par une amplification de leur DNA (RAPD) avec 60 amorces, 418 bandes ont été détectées dont 241 (57,7%) se sont révélées polymorphiques. Des amorces ont été détectées capables de distinguer la plupart des cultivars testés. Les corrélations entre les cultivars ont été évaluées à travers l'analyse par grappes (UPGMA) utilisant les données de la RAPD. Une similitude considérable entre deux paires de cultivars a été mise en évidence et cette observation a confirmé les résultats acquis précédemment. En plus deux agrégations supplémentaires ont été suggérées. La RAPD s'est avérée être un outil efficace pour la classification dans la conservation du germoplasme et l'amélioration génétique.*

Mots-clés : *Amandier,* Amygdalus*, RAPD, diversité génétique, polymorphisme du DNA.*

Introduction

Almond (*Amygdalus communis* L. = *Prunus dulcis* Miller) is a very important species in the Mediterranean basin. In Apulia (South Italy) almond cultivation, although it is now declining, contributed considerably to the agricultural economy. Several interpretations have been put forward to explain the possible causes of such a decline (Carrante, 1958; Ciccarone, 1974; Godini, 1977a,b), including the high number of cultivars (Godini, 1975). Fanelli (1939) published a list of over 100 almond cultivars of Apulian origin, accurately describing 50 of them. Undoubtedly, because of the many synonymous cultivars within the large local almond germplasm, the study of the genetic diversity of Apulian almond cultivars might be important not only for the germplasm conservation but also in parental choice for breeding purposes.

Morphological characters have been used to differentiate almond cultivars (Fanelli, 1939; Fanizza and Bogyo, 1976; Lansari *et al.*, 1994); these types of traits require plant replications and repeated measurements to account for environmental variation. Biochemical characters such as isozymes have also been used in almond for identification of cultivars (Hauagge *et al.*, 1986; Cerezo *et al.*, 1989) or hybrids (Chaparro *et al.*, 1987; Byrne and Littleton, 1988) as well as for genetic analyses

(Arulsekar *et al.*, 1986; Byrne, 1990; Jackson and Clark, 1991). The study of genotypic diversity based on isozyme variation is restricted to a few polymorphic enzyme systems encoded by a small number of loci. The development of molecular biology has provided alternative DNA-based procedures for the detection of polymorphism and genetic variation, and for indirect selection of economically important traits. Molecular markers such as RFLPs (Restriction Fragment Length Polimorphisms) have been used to develop *Prunus* linkage maps (Arus *et al.*, 1995; Foolad *et al.*, 1995) and to infer phylogenetic relationships based on chloroplast variation (Badenes and Parfitt, 1995). However their use has been limited, mainly owing to the level of resources, time and technical expertise they require. An alternative type of genetic marker known as RAPDs (Random Amplified Polymorphic DNAs) overcomes many limitations of RFLP analysis. With this procedure only very small amounts of target DNAs and few hours of work are needed to generate a number of amplification fragments immediately detectable under UV light, the majority corresponding to genetic loci dispersed through the entire nuclear genome. Their use is relatively simple and inexpensive, making RAPDs suitable for the study of genotypic relationships in almond as well as in other perennial tree crops. In this preliminary report the RAPD procedure was used to assess the amount of polymorphism detected among several almond cultivars of Apulian origin and to estimate relationships and similarities, in order to evaluate the potential of these molecular markers in breeding programs as well as in germplasm conservation.

Material and methods

Plant material

Seventeen Apulian almond cultivars (*Amygdalus communis* L. = *Prunus dulcis* Miller) part of the almond cultivar collection at the Istituto di Coltivazioni Arboree at Valenzano, near Bari, were studied. The plant materials represent the regional population and were chosen because of the essential phenological, biological and morphological traits reported in Table 1.

DNA analysis

The genomic DNAs were isolated from mature leaves as in Bowers *et al.* (1993). RAPD materials and conditions of amplification were as in Resta *et al.* (1995). After a few analyses the volume of the amplification samples loaded in the DNA thermal cycler (Hybaid, UK) was scaled down from 20 to 12 µl with no change in the resulting profiles. The sixty random 10-mer primers were commercially available from Operon Technologies (USA), distributed as kits (M, S, and T, 20 primers each, numbered from 1 to 20). Records were kept as print and negatives from Polaroid 665 film. Fragment sizes were determined by comparison with lambda phage DNA digested with one (*Hind*III) or three (*Eco*R1, *Bam*HI, *Hind*III) restriction enzymes.

