

Relationship of genetic structure of *Pseudomonas savastanoi* pv. *savastanoi* populations from Italian olive trees and patterns of host genetic diversity

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A total of 360 *Pseudomonas savastanoi* pv. *savastanoi* isolates obtained from 11 Italian olive (*Olea europaea*) cultivars grown in different provinces were assessed with repetitive PCR using short interspersed elements of the bacterial genome as primers (ERIC, BOX and REP primer sets). The population structure of the isolates was determined by using three different hierarchical clustering algorithms: UPGMA, single-link and complete-link methods. REP primers were the most discriminatory. The various fingerprints obtained from the same cultivar and locality persisted over 2 years of knot sampling. Repetitive PCR and UPGMA analysis, using the three data sets combined, revealed 20 patterns with an overall similarity of 81%, with no grouping of the isolates. The resulting dendrogram shows a bush-like topology. Similar results were obtained with the other two clustering methods. In contrast, data obtained from the literature showed that the genetic structure of olive is characterized by bifurcated dendrograms and clear grouping of cultivars. Therefore it appears that the host plant and its pathogen did not cospeciate. The strict adaptation of the bacterium to olive would represent a case of association by colonization.

Keywords: hierarchical clustering, *Olea europaea*, olive knot disease, repetitive PCR

Introduction

Olive (*Olea europaea*) is among the most ancient cultivated fruit-tree species in the Mediterranean basin (Zohary & Spiegel-Roy, 1975). It was cultivated and traded for its oil by Semitic people in Syria as early as 3000 BC. Subsequently, olive cultivation was spread throughout the Mediterranean basin by Phoenicians, Greeks and Romans (Connell, 1994). Recent studies aiming at elucidating the genetic relationships in olive germplasm have suggested that cultivar selection has occurred in several different areas of the Mediterranean region (multilocal olive selection; Besnard *et al.*, 2001a). In addition, by using different molecular techniques the genetic diversity and interrelationships among olive cultivars have been inferred (Angiolillo *et al.*, 1999; Belaj *et al.*, 2001, 2002, 2003; Besnard *et al.*, 2001a, 2001b; Bronzini de Caraffa *et al.*, 2002; Carriero *et al.*, 2002; Contento *et al.*, 2002).

Olive knot disease is probably the first plant disease to be clearly described in antiquity. Theophrastus (370–286 BC) in *De historia et de causis plantarum* reported that

‘olive suffers from the nail, that some other call it fungus or little bowl’. The causative agent of olive knot, a bacterium, was isolated and described about 2200 years later by Luigi Savastano (1887, 1889) and named *Bacillus oleae tuberculosis*. Later, it was called *Bacterium savastanoi* (Smith, 1908). The current name is *Pseudomonas savastanoi* pv. *savastanoi* (Gardan *et al.*, 1992).

Pseudomonas savastanoi strains have been studied in order to clarify the relationships among the different pathovars, named pvs *savastanoi*, *fraxinii*, *nerii* and *retacarpa*. Strains isolated from olive, ash (*Fraxinus excelsior*), oleander (*Nerium oleander*) and Spanish broom (*Retama sphaerocarpa*) can be defined on the basis of pathogenicity, monoclonal antibodies, fatty acid profiling, production of phytohormones, assimilation of different carbon sources, and DNA similarity (Casano *et al.*, 1987; Janse, 1991; Gardan *et al.*, 1992; Mugnai *et al.*, 1994; Caponero *et al.*, 1995; Alvarez *et al.*, 1998). In addition, recent studies based on DNA–DNA relatedness have led to the inclusion of other phytopathogenic pseudomonads in this species, previously classified as *P. syringae* pathovars, namely *P.s.* pv. *phaseolicola* and *P.s.* pv. *glycinea* (Gardan *et al.*, 1999). However, studies aimed at clarifying possible relatedness between the population structure of *P.s.* pv. *savastanoi* strains and the genetic structure of *O. europaea* cultivars are lacking.

