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Identifying Olive Cultivars by Isozyme Analysis

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Abstract. **Pollen samples of 155 olive *(Olea europaea* L.) cultivars from different origins were analyzed to study isoenzymatic variability in five enzyme systems: alcohol dehydrogenase (ADH), esterase (EST), glucose phosphate isomerase (GPI), leucine aminopeptidase (LAP), and malic enzyme (ME) using starch gel electrophoresis. Polymorphism was observed in all of the isozyme systems. ME, GPI, EST, and LAP were the most useful systems for identification of cultivars. Different combinations of banding patterns of these systems allowed us to identify 85% of the cultivars. The remainder were separated into groups of two or three cultivars that could be identified using morphological characteristics. No intracultivar polymorphisms were observed.**

The olive has been for millenia the most important oleaginous crop of the Mediterranean basin, where 95% of the cultivated world area occurs.

The outcrossing origin of the species and the introgression of genes from wild into cultivated olives (Zohary and Spiegel Roy, 1975) resulted in high diversity of cultivars in all mediterranean countries (Barranco and Rallo, 1984, 1985; Bottari and Spina, 1953; Leitão, 1988; Loussert and Brousse, 1980; Morettini, 1972; Valdeyron and Crossa–Raynaud, 1950). Systems of classification and identification based on quantitative and/or qualitative morphological characters were used by the above cited authors to study this diversity. Although these methods were effective, they presented practical drawbacks due to effect of environmental fluctuations on expression of most morphological traits. The use of biochemical markers such as isozymes overcome these problems since they are little affected by the environment and can easily be detected in a variety of tissues by relatively simple, rapid and inexpensive procedures. However, their use in cultivar identification requires the presence of high intercultivar isozyme polymorphism as well as the absence of intracultivar isozyme polymorphism (Weeden and Lamb, 1985).

Isozymes have been successfully used for cultivar identification in several fruit species as apricots (Byrne and Littleton, 1989), almond (Cerezo et al., 1989), cherimoya (Ellstrand and Lee, 1987; Pascual et al., 1993), mango (Degani and El Batsri, 1990), apple (Weeden and Lamb, 1985), and grape (Parfitt and Arulsekar, 1989; Stavrakakis and Loukas, 1983).

Pontikis et al. (1980) found high isoenzymatic variability in olive pollen samples of 27 cultivars, mostly of Greek origin. All cultivars could be identified with only two out of the sixteen enzyme systems used.

The objective of the present study was to identify a wide range of olive cultivars from 13 countries using isozyme analysis of pollen samples.

Materials and Methods

Pollen samples were collected from 155 cultivars growing at the germplasm collection of the Centro de Investigación y Desarrollo Agrario at Córdoba, Spain. Cultivar names, number of trees sampled, and origins are shown in Table 1.

Pollen was collected according to the method described by Pontikis et al. (1980) and stored at –20C until analyzed. Pollen samples (40 mg) were crushed with 0.15 ml extraction buffer (0.61 M tris HCL, pH 8, containing 1% w/v reduced glutathione).

Electrophoretic analysis was performed on a horizontal slab (gel form: 23 × 20 × 1 cm, 12% Connaught hydrolyzed starch) according to a method described by Shields et al. (1983). The extracts were absorbed into paper wicks (1.5 × 0.3 cm, Whatman no. 3) and inserted into a vertical cut in the gel.

Three gel-electrode buffers were used: tris-citrate, pH 7.8 (TC 7.8); h-is-citrate, pH 7 (TC 7); and histidine, pH 5.7 (H 5.7). The composition of the buffers TC 7.8 and TC 7 are described in Shields et al. (1983). Histidine 5.7 gel buffer was prepared with 0.006 M, L-histidine, pH 5.7. The electrode buffer for H 5.7 gels was prepared with 0.13 M tris and 0.04 M citric acid, pH 7. Electrophoresis was run under constant voltage at 4C. The voltage conditions were 100 V (H 5.7) and 150 V (TC 7.8, TC 7) for the first 35 min. At this point the paper wicks were then removed and the voltage was increased to 200 V (H 5.7) for 3 h and to 300 V (TC 7.8, TC 7) for 4 h.