Analysis of RAPD profiles

Individual RAPD fragments for each primer-genotype combination were scored as 1 (presence) or 0 (absence), and a note of their sizes was made. The set of fragments co-migrating across the 17 cultivars was referred to as a band. The genetic relationships were estimated based on the matrix of polymorphic bands, from the pairwise comparison of cultivars using the simple matching (Ssm) coefficient of similarity (Sneath and Sokal, 1973). Cluster analysis was conducted on similarity coefficients using the unweighted pair group method arithmetic average (UPGMA) and the resulting clusters were expressed as a dendogram. The analysis was carried out on the NTSYS-pc (Rohlf, 1993) software program.

Results and discussion

The essential traits of the cultivars

Table 1 shows the essential traits of the cultivars involved. The chosen varietal sample may be considered representative of the native Apulian almond population. One of the 17 cultivars,

Sannicandro, is known to have originated in the province of Brindisi, whereas the remaining 16 are believed to be from the province of Bari. The sample, with regard to kernel taste, included 4 bitter almond cultivars. The main phenological trait, blooming time, varied from very early blooming to late blooming. With regard to self-compatible biological behaviour, 8 of the 17 cultivars were capable of setting fruit with their own pollen. The resulting nuts possessed very hard or hard shells, depending upon the cultivar, and consequently the shelling percentage ranged from low to medium. The kernel weight was always above 1.0 g and the samples exhibited a unusually high variability in the percentage of double kernels. The cultivars exhibited on average large values of kernel width/length ratios and 9 cultivars out of 17 showed broad ratios. The most extreme values were exhibited by Pizzoantonio, with a very narrow ratio (W/L=39.1), and Sannicandro, with a very broad ratio (W/L=90.1).

Table 1. Essential traits of the Apulian almond cultivars included in the RAPD study

Cultivar	N	Compa-tibility	Kernel taste	Blooming time	Shell type	Nut weight (g)	Kernel weight (g)	Shelling (%)	Doubles (%)	W/L
Falsa Barese	1	S	Sweet	Late	Hard	3.80	1.40	36.8	1.5	72.7
Garibaldina	2	I	Sweet	Medium	Very hard	5.00	1.40	28.0	1.0	63.6
Tuono	3	S	Sweet	Late	Semi-soft	3.80	1.50	39.5	17.5	62.9
Cinquantavignale	4	I	Sweet	Early	Very hard	5.11	1.30	25.4	1.3	54.9
Sannicandro	5	S	Sweet	Very early	Very hard	5.20	1.20	23.1	1.0	90.1
Fragiulio Grande	6	I	Sweet	Late	Hard	5.80	1.70	29.3	10.0	54.2
Filippo Ceo	7	S	Sweet	Medium	Hard	4.60	1.70	36.9	35.0	68.9
Rana Gentile	8	I	Sweet	Late	Hard	4.03	1.46	36.2	45.3	63.8
Padula di Terlizzi	9	I	Bitter	Medium	Very hard	5.47	1.70	31.1	39.0	43.4
Patalina	10	S	Sweet	Late	Very hard	5.90	1.42	24.1	10.0	62.2
Pasola	11	I	Bitter	Medium	Very hard	4.66	1.16	24.9	2.0	50.6
Santoro	12	I	Sweet	Medium	Hard	3.36	1.23	36.6	1.0	51.2
Genco	13	S	Sweet	Late	Hard	4.10	1.40	34.1	2.0	70.6
Pepparudda	14	S	Sweet	Medium	Hard	4.70	1.20	25.5	4.0	72.8
Rachele Grande	15	I	Sweet	Late	Hard	5.27	1.87	35.5	36.0	62.4
Pizzoantonio	16	I	Bitter	Medium	Very hard	4.60	1.39	30.2	14.0	39.1
Andria Amara	17	S	Bitter	Medium	Hard	3.08	1.05	34.1	0.0	58.7

