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R. la Rosa · A. Angiolillo · C. Guerrero · M. Pellegrini · L. Rallo · G. Besnard · A. Bervillé · A. Martin · L. Baldoni

A first linkage map of olive (Olea europaea L.) cultivars using RAPD, AFLP, RFLP and SSR markers

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Abstract The first linkage map of the olive (*Olea europaea L.*) genome has been constructed using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) as dominant markers and a few restriction fragment length polymorphisms (RFLP) and simple-sequence repeats (SSR) as codominant markers. Ninety-five individuals of a cross progeny derived from two highly heterozygous olive cultivars, Leccino and Dolce Agogia, were used by applying the pseudo test-cross strategy. From 61 RAPD primers 279 markers were obtained – 158 were scored for Leccino and 121 for Dolce Agogia. Twenty-one AFLP primer combinations gave 304 useful markers – 160 heterozygous in Leccino and 144 heterozygous in Dolce Agogia. In the Leccino map 249 markers (110 RAPD, 127 AFLP, 8 RFLP and 3 SSR) were linked. This resulted in 22 major linkage groups and 17 minor groups with fewer than four markers. In the Dolce Agogia map, 236 markers (93 RAPD, 133 AFLP, 6 RFLP and 4 SSR) were linked; 27 major linkage groups and three minor groups were obtained. Codominant RFLPs and SSRs, as well as few RAPDs in heteroduplex configuration, were used to establish homologies between linkage groups of both parents. The total distance covered was 2,765 cM and 2,445 cM in the Leccino and Dolce Agogia maps, respectively. The mean map distance between adjacent markers was 13.2 cM in Leccino and 11.9 cM in Dolce Agogia, respectively. Both AFLP and RAPD markers were homogeneously distributed in all of the linkage groups reported. The stearoyl-ACP desaturase gene was mapped on linkage group 4 of cv. Leccino.

Keywords Genome mapping · Amplified fragment length polymorphism (AFLP) · Random amplified polymorphic DNA (RAPD) · Restriction fragment length polymorphism (RFLP) · Simple sequence repeats (SSR)

R. la Rosa · L. Rallo · A. Martin
Dpto. Agronomia – Universidad de Córdoba, Av.da Menendez Pidal, S/N, 14080 Córdoba, Spain

A. Angiolillo · C. Guerrero · M. Pellegrini · L. Baldoni
Istituto di Ricerche sulla Olivicoltura, IRO-CNR, Via Madonna Alta, 128 06128 Perugia, Italy

G. Besnard · A. Bervillé
INRA, UR-GAP, UMR-DGPC, 2 Place Viala, 34060 Montpellier Cedex 1, France

L. Baldoni (✉)
CNR, Institute of Plant Genetics, Research Devision of Perugia, Via Madonna Alta, 130 06128 Perugia, Italy
e-mail: Luciana.Baldoni@igr.cnr.it
Tel.: 39-075-5014866
Fax: 39-075-5014869

Introduction

Olive (*Olea europaea* L.) is one of the most important fruit crops throughout the Mediterranean Basin. The species includes many thousand of varieties, most of them coming from empirical selections by growers throughout the centuries and still under cultivation because of their adaptation to the different local conditions (Besnard et al. 2001). The long generation time severely hinders classical breeding and genetic studies (Angiolillo et al. 1999), the use of forced growing protocols has greatly reduced the length of the juvenile phase, the evaluation of the agronomic traits related to the mature phase still requires at least 5 years (Santos Antunes et al. 1999). Furthermore, the genetic control of the most important traits, including disease and pest resistance, is still unknown.

The use of molecular marker technologies has offered new opportunities to develop early selection strategies in many fruit crops (Janick and Moore 1996; Staub et al. 1996). DNA-based markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers have been widely used for map construction, both combined (Park et al. 2000; Saliba-Colombani et al. 2000) or separately (Gentzbittel et al. 1995; Conner et al. 1997; Keim et al. 1997). Numerous molecular marker linkage maps have been recently constructed in different fruit tree crop species such as peach (Lu et al. 1999), kiwifruit (Testolin et al. 2001) and *Citrus*

(Sankar and Moore 2001), thereby facilitating the localization of genes controlling important traits in grapevine (Dalbo et al. 2000), *Citrus* (Deng et al. 2001) and peach (Jauregui et al. 2001). In apple, saturation mapping has led to the isolation of the apple scab resistance gene *Vf* (Xu and Korban 2000). Quantitative trait loci (QTLs) controlling fruit quality have been mapped in peach (Abbott et al. 1998; Dirlewanger et al. 1999), and molecular markers linked to QTLs governing yield and seed number have been identified in *Citrus* (Garcia et al. 2000).

AFLPs and RAPDs have mainly been used as markers for map construction because they can quickly generate a large number of polymorphisms, produce a large number of scorable loci per single assay and, generally, give significant coverage of the genome (Staub et al. 1996).

