Characterization and Identification of Spanish Olive Germplasm by Means of RAPD Markers

A. Belaj, I. Trujillo, D. Barranco, and L. Rallo
Departamento de Agronomía. ETSIAM. Universidad de Córdoba. Avenida Ménendez Pidal s/n, Apdo 3048, 14080 Córdoba, Spain

Received for publication 29 July 2002. Accepted 29 July 2002.

Abstract. Thirteen randomly amplified polymorphic DNA (RAPD) primers were assayed in 82 Spanish olive cultivars of economical interest. A total of 82 bands were scored giving an average of 6.3 bands per primer. A total of 4 (OPA-01) to 10 bands (OPA-19) was amplified, while the number of polymorphic fragments ranged from 2 (OPK-07) to 9 (OPA-10), indicative of 5.7 polymorphic bands per primer. A total of 70% of the amplification products (73 bands) were polymorphic. The 13 primers yielded 184 banding patterns (14.9 per primer). The number of banding patterns per primer ranged from 4 (OPK-07) to 39 (OPA-19). Fifty-three unique banding patterns were found, the majority of which resulted from different combinations of polymorphic bands. The combination of five primers OPA-19, OPF-06, OPX-01, OPX-03, and OPI-12, allowed identification of all the cultivars. Seventy-four cultivars (90%) were identified only by the combination of the five primers. The addition of the fifth primer (OPI-12) was cost effective for the identification of the eight remaining cultivars (10%). The order of the primers according to their practical discriminating capacity in this study was: OPA-19 > OPF-06 > OPX-01 > OPX-03 > OPI-12. Hence RAPD markers are recommended for olive fingerprinting in order to generate a database for olive cultivar identification.

The olive is a characteristic plant of the Mediterranean basin. Its outstanding economical importance is mainly due to the use of the olive oil as the principal source of edible oil for the people of this area for millenia. Spain is the world’s leading olive producing country. As occurs in other olive producing countries, the distinctive feature of olive plant material in Spain is the abundance of very old cultivars restricted to specific areas where they originally grew (Barranco, 1997).

In an exploration survey of olive cultivars in Spain, carried out between 1972 and 1992, 262 different cultivars were identified (Barranco and Rallo, 2000). These have been conserved in the World Olive Germplasm Bank of the CIF”Alameda del Obispo”, Cordoba, Spain. The homonyms and synonyms have usually been troublesome in cultivar identification and management of olive genetic resources as names traditionally reflect common morphological traits (particularly of the fruit), place of origin or practical utility of the cultivars (Barranco and Rallo, 2000).

The high diversity of cultivars and the confusion in naming require precise methods of discrimination for cultivar identification. Precise and rapid cultivar identification methods are also required in the management of cultivar collections as well as in olive breeding programs. Furthermore, cultivar identification represents a very important aspect for plant certification schedules in nursery industry.

Morphological data are the principal descriptors used to detail the variability found in Spanish olive germplasm (Barranco, 1997; Barranco and Rallo, 1994; Barranco et al., 2000). Although these markers provide a very useful tool for identification, they are generally influenced by environmental factors and require a lengthy and expensive evaluation during the whole vegetative growth period. Enzyme markers have provided an alternative means of olive cultivar identification (Trujillo et al., 1995), but the use of pollen samples limit collection to mature trees at the time of flowering.

The introduction of DNA based markers provides an opportunity for genetic characterization that allows direct comparison of different genetic material independent of environmental influences. Among the different markers, randomly amplified polymorphic DNA (RAPDs) are well suited for fingerprinting genotypes because of their technical simplicity, speed and low cost (Williams et al., 1990). In olive these markers have been widely used as a means of cultivar identification and genetic variability studies (Belaj et al., 2001; Besnard et al., 2001a; Fabbris et al., 1995; Sanz-Cortés et al., 2001; Wiessman et al., 1998).