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Accepted 2 April 2004

Genetic studies on olive germplasm have revealed clear grouping among the major Italian cultivars. Along with cultivars from other Mediterranean countries, different clusters have been identified according to the technique used (Belaj *et al.*, 2001, 2002, 2003; Besnard *et al.*, 2001a, 2001b; Bronzini de Caraffa *et al.*, 2002; Contento *et al.*, 2002). In some cases a relationship between local Italian cultivars and the geographic area of their cultivation is evident (Carriero *et al.*, 2002; Belaj *et al.*, 2003).

Another important aspect to assess in the context of the host plant–pathogen interaction is whether the intimate association between *P.s. pv. savastanoi* and *O. europaea* represents an example of coevolution (the host and the pathogen have cospeciated), or if it represents an adaptation of the microorganism to the plant by means of adaptative strategies (association by colonization). Little is known about this subject.

The objectives of this paper were (i) to assess the population structure of *P.s. pv. savastanoi* isolates obtained from knots of different olive cultivars grown in different areas of Italy; and (ii) to test if any topological relationship exists between the pattern concerning the population structure of *P.s. pv. savastanoi* and the genetic diversity of the host plant.

Materials and methods

Isolation and identification of isolates

Young olive knots taken from olive cultivars listed in Table 1 were used for bacterial isolation. Cultivars were chosen on the basis of the groupings identified in earlier studies of the genetic structure of olive populations. Cultivar Frantoio, utilized throughout Italy, was sampled in several areas representing different provinces. Several

local cultivars were also included in the study. Sampling of diseased twigs was performed at each site during spring and autumn (locality × cultivar, Table 1) in 2001 and 2002. The samples were obtained by randomly collecting knots from five different trees always within the same 500 m² area of the olive orchard. The trees sampled were always the same. Each knot was processed separately. Small fragments (2–3 mm) of knot were cut aseptically with a sterile scalpel and placed in a sterile mortar containing 5 mL sterile saline (SS: 0.85% NaCl in distilled water). Tenfold serial dilutions were made and 0.1 mL aliquots were spread on nutrient agar (NA; Oxoid, Basingstoke, UK) in Petri dishes and incubated at 25–26°C for 72 h. Preliminary biochemical tests – presence of levan and oxidase, potato soft rot, presence of arginine dehydrolase and tobacco hypersensitivity (LOPAT tests; Lelliott *et al.*, 1966) were performed with semitranslucent and flat colonies 1–2 mm in diameter with irregular margins, suspected to belong to *P.s. pv. savastanoi*. The identity of putative isolates was achieved by means of a polyphasic approach (Vandamme *et al.*, 1996) using SDS–PAGE of soluble whole-cell protein extracts as a confirmatory test (Scortichini *et al.*, 2002). A comparison of protein profiles of the isolates was performed including *P.s. pv. savastanoi* type-strain NCPPB 639, isolated from *O. europaea* in the former Yugoslavia, and some other representative strains obtained from international culture collections for comparative purposes (NCPPB 1342 isolated in the USA; NCPPB 3335 from France; PD 912 from Greece). Five isolates per sample (one per knot taken from five different trees = 20 isolates per site) were used for the study, making a total of 360 isolates. In addition, for a subset of isolates representing each cultivar and province, a pathogenicity test was carried out by inoculating stems of cv. Frantoio. A loopful of pure culture grown for 48 h on NA

Olive cultivar	Province	Pattern identification	Isolates per pattern
Frantoio	Rimini (NE) ^a	e (FrRn) ^b	20
Frantoio	Perugia (C)	b (FrPg1); d (FrPg2)	b: 11, d: 9
Frantoio	Rieti (C)	d (FrRi2); g (FrRi1); m (FrRi3)	d: 19, g: 4, m: 6
Frantoio	Pescara (CE)	b (FrPg1); d (FrPr2)	b: 13, d: 7
Frantoio	Grosseto (CW)	p (FrGr)	20
Frantoio	Viterbo (CW)	p (FrVt1), q (FrVt2)	p: 11, q: 9
Frantoio	Bari (SE)	a (FrBa2); f (FrBa1)	a: 12, f: 8
Leccino	Perugia (CE)	h (LecPg)	20
Leccino	Chieti (CE)	h (LecCh)	20
Correggiolo	Rimini (NE)	c (Corr)	20
Carboncella	Rieti (C)	s (Carb1); v (Carb2)	s: 13, v: 7
Moraiolo	Chieti (CE)	l (Mor)	20
Pendolino	Chieti (CE)	r (Pend2); u (Pend1)	r: 11, u: 9
Itrana	Roma (CW)	n (Itr)	20
Rosciola	Roma (CW)	i (Rosc)	20
Ritornella	Roma (CW)	i (Rit)	20
Coratina	Bari (SE)	o (Corat)	20
Carolea	Catanzaro (S)	t (Carol)	20