In outbreeding heterozygous perennial crops, such as olive, F_2 or backcross segregating populations are rarely available, and genetic mapping studies are commonly performed on progenies issued from the cross between two heterozygous parents. In this case, marker data can be analysed as a double pseudo-testcross and a map constructed separately for each parent (Grattapaglia and Sederoff 1994; Nikaido et al. 2000; Seefelder et al. 2000). Many of the maps of tree species were constructed using F_1 cross progenies and markers showing a testcross configuration – i.e. heterozygous in one parent and null in the other parent – leading to single-tree genetic linkage maps (Arcade et al. 1999; Lespinasse et al. 2000). Molecular markers have also been used for combining the genetic maps built from separate progenies (Maliepaard et al. 1998; Chevreau et al. 1999; Wang et al. 2002).

In olive, RAPD (Wiesman et al. 1998; Mekuria et al. 1999; Belaj et al. 2001), AFLP (Angiolillo et al. 1999), RFLP (Besnard et al. 2000) and simple sequence repeat (SSR) markers (Sefc et al. 2000; Cipriani et al. 2002) have been used for genetic diversity studies and cultivar identification, however, to date, there is almost no information available on segregation analysis of genes or molecular makers in an olive cross population. We report the construction of a first olive linkage map using a cross between two highly heterozygous olive cultivars (Leccino and Dolce Agogia) and employing dominant and codominant markers in a pseudo test-cross strategy. Due to the dominant nature of AFLPs and RAPDs and considering the use of a cross population, separate maps have been constructed because gametes segregate independently in each parent. A few RFLPs and SSRs were used to join the parental linkage maps. Additionally, some codominant heteroduplex RAPDs were also identified, as previously described and used in map merging (Davis et al. 1995; Novy and Vorsa 1996).

Materials and methods

Plant material

A progeny set of 100 individuals, derived by crossing two highly heterozygous olive (*Olea europaea* L.) cultivars, Leccino (female parent) and Dolce Agogia (male parent), was chosen for linkage map construction because previous screening performed by RFLP markers (data not shown) indicated that these cultivars were highly genetically distinct. The self-incompatibility of the Leccino cultivar, used as the maternal line, prevented the risk of contamination by self-pollination in the progeny.

This population was generated by the IRO-CNR (Perugia, Italy). Plants, about 15 years old, have been grown in pots and were unable to develop properly; therefore, most of them maintained the juvenile characters and did not flower.

DNA extraction

Total DNA was extracted from fresh leaves of the parental lines and seedlings according to the protocol previously established for olive (Angiolillo et al. 1999).

RAPD markers

RAPD analysis was carried out as described by Belaj et al. (2001) using polyacrylamide gel electrophoresis (PAGE) to resolve polymorphic bands. Eighty-five primers, previously selected for giving good amplification patterns in olive (Belaj et al. 2001), were tested in Leccino and Dolce Agogia and in 22 of their cross descendants. Of these 85, those generating segregating RAPD bands, were used on the entire population. The polymorphic bands were directly scored on the dried gels. Those RAPDs showing extra bands in the mix of parental DNA but not present in the DNA of the parents when separated were analysed as possible sources of heteroduplex codominant RAPDs (Novy and Vorsa 1996).

AFLP markers

AFLP analysis was performed as previously described for olive (Angiolillo et al. 1999). Nine *Eco*RI primers (ACA, AAC, AGC, ACT, AGG, ACG, ACC, AAG, ATC) and eight *Mse*I primers (CTC, CAG, CAC, CAA, CTG, CTT, CAT, CTA) with three selective nucleotides were used. A total of 21 primer combinations was screened (Table 2) among those previously tested because they provided the highest number of heterozygous bands.

RFLP markers

RFLP analysis was performed accordingly to the protocol used by Besnard et al. (2000). Fifty cDNA clones, coming from a lambda ZAP II cDNA library (provided by IRO-CNR) of olive fruit mesocarp at 7 weeks after blooming were used as probes. Both parents and 12 cross seedlings were checked for polymorphism. Probes/restriction enzyme combinations displaying polymorphism were used to characterize the rest of the progeny. In addition, to locate the stearoyl-ACP desaturase gene on the map, we used the olive cDNA clone (Baldoni et al. 1996) of the gene as a probe.

SSR markers

Six microsatellite loci were tested with respect to location on the maps using the following primer pairs: ME3OMS (Brachet et al. 1999), PAATT2, PAGA9, PAGA2, PAGA5 (Saumitou-Laprade et al. 2000) and Faemsatl4 (Lefort et al. 1999). Amplifications were performed following the author's recommendations.

Linkage analysis

Parental individuals and their cross progeny were scored for the presence or absence of given fragments. Polymorphic AFLP and RAPD fragments that were present in one parent, absent in the other and segregating 1:1 in the progeny, as in a testcross progeny, were considered to be mapping markers (Ritter et al. 1990). Only reproducible and well-defined bands were considered. Marker data

were scored as 1 (band presence) or 0 (band absence) jointly with the parental origin of the marker. Two data sets were obtained, one for each parent. For each marker the reversed genotype was also considered because their phase in the gametes was unknown. To allow the detection of these markers, linked in repulsion phase, the data were duplicated and recoded (indicated with R), as described by Grattapaglia and Sederoff (1994).