The present study reports use of RAPD markers on a set of 82 Spanish olive cultivars. Among them, outstanding productive cultivars such as ‘Picual’, ‘Arbequina’, ‘Manzanilla de Sevilla’, ‘Empeltre’, ‘Blanqueta’, ‘Hojiblanca’, ‘Morrison’, and ‘Villalonga’ (Barranco and Rallo, 2000) were included. Furthermore, the cultivars ‘Picual’ and ‘Arbequina’ have been used in an intraspecific breeding program carried out by the Dept. of Agronomy at Cordoba Univ. Our objective was to describe the RAPD diversity among the Spanish olive cultivars, and to determine if RAPD markers can be used to accurately discriminate among the cultivars.

Material and Methods

Plant material and DNA extraction. Eighty-two Spanish olive cultivars from the World Germplasm Bank of the CIF”Alameda del Obispo” in Cordoba, Spain, were studied (Table 1). These cultivars, chosen on the basis of their economical importance and geographic distribution (Barranco, 1997; Barranco and Rallo, 2000), were previously described by means of pomological characterization (Barranco and Trujillo, unpublished data). Genomic DNA was extracted from young leaf tissue collected in Spring 1999, following the method described by Belaj et al. (2001).

Polymerase chain reaction. Thirteen decamer oligonucleotides from kits A, F, I, J, K, P, Q, and X from Operon Technologies (Alameda, Calif.) were used for PCR amplifications. DNA was amplified in 20 µL reaction mixtures containing 20–40 ng of template DNA, 0.05 U polymerase AmpliTaq DNA Stooff fragment (Applied Biosystems, Foster City, Calif.), 0.75 mM each of dNTP (Roche, Basel, Switzerland), 20 µM of the primer, 25 mM MgCl2, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3). The reactions were performed in a thermal cycler (Geneamp PCR System 9600, Perkin-Elmer) programmed for: 1 cycle of 1 min at 94 °C followed by 40 cycles: of 20 s at 94 °C, 20 s at 35 °C, and 2 min at 72 °C, for denaturing, primer annealing, and extension, respectively. The last cycle was followed by incubation for 6 min at 72 °C. All the reactions were conducted three times using DNA from different extracts and different lots of the AmpliTaq DNA polymerase.

Separation and visualisation of the amplification products. Amplification products were separated on polyacrylamide gels as they provide a higher resolution of the amplification fragments in comparison to agarose gels (Belaj et al., 2001). Gels of 18 × 16 cm containing 10% acrylamide, 0.126% piperazine diacrylamide crosslinker in 0.375 M Tris-HCl, pH 8.8, were separated in Tris glycerine (0.025 M Tris, and 0.192 M glycerine) at 30 mA per gel for 3.5 h. The DNA was visualized by silver staining as described by Bassam et al. (1991). Gels were photographed using a digital camera (Kodak DC 120, Eastman Kodak Co., Rochester, N.Y.). Molecular sizes of the amplification products were estimated using a 123-base pair (bp) DNA ladder from Sigma (St. Louis).

Data analysis. RAPD bands were scored as 1 (present) or 0 (absent) in a binary matrix

HortScience, Vol. 39(2), April 2004
for each primer. A conservative criterion for the selection of bands was used. Only reproducible and well-defined bands in each of the three replications were considered. This frequency varied from 0.012 (bands present in only one cultivar of the 82 studied) to 0.988 (absent in only one cultivar of the 82 studied). In general, the average frequency of the above-mentioned bands was high (0.50).

The variability found with the 13 primers were 4 (OPA-01) to 10 bands (OPF-06) were scored giving an average of 6.3 bands (per primer). Four (OPA-01) to 10 bands (OPA-19) were discriminated with a single primer as a result of the presence of 53 unique patterns (14.9 per primer). The number of banding patterns per primer ranged from 4 (OPK-07) to 9 (OPA-19). Fifty-three unique banding patterns were found (Table 3), the majority of them resulted from different combinations of polymorphic bands found and a very few were obtained from the unique RAPD bands.