Table 1 Origin, pattern identification and isolates per pattern of *Pseudomonas savastanoi* pv. *savastanoi* isolates used in this study

^aGeographical areas: C, central Italy; CE, central-east; CW, central-west; NE, north-east; S, south; SE, south-east.

^bAbbreviation corresponds to name of pattern in Fig. 3.

at 25–26°C was diluted in SS to $1-2 \times 10^7$ CFU mL⁻¹. Inoculum (10–20 µL) was injected by hypodermic syringe into the stem of a 1-year-old olive twig in late spring. For each isolate, three twigs were inoculated. Control twigs were injected with SS only. Symptom development was noted for up to 2 months after inoculations.

Repetitive PCR

The technique described by Smith *et al.* (1995) was used. A loopful of cells from a single colony grown for 48 h on NA was suspended in SS and centrifuged at 12 000 g for 2 min. After discarding the supernatant, the pellet was suspended in SS at an optical density corresponding to $1-2 \times 10^8$ CFU mL⁻¹. The suspension was heated in boiling water for 10 min, then stored at –20°C. The repetitive PCR (rep-PCR) method used followed Louws *et al.* (1994). Assessment of genetic diversity was performed using three short interspersed elements as primers: enterobacterial repetitive intergenic consensus (ERIC); the BOXA1R subunit of the BOX element of *Streptococcus pneumoniae* (BOX); and repetitive extragenic palindromic (REP) sequences. The ERIC, BOX and REP primer sets were synthesized by Eurogentec (Seraing, Belgium). Amplification was performed in an MJ Research PTC programmable thermal cycler (Watertown, MS, USA) in 25 µL volumes containing 200 µM deoxynucleoside triphosphate, 2 mM MgCl₂, 1.5 µM primers, *Taq* polymerase 1.0 U and 4 µL template DNA. The PCR mixture was overlaid with 25 µL mineral oil. Thermal cycling was carried out as described by Louws *et al.* (1994): an initial denaturation cycle at 95°C for 7 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C (ERIC), 53°C (BOX) or 40°C (REP) for 1 min, extension cycle at 65°C for 8 min, a single final extension cycle at 65°C for 16 min, and final storage at 4°C. The PCR amplifications were performed in duplicate. Products of PCR were separated by gel electrophoresis on 2.5% agarose (Seakem LE, Rockland, ME, USA) in 1 × TAE buffer at 5 V cm⁻¹ over 5 h, stained with ethidium bromide, visualized with a UV transilluminator Spectroline (Spectronic Corporation, Westburg, NY, USA) and photographed with Polaroid film type 55. *Pseudomonas savastanoi* pv. *savastanoi* type-strain NCPPB 639 was included in the analysis for comparative purposes. The PCR amplifications were performed in duplicate.

Data analysis

For each primer and for each strain, bands were scored as present (1) or absent (0) and the readings were entered in a computer file as a binary matrix. Each distinct banding pattern was regarded as a fingerprint. The fingerprints were compared using Dice's (1945) coefficient which produces output data matrices similar to Nei & Li's (1979) distances (D_{ij}) between pairs of fingerprints (Rohlf, 2000). $D_{ij} = n_{ij}/n_i + n_j$, where n_{ij} is the number of common bands in fingerprints i and j , and n_i and n_j are the number bands in fingerprints i and j , respectively. In addition, as distance measure, Jaccard's coefficient of similarity index was also