Segregation distortion from the expected 1:1 Mendelian ratio was evaluated applying the χ^2 values for goodness-of-fit at either the 5%, 1% and 0.1% levels of probability. Codominant markers (RFLP, SSR and RAPD heteroduplexes) were introduced in both data sets and later used to find homologies between linkage groups of both parents.

Linkage analysis was done using MAPMAKER 3.0 (Lincoln et al. 1992). Marker groups were determined with a LOD > 4.0 and recombination frequency less than 30. The relative order of the markers in each linkage group was determined with the option 'compare'. In those groups with ten or more markers, the nine with the largest LOD scores were linked with the 'compare' option. The remaining markers were introduced with the 'try' option. Finally, the 'ripple 3' and 'ripple 4' commands were used to obtain the best marker order. Linkage gaps longer than 25 cM were confirmed by linkage data of at least two markers on each side of the considered linkage gap (only one marker when the linkage gap was at the end of the linkage group). Lowering the LOD value from 4 to 3 has been attempted in order to reduce the number of linkage groups, but only the association of unlinked markers to the existing groups with a relatively weak linkage was obtained.

The genome length estimation was determined from partial linkage data according to 'method 3' of Chakravarti et al. (1991): G = N(N – 1)X/K, where G is the estimated genome length, N the number of markers analysed, N(N – 1) the number of pairwise comparisons, X the largest distance value (in centiMorgans) between linked markers and K the number of linked markers at different LOD values.

Results

Polymorphism detected

The SSR codominant markers enabled testing of the progeny before mapping and resulted in the exclusion of five individuals from the analysis due to suspected illicit pollination or self-pollination (Rallo et al. 2000).

From the 85 RAPD primers tested, 61 gave bands that were present in one of the parents and segregated 1:1 in the progeny. These primers were used for map construction. The percentage of heterozygous RAPD bands was considerably higher (83%) than that of the AFLPs (66%) in both cultivars (Table 1). They produced a total of 279 RAPD markers, 158 present in Leccino and 121 present in Dolce Agogia (Table 1), segregating 1:1 in the progeny. In the AFLP analysis, each primer combination amplified an average of 80 bands; 213 polymorphic bands were present in Leccino and 186 in Dolce Agogia (Table 1). Only 304 of the polymorphic AFLP bands were scored for genome mapping: 160 heterozygous in Leccino and 144 heterozygous in Dolce Agogia (Table 2). One hundred and forty-three AFLP bands and 87 RAPD bands were present in both parents (Table 1) and segregated 3:1 in the

Table 2 Primer combinations used for the AFLP markers in olive mapping and bands scored for the linkage analysis

Primer pair	A[a]	B[b]	Total no. of bands segregating 1:1
E-ACA/M-CTC	2	1	3
E-AAC/M-CAG	2	4	6
E-AGC/M-CAC	5	1	6
E-ACT/M-CAC	9	6	15
E-ACT/M-CAA	5	6	11
E-AGG/M-CTC	9	10	19
E-AGC/M-CTG	5	6	11
E-ACT/M-CAG	7	9	16
E-AAC/M-CTT	10	4	14
E-AGG/M-CAA	13	8	21
E-ACG/M-CAT	7	15	22
E-AGC/M-CTC	7	10	17
E-ACC/M-CTA	14	9	23
E-ACA/M-CAT	13	8	21
E-AAG/M-CAG	4	3	7
E-ACG/M-CTA	6	8	14
E-AGG/M-CTG	7	4	11
E-ATC/M-CAC	7	5	12
E-AAG/M-CAA	8	5	13
E-ACC/M-CTT	11	7	18
E-ACA/M-CTA	9	15	24
Total	160	144	304

[a] B and present (heterozygous) in Leccino, absent in Dolce Agogia and segregating 1:1 in the F_1 progeny
[b] B and present (heterozygous) in Dolce Agogia, absent in Leccino and segregating 1:1 in the F_1 progeny

Table 1 Total number of AFLP and RAPD bands obtained

	AFLPs	Percentage	RAPDs	Percentage
A-Bands present in Leccino, absent in Dolce Agogia and segregating 1:1 in the progeny	213[a]	17.8%	158	34.0%
B-Bands present in Dolce Agogia, absent in Leccino and segregating 1:1 in the progeny	186[a]	15.5%	121	26.0%
C-Bands present in both parents and segregating 3:1 in the progeny	143	11.9%	87	19.0%
D-Bands homozygous in Leccino, absent in Dolce Agogia and present in the entire progeny	32	2.7%	22	5.0%
E-Bands homozygous in Dolce Agogia, absent in Leccino and present in the entire progeny	22	1.8%	12	3.0%
F-Bands present in both parents (one parent homozygous and the other homozygous or heterozygous) and in the entire progeny	601	50.2%	70	15.0%
– Total heterozygous bands in Leccino (A + C + 1/2F)	656	66.4%	280	83.0%
– Total heterozygous bands in Dolce Agogia (B + C + 1/2F)	629	66.1%	243	83.8%
– Total bands in Leccino (A + C + D + F)	989		337	
– Total bands in Dolce Agogia (B + C + E + F)	952		290	