Optimal resolution was obtained when using TC 7.8 gels and corresponding electrode buffers for the staining of ADH (E.C. 1.1.1.1), EST (E.C. 3.1.1.–), LAP (E.C. 3.4.11.1), TC 7 for GPI (E.C. 5.3.1.9), and H 5.7 for ME (E.C. 1.1.1.40).

The gels were sliced horizontally after electrophoresis and immediately stained for enzyme activity according to the methods described for ADH and GPI by Vallejos (1983) and for EST and LAP by Mora and Serradilla (1985). ME gels were stained in 90 ml 0.1 M tris pH 8, 10 mg nicotinamide adenine dinucleotide phosphate (NADP), 20 mg methyl thiazolyl tetrazolium (MTT), 5 mg phenazine methosulfate (PMS), 10 ml 1 M sodium L-malate, pH 7.5 and 2 ml 10% $MgCl_2$.

Results and Discussion

Polymorphism was observed in all enzyme systems for the cultivars studied. On the other hand, the banding patterns obtained in samples collected from the same trees in different years or from several trees of the same cultivars either from the same or different origin were consistent and showed no differences.

The zones of activity and bands detected were numbered according to their proximity to the anodal end. The intensity of the

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Table 1. Isozyme phenotypes of 155 olive cultivars.

Cultivar	Origin[y]	Banding patterns					Identification [x]	
		ADH	ME	EST	GPI	LAP	SO	c o
Aglandou	Fr	A	S	B	E	A	*	*
Alameño Blanco	Sp	A	B	D	C	C	*	*
Alameño de Cabra	Sp	A	B	F	M	H	*	*
Alameño de Marchena'	Sp	A	D	D	C	D	*	*
Alameño de Montilla (3)	Sp	A	G	G	N	C	*	*
Amigdaloia	Gr'	A	E	E	D	A	*	*
Arbequina (2)	Sp	A	A	M	E	C	*	*
Ascolana Tenera	It	A	A	G	A	I	*	*
Ayrouni	Lb	A	A	A	C	C	f	*
Azeitem	Pt	A	E	D	D	C	*	*
Azul	Sp	A	I	F	N	C	*	*
Beladi	Lb	A	H	B	G	B	*	*
Blanqueta (2)	Sp	A	J	F	G	B	*	*
Bouteillan	Fr	A	G	B	I	A	*	*
Buidiego	Sp	A	Q	R	C	D	*	*
Caballo	Sp	A	T	A	D	C	*	*
Callosina	Sp	A	I	C	M	H	*	*
Campanil	Sp	A	G	F	M	D	*	*
Caninese	It	B	J	A	V	A	*	*
Cañivano Blanco	Sp	A	B	D	C	D	a	*
Cañivano Negro	Sp	A	A	B	D	D	b	b
Carboncella	It	A	C	H	F	H	*	*
Carrasqueño de Alcaudete	Sp	A	A	E	D	D	*	*
Carrasqueño de Elvas	Pt	A	A	D	C	D	*	*
Carrasqueño de la Sierra	Sp	A	O	A	D	H	*	*
Carrasqueño de Lucena	Sp	A	A	P	G	D	*	*
Cellina	It	A	J	N	Q	F	*	*
Cerezuela	Sp	A	I	B	G	D	**	*
Cipresino	It	A	K	D	I	G	*	*
Cobrançosa	Pt	A	E	D	C	C	b	*
Coratina	It	A	J	T	J	G	*	*
Cordovil de Serpa	Pt	A	B	E	D	D	k	*
Cornezuelo (3)	Sp	A	v	A	G	D	*	*
Cornicabra (4)	Sp	A	G	F	G	D	*	*
Cornicabra Parda de Villena	Sp	A	E	K	N	H	*	*
Chalkidiki	Gr	A	V	B	G	H	*	*
Chami	Sr	A	D	B	W	A	*	*
Changlot Real	Sp	A	J	B	D	D	c	c
Chetoui	Tn	A	C	s	J	A	*	*
Chorrúo (2)	Sp	A	G	L	D	C	*	*
Chorrúo de Fruto Redondo	Sp	A	G	A	U	D	*	*
Dam	Sr	A	D	B	U	A	*	*
Dolce Agogia	It	A	J	I	L	B	*	*
Domat	Tq	A	U	B	K	D	*	*
Dulzal	Sp	A	—	B	U	c	*	*
Dulzal-1	Sp	A	R	E	G	c	*	*
Edremit Yaglik	Tq	A	u	B	w	c	*	*
Empeltre (2)	Sp	A	I	B	c	D	*	*
Escarabajuelo-1	Sp	A	A	D	D	c	*	*
Escarabajuelo de Ubeda	Sp	A	K	I	D	D	*	*
Frantoio	It	A	J	I	Q	F	*	*
Frantoio A. Corsini	It	A	J	I	F	F	*	*
Galego (2)	Pt	A	S	K	G	C	*	*
Genovesa	Sp	A	J	B	D	D	c	c
Golbina de Belchite	Sp	A	C	E	F	H	*	*
Gordal Sevillana (5)	Sp	A	L	F	D	c	d	*
Habichuelero	Sp	A	B	D	D	D	*	*
Hojiblanca (3)	Sp	A	I	C	B	c	*	*
Hrai Souni	Sr	A	B	G	O	c	e	*