The minimum number of necessary primers for the identification of each one of the varieties (considering only the five above-mentioned markers) is presented in Table 3. The number of polymorphic fragments ranged from 2 (OPK-07) to 9 (OPA-19) with a mean of 5.7 polymorphic bands per primer. A total of 89% of the amplification products were polymorphic (73 bands).

The variability found with the 13 primers used made possible the identification of all the cultivars included in the study. The combination of five primers OPA-19, OPF-06, OPX-01, OPX-03, and OP-12 allowed identification of all the cultivars (Table 1). Other combinations of primers could provide additional possibilities for identification of the cultivars.

The minimum number of necessary primers for the identification of each one of the varieties (considering only the five above-mentioned primers) is presented in Table 3. Thirty-eight cultivars (≈45% of the total) were discriminated with a single primer as a result of the presence of 53 unique patterns for these cultivars (Table 3). Twelve out of the 24 major Spanish olive cultivars (Table 1) are included in this group. Furthermore, the cultivars ‘Menya’, ‘Pequeña de Casas Húehe’, ‘Canetana’, and ‘Manzanilla del Piquto’ were identified by unique patterns obtained by presence or absence of unique bands.

For the identification of the rest of the varieties the combination of bands was used. A total of 91% with a mean of 0.69 monomorphic bands per primer. The number of polymorphic fragments ranged from 2 (OPK-07) to 9 (OPA-19) with a mean of 5.7 polymorphic bands per primer. A total of 89% of the amplification products were polymorphic (73 bands).
BREEDING, CULTIVARS, ROOTSTOCKS, & GERmplasm RESOURCES

Fig. 1. PCR-RAPD products and schematic representation of some of the banding patterns obtained with the primer OPA-19 (a, b), the selected amplification products are indicated with arrows. The letters at the bottom of the photographs correspond to the banding patterns (b). The letters MP indicate the molecular marker. The molecular weight of the selected bands is indicated on the Y-axis (a).

Fig. 2. Percentage of the cultivars discriminated by a certain number of primers combination. 1 = OPA-19; 1 + 2 = OPA-19 + OPF-06; 1 + 2 + 3 = OPA-19 + OPF-06 + OPX-01; 1 + 2 + 3 + 4 = OPA-19 + OPF-06 + OPX-01 + OPX-03; 1 + 2 + 3 + 4 + 5 = OPA-19 + OPF-06 + OPX-01 + OPX-03 + OPI-12.

Table 2. Primers used, the total number of bands (T), polymorphic bands (P) and the number of banding patterns (BP) obtained for each primer.

| Primer | T | P | BP |
|--------|---|---|----|
| OPA-01 | 4 | 4 | 6  |
| OPA-03 | 6 | 4 | 11 |
| OPA-19 | 10 | 9 | 39 |
| OPF-06 | 7 | 7 | 25 |
| OPI-12 | 6 | 5 | 13 |
| OPI-14 | 6 | 6 | 12 |
| OPI-18 | 5 | 5 | 10 |
| OPI-07 | 5 | 2 | 4  |
| OPI-17 | 7 | 6 | 12 |
| OPI-19 | 6 | 6 | 13 |
| OPI-15 | 6 | 6 | 14 |
| OPIX-01 | 8 | 7 | 11 |
| OPIX-03 | 6 | 6 | 17 |
| Total: 82 | 73 | 184 |

*Information about the fingerprinting data obtained by each primer can be obtained from the authors upon request.

Discussion

The olive tree (Olea europaea L.) is considered a highly heterozygous species, therefore the polymorphism found in this study is expected. The autochtonous origin and very low pressure of selection throughout history of olive cultivation have influenced to the high diversity of olive cultivars in Spain (Barranco and Rallo, 1984; 2000). In terms of identified polymorphisms, our results agree with previous studies obtained by means of RAPD markers in olive (Belaj et al., 2001; Besnard et al., 2001b; Sanz-Cortés et al., 2001). To our knowledge, this study is the most complete study of Spanish olive cultivars in terms of plant material and it also represents the first attempt to create a fingerprinting database by means of RAPD markers for these cultivars.