[a] From these total AFLP bands only those indicated in Table 2 were included in the maps

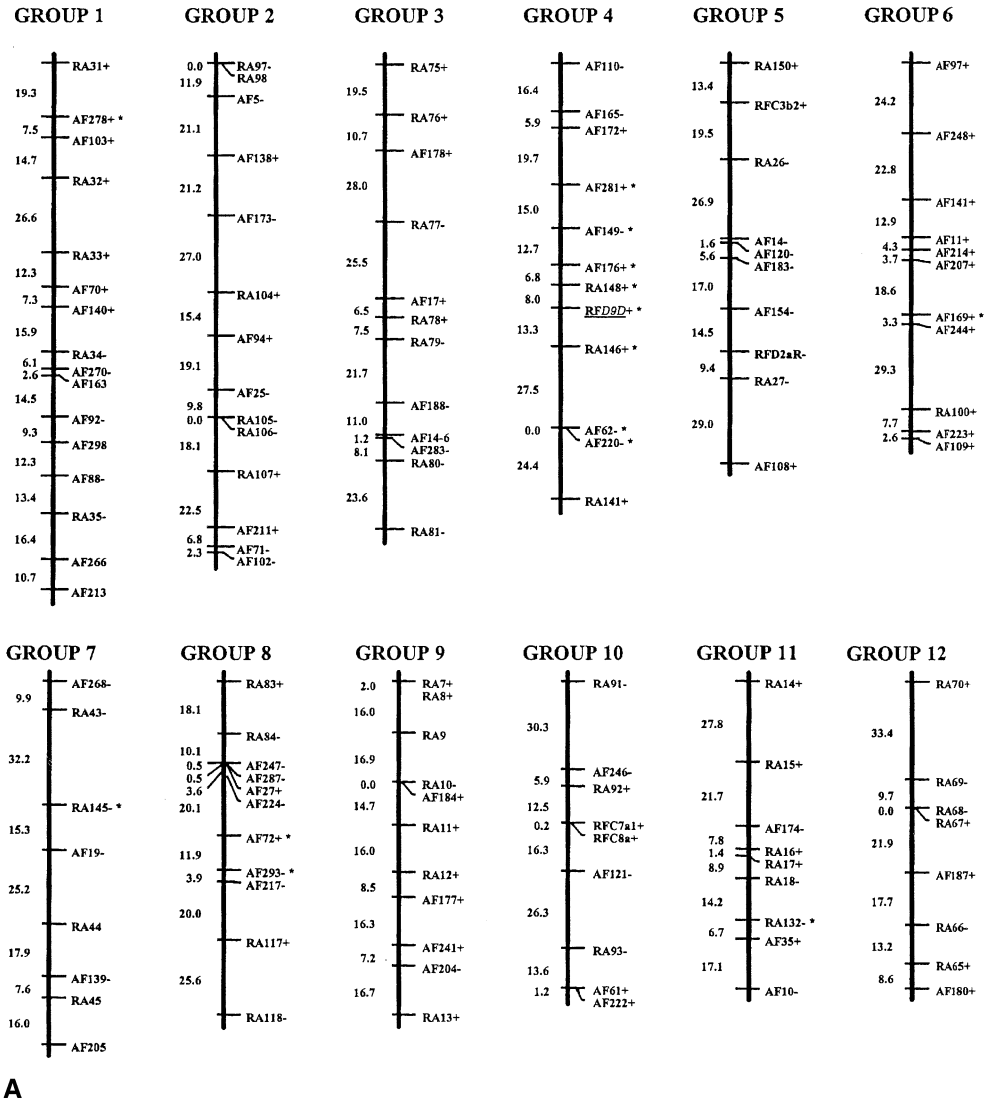


Fig. 1 Genetic linkage map of cv. Leccino. To the *right* of each linkage group is the marker name, to the *left* the distance (in centiMorgans) between markers. The type of marker is indicated by the *first two* letters of its name: *RA* RAPD, *AF* AFLP, *RF* RFLP, *SS* SSR. The linkage phase is indicated by: +coupling, –repulsion phase. *Underlined marker* (RF*D9D*) represents the stearoyl-ACP desaturase gene. Markers with * do not fit 1:1 Mendelian ratio ($P < 0.05$)

progeny, because both parents were heterozygous at those loci. These bands were not included in the screening because it was impossible to distinguish the heterozygous from the homozygous progeny carrying the band, and these less informative markers could not be used with MAPMAKER to join the maps of the parentals.

Only 13 out of 50 RFLP cDNA probes displayed segregating polymorphisms, and nine of these performed as codominant markers and thus were located on the maps. Four showed alleles simultaneously segregating in both parents, and these were used for map combination. In addition, the probe/enzyme combination stearoyl-ACP desaturase/*Hind*III led to a segregating RFLP allele derived from the Leccino parent.

Four out of five SSR loci showed alleles segregating in at least one of the parents, and these were located on the maps. No homologies were established with SSRs between linkage groups of both cultivars.