N: Numbers from 1 to 17 identifying the cultivars in Fig. 1a,b
S: Self-compatible; I: Self-incompatible (Source: Godini, 1977b; Reina *et al.*, 1985; Godini *et al.*, 1992)
W/L: Width/Length kernel ratio x 100 (Source: Kester and Asay, 1975)
Blooming time (Source: Fanelli, 1939; Godini *et al.*, 1977)
Shell time (Source: Anonymous, 1981)

Band size and polymorphism

A total of 418 bands were scored from the comparison of amplifications with 60 primers of DNAs from 17 Apulian almond cultivars. Two to 23 bands per primer of variable lengths (0.3 to 2.5 kb) were detected. More than two thirds of the bands were larger than 350 but smaller than 1400 base pairs, and less than 7% larger than 2100 base pairs, i.e., mostly of small and medium size. The polymorphic bands were 241 (57.7%), and in average the band polymorphism per genotype was 3.4%. One general indication of the efficiency of a marker system is provided by the number of loci uncovered over a target group of genotypes by a random set of such markers. Several factors can affect the number of bands per primer detected, including: the species under study and the number of genotypes compared, the primer sequences, minor variations in the amplification protocol, and the scoring. Of the 60 primers used in this study, one failed to amplify (T-10), eight amplified profiles unsuitable for scoring (S-6, S-18, S-19, S-20, T-9, T-16, T-19), and four (S-14, S-16, T-8, T-13) amplified profiles with 12 bands, all invariant. Thus only 51 out of 60 primers were scored. The

average number of bands per primer detected over all the 60 primers tested was 4.0 polymorphic and 7.0 total (invariant plus polymorphic).

In comparable, reports the total bands per tested primer were: 14.7 in 23 *Brassica juncea* genotypes using 34 primers (Jain *et al.*, 1994), 10.5 in 19 *Brassica* accessions using 41 primers (Thorman and Osborne, 1992), 5.05 in 25 *Malus* rootstocks using 20 primers (Landry *et al.*, 1994), 4.3 in 15 *Pistacia vera* cultivars using 33 primers (Dollo *et al.*, 1994), and 3.1 in 21 *Vitis vinifera* cultivars using 60 primers (Resta *et al.*, 1995). One half (57.7%) of the 418 bands detected polymorphism in the almond cultivars analysed, a relevant fraction considering the presumably narrow gene pool. The genetic loci which were invariant are important for their potential to detect polymorphism in other *Prunus* genotypes.