Table 1. (continued).

Cultivar	Origin	Banding patterns					Identification[x]	
		ADH	ME	EST	GPI	LAP	SO	CO
Imperial	Sp	A	U	A	C	H	*	*
Imperial de Jaén	Sp	A	G	A	G	C	*	*
Itrana	It	A	A	E	N	C	*	*
Jabaluna	Sp	A	K	U	D	D	*	*
Jaropo	Sp	A	B	E	D	C	*	*
Kaesi	Sr	A	D	B	P	A	*	*
Kalamon	Gr	B	N	L	R	C	*	*
Kelb et Ter	Sr	A	–	F	R	H	*	*
Kelb et Ter–10/13	Sr	A	B	C	O	C	*	*
Koroneiki	Gr	A	M	B	W	H	*	*
Leccino	It	A	T	B	J	E	*	*
Leccio di Corno	It	A	Y	B	I	E	*	*
Lechín de Granada	Sp	A	W	A	N	C	*	*
Lechín de Sevilla (6)	Sp	A	B	G	O	C	e	*
Limoncillo (2)	Sp	A	A	A	C	D	*	*
Macho de Jaén	Sp	A	B	B	C	H	*	*
Madural	Pt	A	B	E	D	D	k	k
Manzanilla de Jaén (3)	Sp	A	B	I	C	D	*	*
Manzanilla del Piquito	Sp	A	A	A	C	C	f	f
Manzanilla Prieta	Sp	A	G	A	C	C	*	*
Manzanilla de Sevilla (7)	Sp	A	B	F	G	C	*	*
Manzanilla de Tortosa	Sp	A	N	B	E	C	*	*
Maoui Stambouli	Sr	A	B	F	O	C	*	*
Marteño–10/14	Sp	A	G	G	M	C	*	*
Maurino	It	A	T	I	F	E	*	*
Megaritiki	Gr	B	J	T	V	H	*	*
Memecik	Tq	A	C	B	A	A	*	*
Merhavia	Is	A	–	F	D	D	g	*
Meski	Tn	A	P	S	D	H	*	*
Morejona	Sp	A	E	L	G	D	*	*
Morona	Sp	A	E	E	G	C	*	*
Morruda	Sp	A	N	B	W	C	*	*
Nabali	Is	A	B	B	C	D	i	*
Negral	Sp	A	C	B	F	H	*	*
Negrillo de Arjona	Sp	A	–	E	B	D	*	*
Negrillo de Estepa	Sp	A	B	F	N	H	*	*
Negrillo de la Carlota	Sp	A	G	I	N	C	*	*
Nevadillo Blanco de Jaen	Sp	A	A	C	M	C	*	*
Nevadillo Blanco de Lucena	Sp	A	B	A	C	C	*	*
Nevadillo Blanco de Osuna	Sp	A	G	C	N	C	*	*
Nevadillo Negro de Jaén	Sp	A	Y	E	D	D	*	*
Nevadillo–2	Sp	A	O	L	D	D	*	*
Nevado Azul (2)	Sp	A	B	B	C	C	*	*
Nevado Basto	Sp	A	A	A	C	C	f	f
Oblonga	U.S	A	J	G	Q	F	*	–
Ocal	Sp	A	B	F	D	D	g	*
Ocal–1	Sp	A	I	B	D	H	*	*
Olivo de Maura	Sp	A	W	B	M	C	*	*
Olivo Macho–3	Sp	A	A	B	D	D	b	b
Ouslati	Tn	A	A	U	J	H	*	*
Pajarero de Lucena	Sp	A	A	F	G	C	*	*
Pavo	Sp	A	O	F	C	H	*	*
Pendolino	It	A	E	B	I	H	*	*
Perillo de Jaén	Sp	A	E	C	M	H	*	*
Pico Limón	Sp	A	O	A	N	D	*	*
Picual (29)	Sp	A	A	A	G	D	*	*
Picual de Estepa	Sp	A	B	C	D	D	*	*
Picudo (5)	Sp	A	E	P	C	C	*	*
Picudo Blanco	Sp	A	B	L	M	C	*	*