Three RAPD heteroduplexes were also identified. They were represented by different codes in the two maps (Table 4) because they had been previously identified as separate markers in each map and the original codes were still maintained after their relationship was established.

Marker segregation

The results of the AFLP and RAPD segregation at either the 5%, 1% or 0.1% level of probability applying the χ^2 values for goodness-of-fit to 1:1 Mendelian ratio are reported in Table 3. A total of 17 RAPD (6.6%) and 61 AFLP markers (16.8%) were significantly skewed from the 1:1 Mendelian ratio. To construct the map backbones, we used only the non-distorted markers. Subsequently, those showing skewed segregation ratios were added. A direct inclusion of all the markers at the same time did not change the position of the markers nor of the observed linkage groups, except for two (RA145 and RA131) in Group 7 and Group 24 of Leccino, respectively. In these

Fig. 1 (continued)

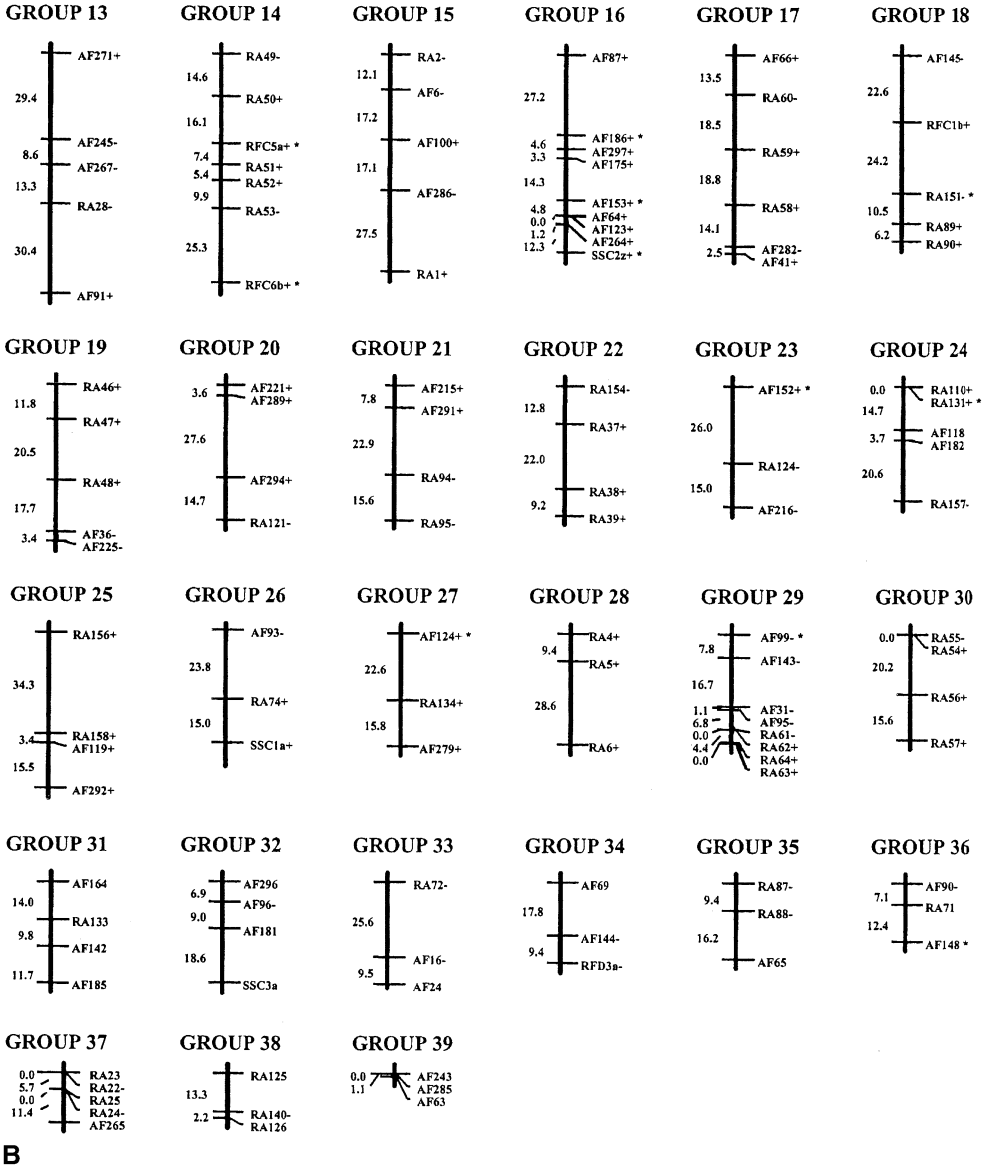


Table 3 Distribution of χ^2 values showing the distortion from the expected 1:1 Mendelian segregation ratio

	AFLP (%)	RAPD (%)
Markers segregating 1:1	83.2	93.4
Markers distorting at 5% level of probability	8.4	3.9
Markers distorting at 1% level of probability	5.4	1.8
Markers distorting at 0.1% level of probability	3.0	0.0

cases, an inversion in the position of two neighbouring markers was noted but, due to relative insignificance of these minor differences, all the skewed markers were included in the maps.