used (Jaccard, 1908): $a/(n - d)$, where a is the number of bands in common between two fingerprints, n is the total number of possible matches and mismatches for the two fingerprints, and d is the number of negative matches between the two fingerprints. For a better comparison of the data, cluster analysis was performed using three different hierarchical clustering methods: the unweighted pair-group method using arithmetic averages (UPGMA), single-link and complete-link clustering. In single-link clustering the distance between groups is defined as the distance between the closest pair of objects, where only pairs consisting of one object from each group are considered. In the complete-link method the distance between every possible object pair (i, j) is computed and the maximum value of these distances is said to be the distance between clusters r and s . Here the distance between two clusters is given by the value of the longest link between the clusters. The NTSYS software, PC version 2.11j (Exeter Software, New York, NY, USA; Rohlf, 2000) was used. Dendrograms were constructed with the tree display option (TREE). A cophenetic value matrix was calculated using the COPH option and compared with the original similarity matrix using the MXCOMP option to test the goodness of fit of the cluster analysis.

Results

Isolation and identification of bacterial isolates

All *P.s.* pv. *savastanoi* isolates were: levan-negative, oxidase-negative, potato soft rot-negative, arginine dehydrolase-negative and tobacco hypersensitivity-positive. Concerning the presence of levan, 21 isolates out of 360, obtained from cv. Frantoio grown in Rimini and Grosseto provinces, were levan-positive. These isolates were obtained from olive trees also hosting levan-negative isolates. The comparison (by SDS-PAGE) of whole-cell protein extract profiles of representative *P.s.* pv. *savastanoi* strains with those isolated in the present study revealed a substantial similarity, with only slight differences in band intensity. In the pathogenicity test, typical knots were formed along inoculated twigs within 1 month of inoculation. Based on these tests it was concluded that the 360 isolates obtained from olive knots that were analysed in this study belong to *P.s.* pv. *savastanoi*.

Repetitive PCR and clustering

DNA fingerprints of *P.s.* pv. *savastanoi* isolates obtained from different Italian olive cultivars grown in different areas were determined using repetitive PCR with ERIC, BOX and REP primer sets. Reproducible genomic PCR profiles consisted of bands ranging in size from ≈ 100–1500 bp. Representative fingerprints with REP primers are shown in Figs 1 and 2. For the UPGMA, single-link and complete-link clustering analysis, a total of 40 reproducible, clearly resolved bands were scored. REP primers were the most informative of the three primer sets used, as more discriminatory bands were present in the resulting

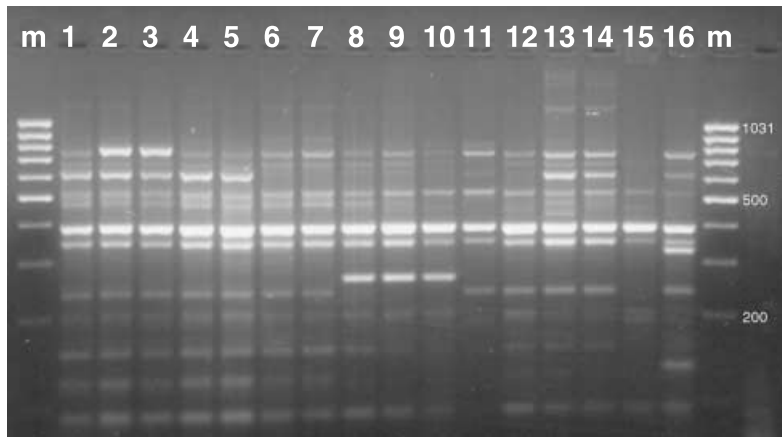


Figure 1 Repetitive PCR fingerprints, using REP primers set, of 16 different *Pseudomonas savastanoi* pv. *savastanoi* isolates obtained from different olive cultivars and provinces in Italy (Table 1; Fig. 3). Lane m: molecular size marker (100 bp DNA ladder; Genenco). Lane 1: *P.s.* pv. *savastanoi* type-strain NCPPB 639; lane 2: Frantoio/Rimini (FrRn); lane 3: Frantoio/Rimini (FrRn); lane 4: Frantoio/Perugia (FrPg1); lane 5: Frantoio/Pescara (FrPe1); lane 6: Frantoio/Bari (FrBa1); lane 7: Frantoio/Bari (FrBa1); lane 8: Coratina (Corat); lane 9: Coratina (Corat); lane 10: Coratina (Corat); lane 11: Leccino/Chieti (LecCh); lane 12: Moraiolo/Chieti (Mor); lane 13: Rosciola/Roma (RosC); lane 14: Ritornella/Roma (Rit); lane 15: Itrana/Roma (Itr); lane 16: Carboncella/Rieti (Carb1).