Table 1. (continued).

Cultivar	Origin	Banding patterns					Identification[x]	
		ADH	ME	EST	GPI	LAP	SO	CO
Picudo de Fruto Rojo	Sp	A	E	D	C	C	h	*
Picholine	Fr	A	A	B	A	A	*	*
Picholine Marrocaine	Mr	A	B	D	C	D	a	–
Racimal de Jaén	Sp	A	I	B	D	D	*	*
Rapasayo	Sp	A	B	F	N	C	*	*
Razzola	It	A	J	G	F	F	*	*
Real Sevillana	Sp	A	G	L	M	C	*	*
Redondil	Pt	A	B	B	G	C	*	*
Redondilla de Logroño	Sp	A	A	E	D	C	*	*
Rosciola	It	A	A	J	W	H	*	*
Royal de Calatayud	Sp	A	U	H	M	C	*	*
Salgar Redondo	Sp	A	A	F	C	C	*	*
San Agostino	It	A	A	I	U	A	*	*
Santa Caterina	It	A	L	F	D	C	d	*
Sefraoui	Sr	A	D	F	I	A	*	*
Sevillenca	Sp	A	N	F	T	H	*	*
Sigoise	Ar	A	B	D	A	D	*	---
Sourani	Sr	A	B	C	H	C	j	*
Tanche	Fr	A	---	E	K	A	*	*
Tempranillo de Calatayud	Sp	A	C	B	J	H	*	*
Tempranillo de Lucena	Sp	A	I	C	G	C	*	*
Temprano	Sp	B	K	O	T	C	*	*
Torcio de Huelma	Sp	A	R	O	U	C	*	*
Trylia	Tq	A	N	B	I	C	*	*
Uovo di Piccione	It	A	V	F	C	C	*	*
Uslu	Tq	A	F	A	A	D	*	*
Valanolia	Gr	A	N	B	D	C	*	*
Verdale	Fr	A	S	F	C	C	*	*
Verdial de Alcaudete (2)	Sp	A	B	B	C	D	i	*
Verdial de Badajoz	Sp	A	G	C	C	D	*	*
Verdial de Huevar (2)	Sp	A	A	D	C	C	*	*
Verdial de Vélez Málaga	Sp	A	Z	A	G	D	*	*
Verdilla de Calatayud	Sp	A	A	O	F	D	*	*
Villalonga	Sp	A	---	V	---	C	*	*
Zalmati	Tn	A	U	Q	S	E	*	*
Zarza	Sp	A	B	C	H	C	j	*
Zorzariega	Sp	A	G	I	A	C	*	*
Total identified cultivars							132	144
Total unidentified cultivars							23	8

[z]Cultivar: when there is more than one tree Per cultivar it is indicated between parentheses.
[y]Origin: Ar = Argelia; Sp = Spain; Fr = France; Gr = Greece; Is = Israel; It = Italy; Lb = Lebanon; Mr = Morocco Pt = Portugal; Sr = Syria; Tn = Tunisia; Tq = Turkey; U.S. = United States.
"Identification: the same letters indicate identical combinations of banding patterns; SO = identification without considering the country of origin; CO = identification considering the country of origin; * = cultivars identified, unique combinations of banding patterns; a-k = cultivars not identified.

bands for enzymatic systems was illustrated with different strips: black (full-colored), white (medium-colored), and dotted (low-colored), which indicate high, medium, and low activities, respectively. The banding patterns obtained for each enzymatic system are described below.