RFLP and SSR markers displayed no significant deviation from 1:1 ratio.

Linkage analysis

The map was constructed following the pseudotest-cross strategy (Grattapaglia and Sederoff 1994): two maps were generated with the markers polymorphic in each of the parents (Figs. 1 and 2).

In the Leccino map, 248 markers (110 RAPD, 127 AFLP, eight RFLP and three SSR) were linked out of the 332 identified in this parent. This resulted in the establishment of 22 linkage groups consisting of more than five markers and 17 minor linkage groups with three or four markers. In the Dolce Agogia map, 236 markers (93 RAPD, 133 AFLP, six RFLP and four SSR) were linked out of the 278 found for this parent. In this case, 27 linkage groups were obtained, with only three minor groups. Common RFLP, SSR and RAPD codominant markers enabled the establisment of homologies between linkage group pairs of both parents (Table 4).

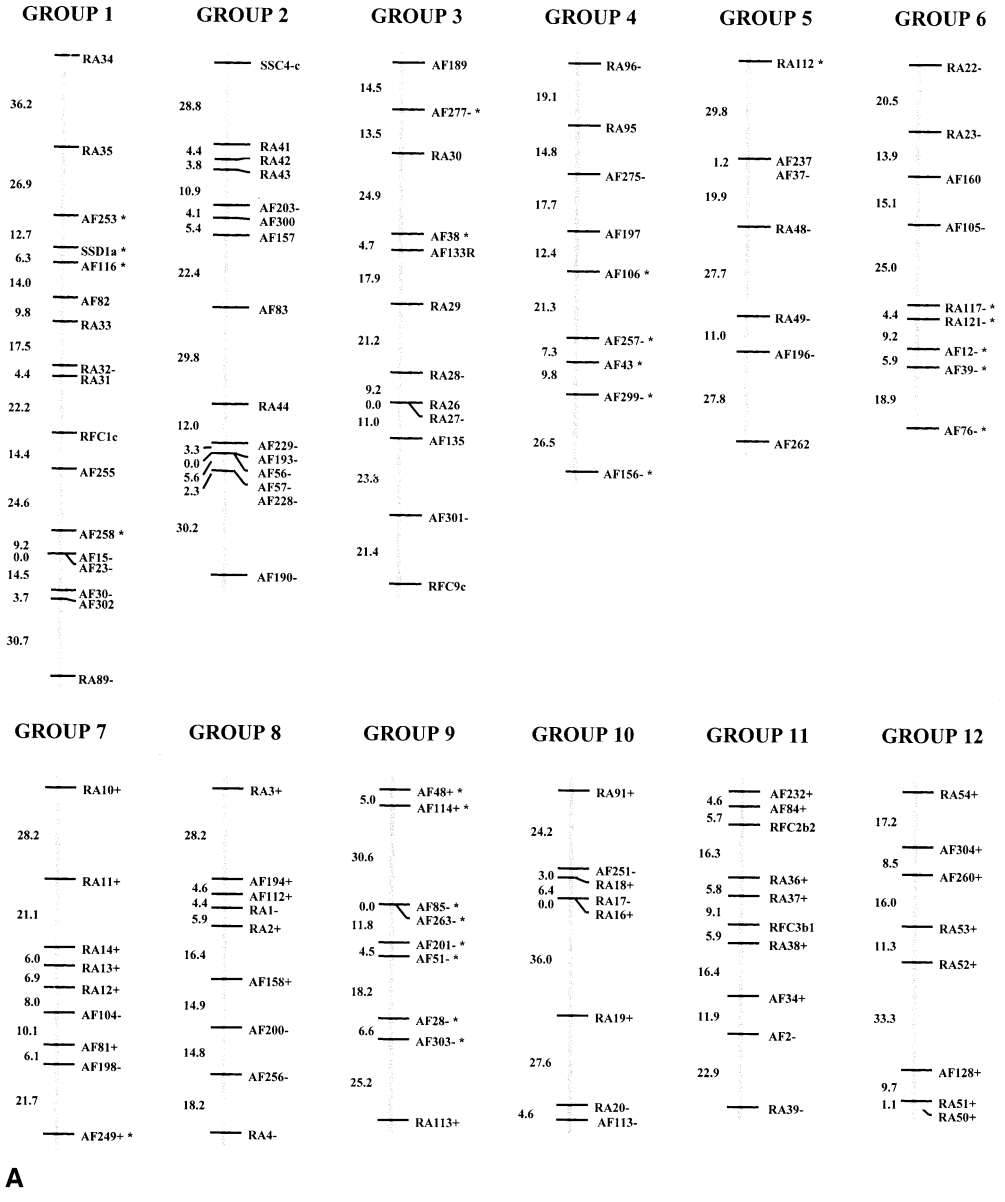


Fig. 2 Genetic linkage map of cv. Dolce Agogia. To the *right* of each linkage group is the marker name, and to the *left* the distance (centiMorgans) between markers. The type of marker is indicated by the first two letters of its name: *RA* RAPD, *AF* AFLP, *RF* RFLP, *SS* SSR. The linkage phase is indicated by: +coupling, –repulsion phase. Markers with * do not fit 1:1 Mendelian ratio ($P < 0.05$)