The identification of cultivars is a very important application of molecular markers in olive. Therefore the potential of RAPD markers to yield different banding patterns for as many cultivars as possible is of great interest. And selection of the most discriminating primers reduces the cost of analysis (i.e. the number of primers and thus the number of amplifications) for reliable cultivar distinction (Tessier et al., 1999).

The combination of only four primers (OPA-19, OPF-06, OPX-01, and OPX-03) made possible the distinution of 90% of the cultivars included in the study. The discrimination power of each primer depends not only on the number of banding patterns generated but also on their frequency. The last primer (OPI-12) was kept for its ability to discriminate the few nondistinguishable pairs of cultivars and not for its efficiency. Identification of the majority of the cultivars (93.7%) by means of unique banding patterns or different combinations of the banding patterns provided by two primers is clear evidence of the high discrimination capacity of RAPD markers. However, discrimination of some of the most important
Spanish olive cultivars by cultivar-specific RAPD banding patterns provides the nursery industry with a powerful tool to certify their plant material. Previous studies carried out in olive by means of RAPD markers have reported the utility of these markers for reliable identification of a relatively high number of cultivars using a small number of primers (Belaj et al., 2001; Besnard et al., 2001b; Sanz-Cortés et al., 2001, Belaj, unpublished data). However, RAPDs present limitations when interpreting the results. The lack of reproducibility of the technique requires the following of a well-established protocol for routine use in the laboratory as well as a conservative criteria at the selection of the amplification products (Belaj et al., 2001).

As in previous investigations carried out by means of morphological (Barranco, 1997; Barranco and Rallo, 1984) and DNA markers (Belaj et al., 2001; Mekuria et al., 1999; Wiesman et al., 1998), our results confirm the hypothesis that generic names such as 'Gordal', 'Lechín', 'Manzanilla', 'Verdial', etc., include different cultivars. No differences were found among the amplification profiles obtained from individuals trees of the same cultivar (data not shown). This results suggest the usefulness of different markers for an effective conservation and use of olive genetic resources in germplasm banks. Despite their great power of discrimination, molecular techniques should not be considered as substitutes of morphological characterization, but as complementary tools for the study of genetic resources (Karp et al., 1997).

Table 3. Identification of cultivars by a minimum number of primers.

| Cultivar name | Primer |
|---------------|--------|
| **Cultivars identified by unique banding patterns (one primer)** | |
| Arbequina | OPA-19 (D); OPF-06 (D) |
| Argüelo | OPA-01 (D) |
| Asnal | OPA-19 (A) |
| Blanqueta | OPA-19 (AF); OPF-06 (F); OPX-03 (F) |
| Bolvino | OPF-06 (Y) |
| Buitrago | OPF-01 (K) |
| Calabujo | OPF-06 (F) |
| Callosina | OPA-19 (O) |
| Canéter | OPA-19 (Z); OPX-01 (F); OPF-06 (H) |
| Cathayano Blanco | OPA-19 (AL) |
| Castellana | OPA-19 (H) |
| Cornicabra | OPF-03 (H) |
| Empeltre | OPA-19 (J) |
| Genovesa | OPA-19 (K) |
| Gordal de Granada | OPA-19 (L) |
| Imperial | OPX-03 (J) |
| Lechin de Granada | OPA-19 (N) |
| Lechin de Sevilla | OPA-19 (P); OPX-03 (K) |
| Llimeta | OPA-19 (AG); OPF-06 (U); OPX-03 (P) |
| Lozama | OPA-19 (Q) |
| Lucio | OPA-19 (R); OPF-06 (J) |
| Manzanilla de Jaén | OPF-06 (Y) |
| Manzanilla del Piquito | OPX-01 (H) |
| Mencia | OPA-19 (U); OPF-06 (L); OPI-12 (N) |
| Morisca | OPF-19 (V) |
| Morot | OPF-06 (N) |
| Negrillo de Estepa | OPF-06 (N) |
| Ojo de Liebre | OPA-19 (A) |
| Palomar | OPA-19 (X); OPX-03 (L) |
| Pequeña de Casas Ibáñez | OPF-01 (I) |
| Pical | OPF-06 (P) |
| Raguay | OPF-06 (Q); OPX-03 (M) |
| Royal de Calatayud | OPF-06 (R) |
| Royal de Cazorla | OPA-19 (AC) |
| Vallesa | OPA-19 (AK); OPX-03 (N) |
| Verdial de Huevo | OPA-19 (AE) |
| Verdel | OPA-19 (AN) |
| Villalonga | OPF-06 (T); OPX-03 (O) |
| **Total** | 38 cultivars |