ADH. Only one zone of activity with either one-banded (A) or a two-banded pattern (B) was observed for ADH (Fig. 1). The two-banded pattern was observed only in 'Caninese', 'Kalamon', 'Megaritiki', and 'Temprano'. The rest of the cultivars exhibited the one-banded pattern. These patterns would suggest that ADH is controlled by two alleles at one locus. This needs confirmation by progeny analysis.

ME. Twenty-five diffent banding patterns composed of four to eleven bands were recorded for this enzymatic system (Fig. 2). Unique banding patterns were observed for 'Uslu'(F'), 'Beladi' (H), 'Koroneiki' (M), 'Me&i'(P), 'Buidiego'(Q), and 'Verdial de Vélez Málaga' (Z).

EST. The zymograms of EST consisted of two band groups (EST-1 and EST-2) (Fig. 3). The most-anodal EST- 1 group included five bands and the least-anodal EST-2 group included three bands. Variability was observed for both zones. Six out of the 22 patterns obtained were unique: J for 'Rosciola', M for 'Arbequina', N for 'Cellina', Q for 'Zalmati', R for 'Buidiego', and V for 'Villalonga'.

GPI. The zymograms showed two areas of activity. The more-anodal zone (GPI-1) showed variability but was not consistently storable and it was not included in this study. Considerable polymorphism was observed in the GPI-2 zone, where the bands were consistent and repeatable. Twenty-three different banding patterns were observed among the cultivars tested (Fig. 4). Pat-

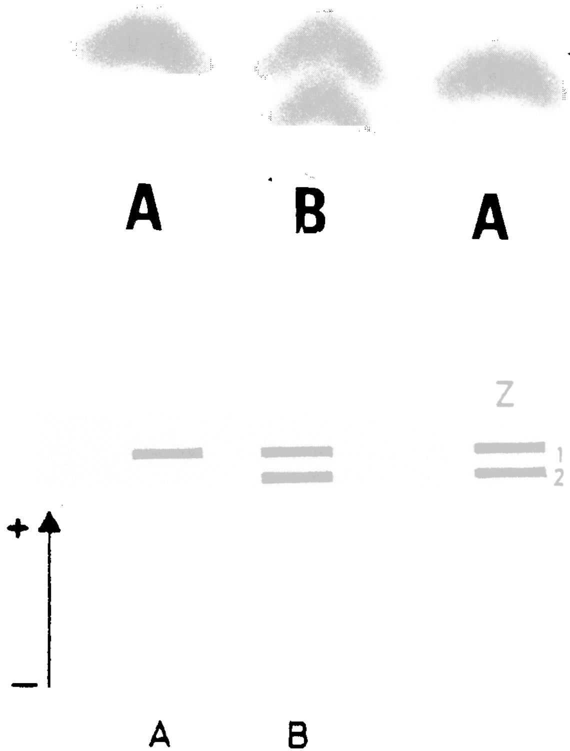

Fig. 1. Photograph and schematic illustration of bands (Z) and banding patterns for ADH.

terns L, P, and S were unique to ‘Dolce Agogia’, ‘Kaesi’, and ‘Zalmati’, respectively.

LAP. Two zones of activity, LAP-1 and LAP-2, were found for this isozyme system. Only the more-anodal (LAP-1) zone showed variability displaying either a single or double-banded pattern (Fig. 5). The LAP-2 zone consisted of two bands present in all cultivars studied. The pattern I was observed only in ‘Ascolana Tenera’.