Table 4 Homologies established between linkage groups of both parents based upon codominant markers

Codominant markers	Leccino linkage group number	Dolce Agogia linkage group number
RFC1	18	1
RFC3b	5	11
RFC7a	10	25
RFC8	10	25
RA69-RA53	12	12
RA150-RA38	5	11
RA7-RA117	9	6

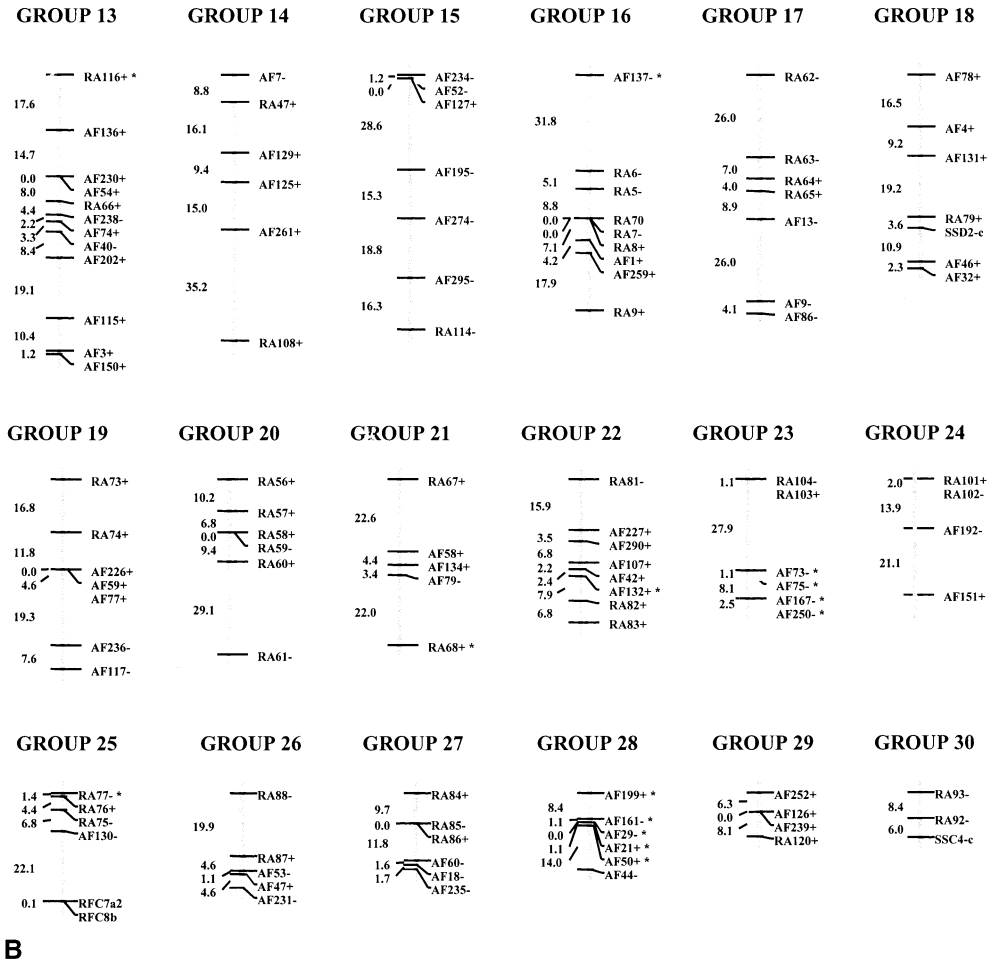
The total distance covered was 2,765.3 cM and 2,445 cM in the Leccino and Dolce Agogia maps, respectively. According to the Chakravarti formula the genome length was estimated as 6,465 cM for Leccino and 6,608 cM for Dolce Agogia.

Mean map distance between adjacent markers was 13.2 cM in Leccino and 11.9 cM in Dolce Agogia, and the size of the linkage groups was 70.9 cM and 81.5 cM, respectively. Both AFLP and RAPD markers were homogeneously distributed in all of the linkage groups in both the coupling and repulsion phase (Figs. 1 and 2). The stearoyl-ACP desaturase gene was linked to the Leccino Group 4 (Fig. 1).

Discussion

Olea europaea has a high number of chromosomes ($2n = 46$), and their size is so small that even the position of the centromere cannot always be recognized with certainty (Minelli et al. 2000). Nevertheless, most of the chromosome pairs have been distinguished (Falistocco and Tosti

Fig. 2 (continued)



B

1996), and structural heterozygosity was observed in chromosome pairs I, V and VII of cv. Coratina (Minelli et al. 2000). This was most likely due to redundancy of some repeated DNA sequences, related to the origin of the cultivars from crosses among different forms of the olive species. The DNA content per haploid nucleus was estimated in cv. Leccino to be 2.26 pg (Rugini et al. 1996), corresponding to a total estimated genome size of 2,200 Mb.