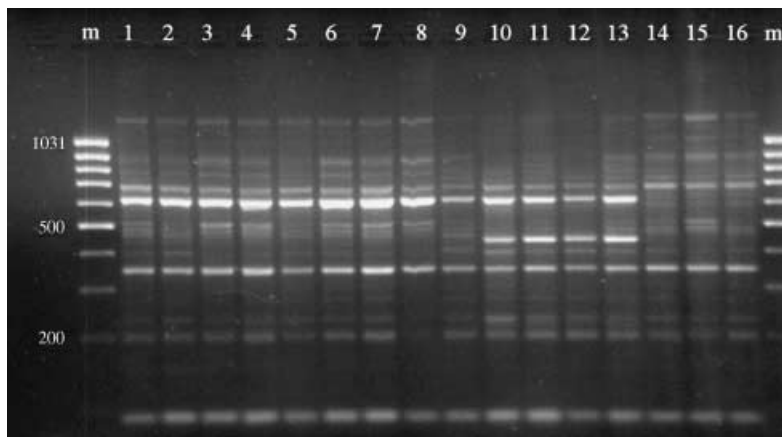


Figure 2 Repetitive PCR fingerprints, using REP primer sets, of 16 different *Pseudomonas savastanoi* pv. *savastanoi* isolates obtained from olive cv. Frantoio grown in different provinces in Italy (Table 1; Fig. 3). Lane m: molecular size marker (100 bp DNA ladder; Genenco). Lane 1: *P.s.* pv. *savastanoi* NCPPB 639 type-strain; lane 2: Frantoio/Rimini (FrRn); lane 3: Frantoio/Rimini (FrRn); lane 4: Frantoio/Perugia (FrPg1); lane 5: Frantoio/Perugia (FrPg1); lane 6: Frantoio/Pescara (FrPe1); lane 7: Frantoio/Pescara (FrPe1); lane 8: Frantoio/Rieti (FrRi2); lane 9: Frantoio/Rieti (FrRi1); lane 10: Frantoio/Grosseto (FrGr); lane 11: Frantoio/Grosseto (FrGr); lane 12: Frantoio/Viterbo (FrVt1); lane 13: Frantoio/Viterbo (FrVt1); lane 14: Frantoio/Bari (FrBa1); lane 15: Frantoio/Bari (FrBa1); lane 16: Frantoio/Bari (FrBa1).

banding pattern compared with ERIC and BOX primers. A cophenetic value of > 0.90, 0.84 and 0.81 was determined for the three similarity matrixes, UPGMA, single-link and complete-link, respectively, indicating a high goodness of fit for the cluster analysis. Dice's coefficient and Jaccard's similarity index yielded very similar dendrograms (data not shown). Only the results obtained with Dice's coefficient are discussed here.

UPGMA analysis of the combined data sets (Fig. 3) using Dice's coefficient mainly revealed a high similarity among the *P.s.* pv. *savastanoi* isolates from different cultivars and provinces of Italy, but also the occurrence of different patterns. In some areas (the provinces of Bari, Perugia, Pescara, Rieti and Viterbo), isolates showing different fingerprints were obtained from cv. Frantoio (Table 1; Fig. 2). In particular, three different fingerprints out of 20 isolates were found from this cultivar in the province of Rieti. Isolates obtained from cvs Carboncella and Pendolino also showed variability, and revealed two patterns. However, the overall similarity among all the isolates was 81%, and this high similarity, as well as the kind of branching pattern of the dendrogram (a bush-like

topology), did not allow grouping of the isolates. In total, 20 different patterns (assigned letters a to v, excluding j and k) were obtained (Table 1). The type-strain NCPPB 639 showed distinct fingerprinting and represents a different pattern, showing 93% similarity with the pattern characterizing some isolates obtained from the cv. Frantoio in the province of Bari. The isolates obtained from cvs Carolea, Coratina, Correggiolo, Itrana and Moraiolo showed distinct fingerprints, whereas the isolates from cvs Rosciola and Ritornella growing in the same area yielded the same fingerprint (Fig. 1, lanes 13 and 14), similar to the isolates from cv. Leccino obtained from either Perugia or Chieti.