| Cultivars identified by the banding patterns obtained by two primers | |
|-----------------------------|---------------------|
| Alameño de Cabra | OPA-19 (A) + OPF-06 (A); OPA-19 (A) + OPX-03 (A) |
| Alameño de Montilla | OPA-19 (B) + OPX-01 (B); OPA-01 (B) + OPX-03 (B) |
| Almorin | OPX-01 (A) + OPX-03 (C) |
| Azul | OPA-19 (F) + OPF-06 (B); OPA-19 (F) + OPX-03 (B) |
| Bical | OPA-19 (G) + OPF-06 (E); OPA-19 (G) + OPX-03 (B); OPF-06 (E) + OPA-01 (A) |
| Carrasqueño Alcaudez | OPA-19 (F) + OPF-06 (A); OPA-19 (F) + OPX-03 (G) |
| Chattol Real | OPA-19 (G) + OPF-06 (B); OPA-19 (G) + OPX-01 (E); OPA-19 (G) + OPX-03 (G) |
| Chesna | OPF-06 (O) + OPX-01 (B) |
| Chorro | OPF-06 (G) + OPX-03 (H) |
| Corbella | OPF-06 (O) + OPF-01 (A); OPF-06 (E) + OPX-03 (H) |
| **Total** | 40 cultivars |

| Cultivars identified by the banding patterns obtained by three primers | |
|-----------------------------|---------------------|
| Aliforma | OPA-19 (A) + OPX-02 (I); OPA-19 (A) + OPX-03 (A) |
| Arbequina | OPA-19 (B) + OPX-06 (B); OPA-19 (B) + OPX-03 (A); OPA-19 (B) + OPX-03 (A) |
| Arbik | OPF-06 (J) |
| Arles | OPX-01 (G) + OPF-06 (B); OPA-19 (G) + OPX-03 (B) |
| Arzal | OPF-06 (A) + OPX-03 (A) |
| Biral | OPA-19 (G) + OPX-03 (B) |
| Bosca | OPA-19 (A) + OPX-03 (B) |
| Buitrago | OPA-19 (Z); OPX-01 (F); OPF-06 (H) |
| Cachala | OPA-19 (H) |
| Calavera | OPF-06 (O) + OPX-01 (B) |
| Cando | OPF-06 (G) + OPX-03 (H) |
| Caequeña | OPA-19 (F) + OPX-01 (A); OPF-06 (E) + OPX-03 (H) |
| **Total** | 4 cultivars |
The development in this research of RAPD markers/fingerprints of the main and industrially important olive cultivars from Spain may help for generating a molecular database to reliably catalogue the cultivars. The utilization of this information in a systematic way will facilitate the management of cultivar collections and control the trade of plant material. In order to complete and refine the RAPD data obtained in this study, further studies with larger olive samples and other types of molecular markers, which can permit the interchange of data between different scientific groups and laboratories, SSRs, (de la Rosa et al., 2002; Rallo et al., 2000) are needed. SSRs are currently being used for the characterization and identification of the Germplasm Bank of Cordoba.