We have constructed the first linkage maps reported for the olive species, including AFLP, RAPD and a few RFLP and SSR markers. Dominant AFLP and RAPD markers were used to construct the main frame of the linkage maps applying the pseudo-test cross strategy, and they were included in the Leccino and Dolce Agogia linkage groups, in coupling and repulsion phase, giving consistency to the maps.

The use of an intraspecific cross progeny did not affect the map construction because the level of polymorphism between the cultivars was high (38%), as was the percentage of heterozygous loci, 66% for AFLP and 83% for RAPDs in both parents. Therefore, although the total number of bands scored for AFLPs was considerably higher (more than double) than that scored for RAPDs, the lower frequency of heterozygous (and therefore mappable) bands was similar, making both techniques suitable for the construction of linkage maps in olive.

The suitability of AFLP markers for high-throughput mapping has been well-documented for other crop species. When AFLPs were used to integrate previous RFLP maps they were able to expand the total map distance up to 40%, such as in the case of doubled haploid populations of barley (Becker et al. 1995) and rice (Maheswaran et al. 1997). In addition, when used to construct maps in fruit crop species, they were able to cover 80% of the estimated genome length in peach (Zhen-Xiang et al. 1998) and 46% in kiwifruit (Testolin et al. 2001). Furthermore, in olive the number of AFLP fragments was not influenced by the selective nucleotides of the primers, unlike what was reported for peach (Zhen-Xiang et al. 1998). This is probably due to the differences in the repetitive parts of the genomes of the different species.

The percentage of cDNA probes suitable for locating RFLPs on the maps was low, and even lower was the percentage of those probes (4 out of 50) able to establish homologies between linkage groups of both parents (with alleles segregating in both cultivars). Due to these poor results, the suitability of this kind of marker to link the maps was considered questionable and, thus, they were

not identified (unless the stearoyl-ACP desaturase cDNA clone) and their putative function not verified. On the contrary, the use of SSR markers for this kind of purpose is emphasized.

The percentage of AFLP markers showing skewed segregation (16.8%) was similar to that obtained in peach (15%, Zhen-Xiang et al. 1998) but higher than that reported in *Populus* (7%, Wu et al. 2000), while that of RAPDs (6.6%) was considerably lower than that observed in numerous tree species, such as *Pinus* (14–15%, Kubisiac et al. 1995) and *Quercus* (18%, Barreneche et al. 1998). One SCAR marker generated from a RAPD has also shown regular Mendelian segregation in olive (Hernandez et al. 2001).

The reason of the higher distorted segregation for AFLPs in olive is unknown. Distorted segregation ratios, which occur in several annual and perennial plants, may be attributed to the presence of incompatibility or lethal alleles, as in sugar beet (Pilien et al. 1993), or may result from competition among gametes or from abortion of the gamete or zygote, such as in *Aegilops* (Faris et al. 1998). Nevertheless, the inclusion of skewed markers in the olive maps did not affect the linkage arrangement, and they were distributed along the linkage groups (Figs. 1 and 2) with normally segregating loci. Some of them were linked together, such as the case of five AFLP, two RAPD and one RFLP (allele of the stearoyl-ACP desaturase gene) which were linked to the Leccino Group 4 (Fig. 1), while eight, five and five AFLP loci were respectively linked to Groups 9, 4, and 28 of the Dolce Agogia male parent map (Fig. 2). Markers with skewed ratios distributed in different linkage groups could be due to heterogeneous transmission of chromosome fragments to progenies (Quillet et al. 1995), while those grouped together could be linked to genes controlling microspore embryogenesis, as has been recently described in maize (Dufour et al. 2001).

The maps obtained in this study linked 249 and 236 markers in Leccino and Dolce Agogia and covered 2,765.3 cM and 2,445 cM in each cultivar, which represent 42.7% and 37.0% coverage of the estimated genome length, respectively. However, the high number of olive chromosomes and their small size (Minelli et al. 2000) may have caused an oversizing of the estimated length as calculated with the Chakravarti formula and, as a consequence, the percentage of genome coverage could be higher than that reported here. The maps have still not coalesced into the 23 linkage groups expected for *O. europaea* because of the presence of minor linkage groups and unlinked markers. This suggests that the markers identified are not randomly sampling the genome or that hot spots of recombination exist (Wu et al. 2000) or, finally, the population is too small.

No phenotypic traits were located on these maps due to the fact that the progeny under study was still growing in pots and most of the plants had not yet reached sexual maturity, in spite of their age. Consequently it was impossible to evaluate the segregation of any character.

During the last few years different cross progeny populations have been generated for genome mapping purposes, and their segregation for many traits is still under evaluation. The availability of the present linkage maps, besides providing new insight into the genetic structure of the species, will serve as a reference to increase the resolution of future maps and will be very useful in consolidating linkage groups and detecting possible chromosomal rearrangements between parental lines. Thus, with further work the map should become a valuable reference tool for olive breeding and for fundamental research into the genetics of this agriculturally and environmentally important species of the Mediterranean area.

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