This enzyme is known to be monomeric in other crops (Byrne and Littleton, 1988; Degani and Et–Batsri, 1990; Gottlieb, 1981; Hauagge et al., 1987). LAP-1, based on the banding patterns observed, appears to be controlled by 4 alleles *(1-4)* at one locus. Under this hypothesis, the patterns B, D, G, and H would correspond to homozygous genotypes for alleles *3, 1, 4,* and *2,* respectively. Patterns A, C, E, and F would be heterozygous for alleles *1/3; 1/2; 2/4,* and *3/4,* respectively. The heterozygote for alleles *2/3* was not observed among the cultivars examined.

Isozyme variability among cultivars. A high level of polymorphism for isozyme phenotypes was observed among the cultivars studied (Table 1). ME, GPI, EST, and LAP were very useful enzymatic systems for varietal identification due to the high number of different banding patterns they yielded. Combinations of these banding patterns allowed the identification of 132 out of 155 cultivars. When the country of origin was considered, 144 out of 155 cultivars could be individually identified. Thus, the method was effective to identify cultivars irrespective of their origin. Therefore, the method should be of great value to identify cultivars in olive germplasm collections.

The relevant enzymatic polymorphism observed among the 155 cultivars tested agrees with the high heterozygosity observed in the species by consequence of their allogamy and with the introgression of genes from wild olives into cultivated forms (Zohary and Spiegel Roy, 1975).

Isoenzymatic variability in olive is comparable to other predominantly outcrossing species like grape (Parfitt and Arulsekar, 1989; Stavrakakis and Loukas, 1983), almond (Cerezo et al., 1989; Hauagge et al., 1987), apple (Weeden and Lamb, 1985), or pineapple (Dewald et al., 1988).

The zymograms obtained for EST, ME, and LAP in this work

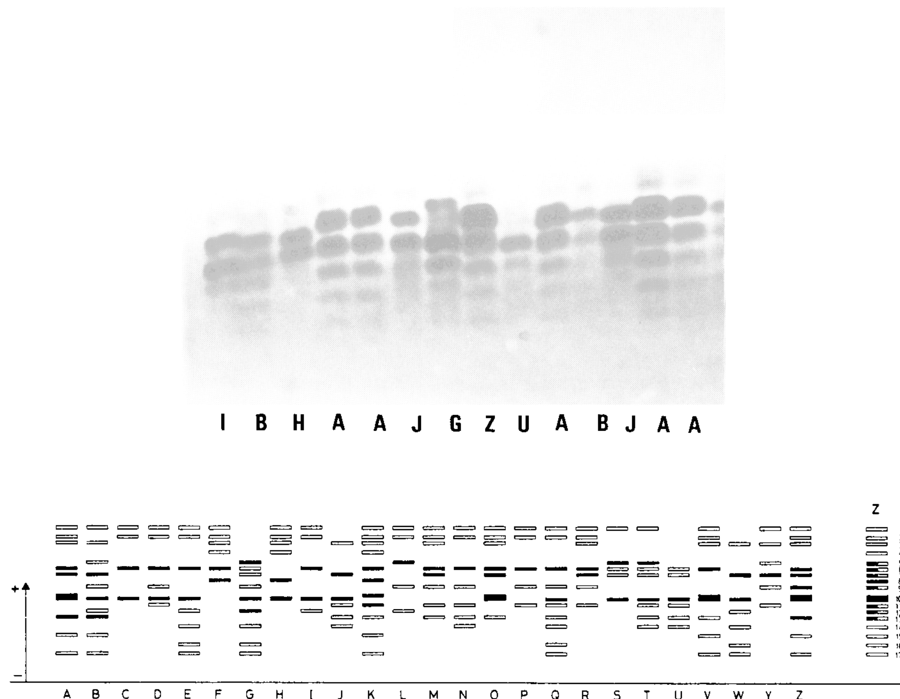

Fig. 2. Photograph and schematic illustration of bands (Z) and banding patterns for ME.