Literature Cited

Barranco, D. 1997. Variedades y patrones. p. 59–80. In: D. Barranco, R. Fernández-Escobar, and L. Rallo (eds.). El cultivo del olivo. Mundiprensa y Junta de Andalucía, Madrid, Spain.

Barranco, D. and L. Rallo. 1984. Las variedades de olivo cultivadas en Andalucía. M.º de Agricultura, Junta de Andalucía, Madrid, Spain.

Barranco, D. and L. Rallo. 2000. Olive cultivars in Spain. HortTechnology 10:107–110.

Barranco, D., A. Cimato, P. Fiorino, L. Rallo, A. Touxani, C. Castañeda, F. Serafin, and I. Trujillo. 2000. World catalogue of olive varieties. Int. Olive Oil Council. Madrid, Spain.

Bassam, B.J., G. Caetano-Anollés, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 80:81–84.

Belaj, A., I. Trujillo, R. de la Rosa, L. Rallo, and M.J. Giménez. 2001. Polymorphism and discriminat- ing capacity of randomly amplified polymorphic markers in an olive germplasm bank. J. Am. Soc. Hort. Sci. 126:64–71.

Besnard, G., P Baradat, and A. Bervillé. 2001a. Genetic relationships in the olive (Olea europaea L.) reflect multilocal selection of cultivars. Theor. Appl. Genet. 102:251–258.

Besnard, G., C. Breton, P. Bérardat, B. Khadari, and A. Bervillé. 2001b. Cultivar identification in olive based on RAPD markers. J. Am. Soc. Hort. Sci. 126:668–675.

De la Rosa, R., C. James, and K.R. Tobutt. 2002. Isolation and characterization of polymorphic microsatellite in olive (Olea europaea L.) and their transferability to other genera in the Oleaceae. Primer note. Mol. Ecol. Notes. 2:265–267.

Fabbri, A., J.I. Hormaza, and V.S. Polito. 1995. Random amplified polymorphic DNA analysis of olive (Olea europaea L.) cultivars. J. Amer. Soc. Hort. Sci. 120:538–542.

Karp, A., S. Kresovich, K.V. Bhat, W.G. Ayad, and T. Hodgkin. 1997. Molecular tools in plant genetic resources conservation: A guide to the technologies. IPGRI Tech. Bul. No. 2. Intl. Plant Genetic Res. Inst., Rome.

Mekuria, G.T., G.G. Collins, and M. Sedgley. 1999. Genetic variability between different accessions of some common commercial olive cultivars. J. Hort. Sci. Biotechnol. 74:309–314.

Rallo, P., G. Dorado, and A. Martin. 2000: Development of simple sequence repeats (SSRs) in olive tree (Olea europaea L.). Theor. Appl. Genet. 101:984–989.

Sanz-Cortés, F., M.L. Badenes, S. Paz, A. Iliáquez, and G. Llácer. 2001. Molecular characterization of olive cultivars using RAPD markers. J. Am. Soc. Hort. Sci. 126:7–12.

Tessier, C., J. David, P. This, J.M. Boursiquot, and A. Charrier. 1999. Optimization of the choice of molecular markers for varietal identification in Vitis vinifera L. Theor. Appl. Genet. 98:171–177.

Trujillo, I., L. Rallo, and P. Arus. 1995. Identifying olive cultivars by isozyme analysis. J. Amer. Soc. Hort. Sci. 120:318–324.

Wiesman, Z., N. Avidan, S. Lavee, and B. Quebedeaux. 1998. Molecular characterization of common olive varieties in Israel and the West bank using randomly amplified polymorphic DNA (RAPD) markers. J. Amer. Soc. Hort. Sci. 123:837–841.

Williams, J.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tinge. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531–6535.