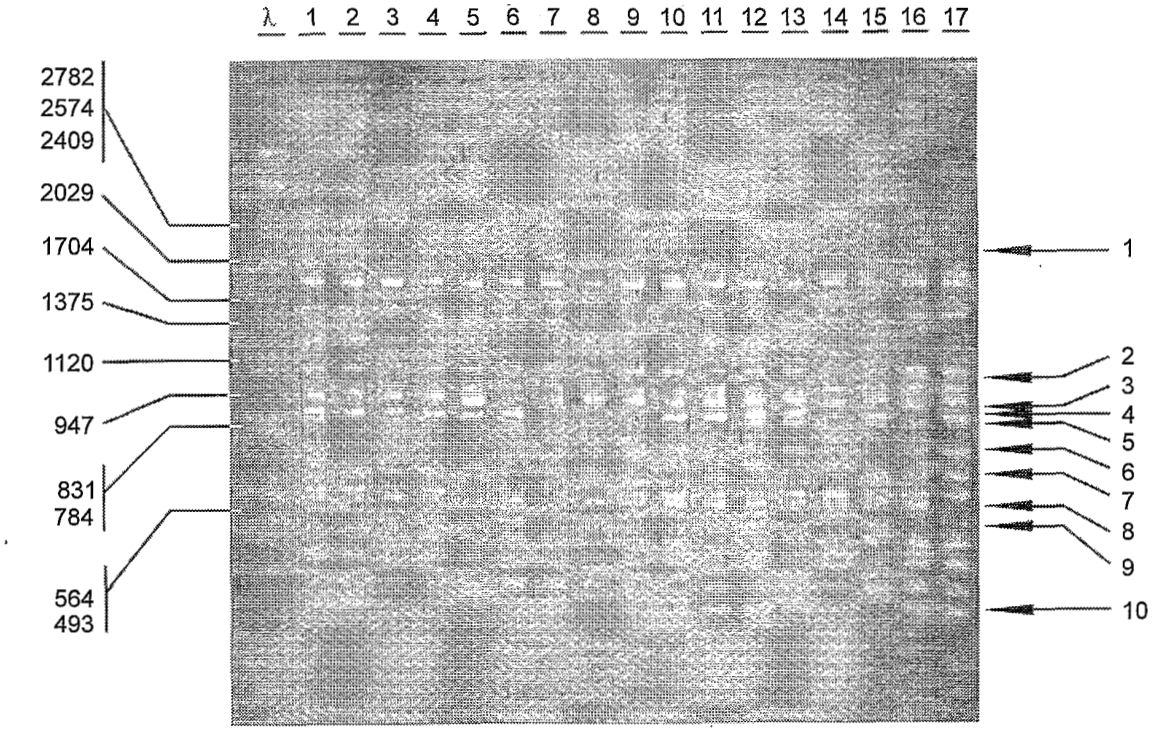
The use of primers selected for faithful reproduction of higher polymorphism in the target group of genotypes could further increase the efficiency and the applications of the RAPD approach. One example of more polymorphic primers (T-4) is in Fig. 1a, which shows the 17 cultivar profiles, each consisting of a number of amplified fragments. Each fragment was scored 0 or 1 in any single cultivar depending on absence or presence, as described in Material and Methods. The comparison of these scores to the scores previously assigned to a separate amplification reaction with the same primer showed that 15 bands were scored identically over the two experiments and one identically except for the attribution to one of the 17 genotypes. Since 6 of the 16 bands were invariant over all genotypes, Fig. 1b shows only the scoring of the remaining 10 polymorphic bands. Based on the scores, produced by the T-4 primer, only three pairs of cultivars resulted identical: Padula di Terlizzi and Pizzoantonio (cultivars numbered 9 and 16 in Table 1 and Figs 1a and 1b), Falsa Barese and Genco (1 and 13), Cinquantavignale and Fragiulio Grande (4 and 6). Thus with a single amplification it was possible to distinguish a total of 14 almond cultivars from the 17 tested, all from the same geographical region. Different combinations of two or three of such highly polymorphic primers were apparently able to distinguish more of the tested cultivars (not shown). It is worth mentioning that in the analysis of the genetic relationship, based on the comparison of 241 polymorphic bands (Fig. 2, and next paragraph), the two pairs Padula di Terlizzi and Pizzoantonio, and Falsa Barese and Genco clustered very close together, a clear indication that primer T-4 was very efficient in detecting polymorphism. Because of concerns over the reproducibility of RAPDs, further research is needed to assess its potential in cultivar identification and characterization of local germplasm in almond and other fruit tree crops.

Cultivar relationships

The dendrogram (Fig. 2) constructed on the basis of RAPD profiles provided some considerations. The first is that some cultivars appeared very similar, a close relationship being detected between the cultivars Padula di Terlizzi and Pizzoantonio (Similarity simple matching coefficient, Ssm=0.94) as well as between Genco and Falsa Barese (Ssm=0.91). In particular the biological and morphological trait similarities between Padula di Terlizzi and Pizzoantonio had been previously observed (Godini *et al.*, 1992). Moreover the two cultivars seem to originate from the same restricted area (Terlizzi, a town 20 km North-West of Bari). Thus, previous observations and the RAPD assay are consistent in showing that these two cultivars could have a similar or identical genotype. As for the pair Falsa Barese and Genco, they also seem to originate from a restricted area, located between the towns of Conversano and Castellana Grotte, 30 km South-East of Bari. Although not previously reported, a comparison of some of their essential traits (Table 1) does not contrast with the high similarity detected with the RAPD assay, thus indicating the possibility that these two cultivars are collateral and/or descendant.

A second consideration is that the dendrogram did not reveal other distinct clusters, even in the presence of relevant DNA polymorphism. The pseudo F and t^2 test were carried out to estimate the clustering of the aggregations revealed by the dendrogram. These tests indicated that the dendrogram might be truncated at the level of 0.75 (Fig. 2), where most of the cultivars tended to aggregate. The small differences among the coefficients of similarities (Ssm ranged from 0.64 to 0.78) caused a poor resolution of the aggregations. This result was expected since the cultivars grown in Apulia very likely originated from a narrow gene pool and were propagated from seedlings obtained by open pollination. Inclusion in the matrix of data originated from a larger gene pool or from an outgroup could be done in order to test this hypothesis.

a



b

Band No.	Cultivar No.																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1
2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
4	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1	0	1
5	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
6	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
7	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1
8	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0
9	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0
10	1	1	1	0	0	0	1	0	1	0	1	1	1	0	0	1	1

Fig. 1. (a) RAPD profiles of Apulian almond cultivars (1-17) using primer T-4; at the left base-pair size of Lambda DNA (λ) marker bands, at the right polymorphic bands numbered 1 to 10; number correspondence to cultivars is in Table 1. (b) Band score (0 = absence, 1 = presence) in the 17 cultivars of Fig. 1a.