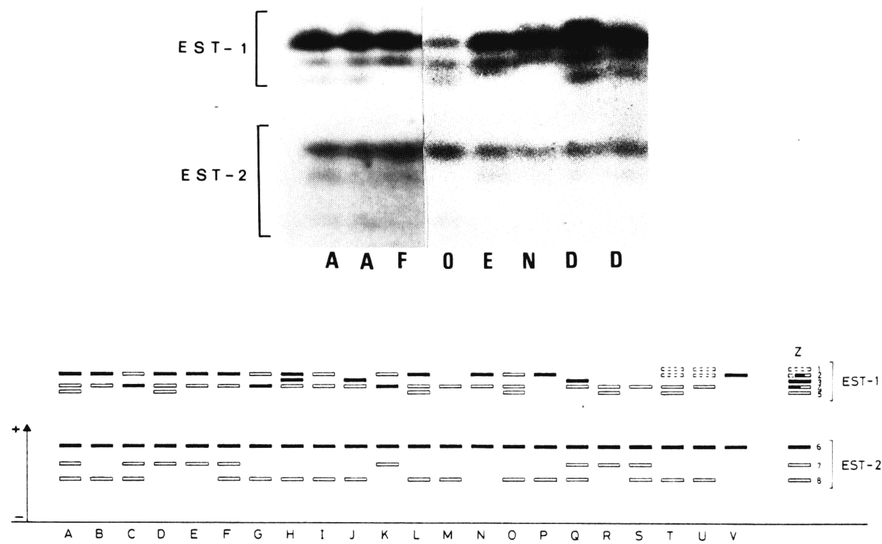

Fig. 3. Photograph and schematic illustration of bands (Z) and banding patterns for EST.

and those previously obtained in olive by Pontikis et al. (1980) are not directly comparable due to the different methodologies used (extraction, gel-electrode buffers, and staining). However both studies report a high level of isozyme polymorphism.

The electrophoretically identical cultivars for all the systems examined could be grouped into ten groups of two cultivars each ('Cañivano Blanco' – 'Picholine Marrocaine'; 'Cañivano Negro'–'Olivo Macho'; 'Changlot Real' – 'Genovesa'; 'Gordal Sevillana'–'Santa Caterina'; 'Hrai souni' – 'Lechín de Sevilla'; 'Madural'–'Cordovil de Serpa'; 'Merhavia' – 'Ocal'; 'Cobrançosa' – 'Picudo de Fruto Rojo'; 'Nabali'–'Verdial de Alcaudete' and 'Sourani'-'Zarza') and one group of three cultivars ('Ayrouni'–'Manzanilla del Piquito'–'Nevado Basto'). In all cases, when isozyme phenotypes were supplemented with a few precise morphological char-

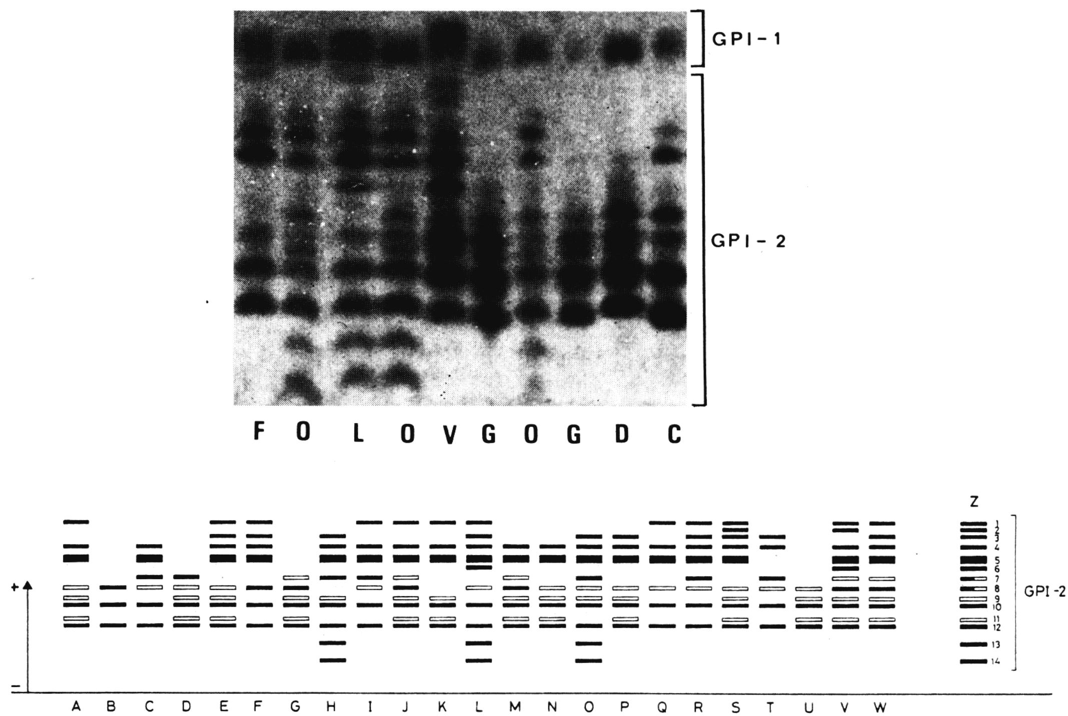

Fig. 4. Photograph and schematic illustration of bands (Z) and banding patterns for GPI.

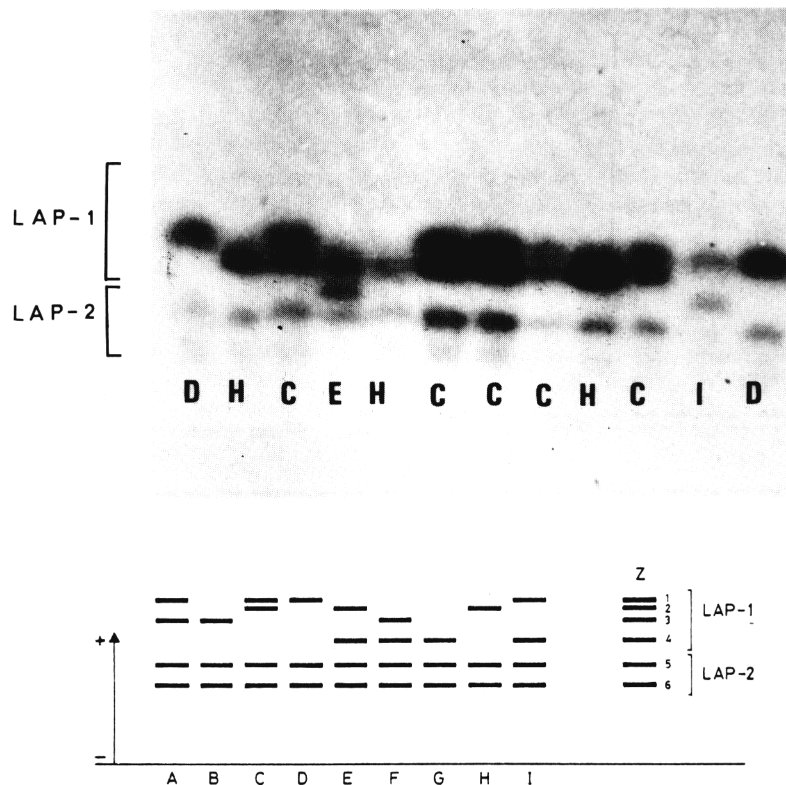

Fig. 5. Photograph and schematic illustration of bands (Z) and banding patterns for LAP.

acteristics of endocarps, leaves, and inflorescence, complete identification of all of the cultivars studied was possible except for 'Madural' and 'Cordovil de Set-pa'. However, our morphological description of these two cultivars disagrees with those obtained by Leitao (1988), suggesting a possible mislabelling in our collection.

Isozyme variability within cultivars. As in other fruit trees, olive cultivars are vegetatively propagated and the only possible intracultivar genetic variation is due to mutation.

Isozymes have been generally unable to detect the genetic changes produced by somatic mutations (DeWald et al, 1988; Tao and Sugiura, 1987; Weeden and Lamb, 1985). However, Loukas and Pontikis (1981) detected slight differences for esterases in two olive samples of the same cultivar from different geographic locations.

We electrophoretically analyzed 90 pollen samples from 21 cultivars collected from the same or different geographic locations. In all cases, no intracultivar polymorphism was observed for the five enzyme systems used. These results provide additional evidence on the lack of intraclone variation for isozyme polymorphisms. Moreover, they strongly support the consistency of the banding patterns obtained with the systems assayed and their suitability for the identification of olive cultivars.

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