A possible aggregation among a few almond cultivars was suggested by the principal coordinate analysis (not shown). One group might be formed including: Santoro, Cinquantavignale, Fragiulio Grande and Tuono, which are frequently found in the area South-East of Bari. A second group could be formed including: Padula di Terlizzi, Pizzoantonio, Pasola and Garibaldina, all self-incompatible cultivars spread in the North-West Bari area. The indication of such aggregations has a potential value to optimize parental choice in breeding crosses and for germplasm conservation programs.

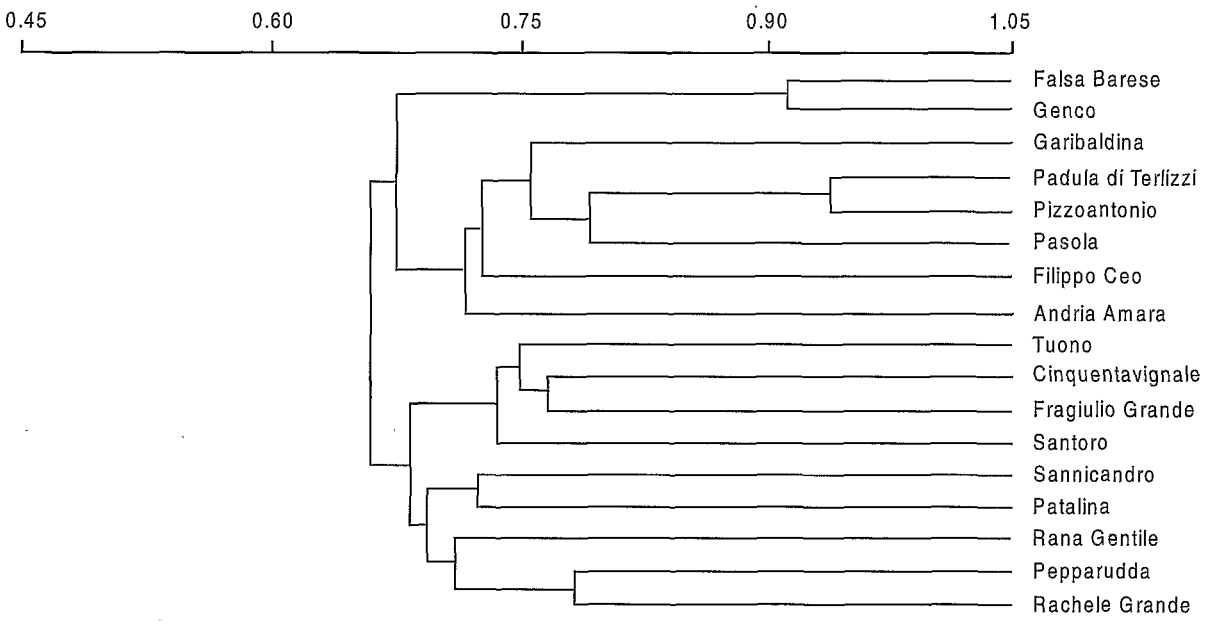


Fig. 2. Dendrograms of 17 Apulian almond cultivars from cluster analysis (UPGMA) of the genetic distances obtained with simple matching similarity coefficients, on the basis of RAPD data.

Conclusions

In conclusion, the results of this preliminary study on almond cultivars grown in Apulia were: (i) the detection of considerable variation at the DNA level; (ii) the selection of primers highly polymorphic; (iii) the revelation of high similarity between two pairs of cultivars; (iv) the indication of the absence of strong diversity trends and the possibility that two groups of cultivars are more closely related; and (v) the consistency of RAPD data with independent observations in estimating genetic relationships.

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