The single-link clustering method, like UPGMA, suggested a high level of similarity between isolates. The isolates from cv. Carolea, which deviated the most, generally showed 91% similarity with the others. The complete-link clustering analysis revealed a similar dendrogram structure to the previous methods.

There were no differences in banding patterns shown by isolates from the same locality obtained in either spring or autumn.

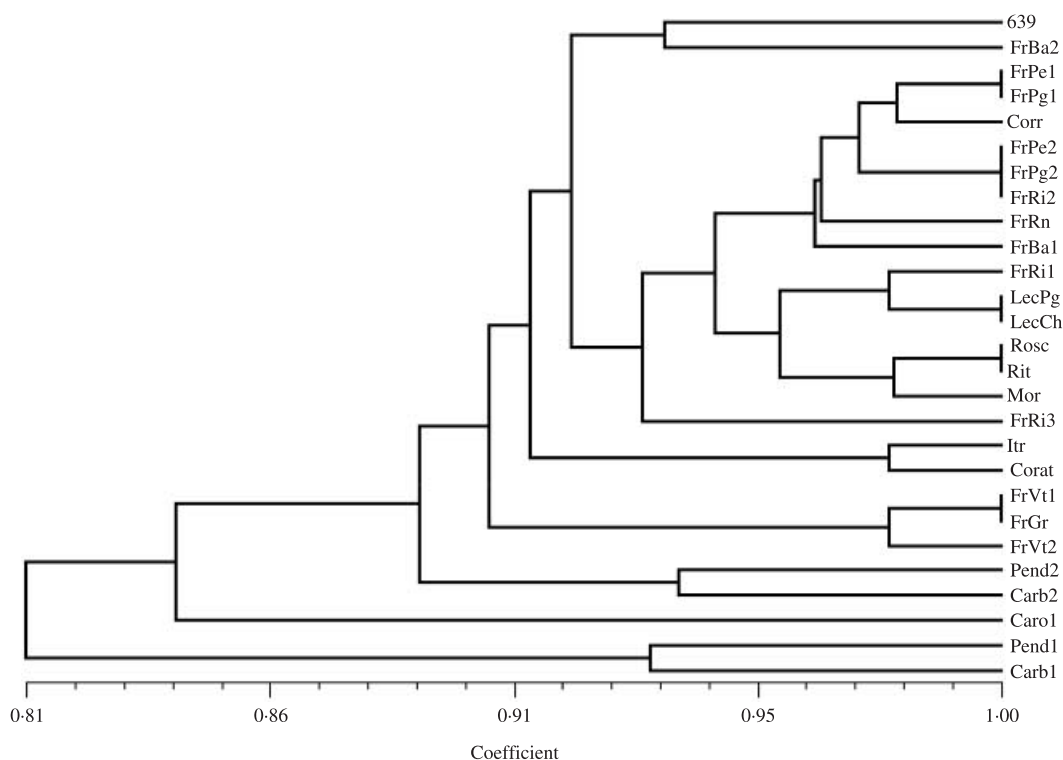


Figure 3 Dendrogram of genetic similarity generated by 360 *Pseudomonas savastanoi* pv. *savastanoi* isolates from olive in Italy. The similarity is the result of the combined data set of ERIC, BOX and REP primer sets using UPGMA analysis and Dice's coefficient. For each pattern the cultivar and province are indicated (Table 1). 639: *P.s.* pv. *savastanoi* NCPPB 639, type strain.

Discussion

This study examined genetic variability among 360 isolates of *P.s.* pv. *savastanoi* obtained from knots of several olive cultivars grown in different regions of Italy. The UPGMA analysis of DNA fingerprints, obtained with repetitive PCR and ERIC, BOX and REP primer sets revealed the presence of 20 patterns among the isolates tested. However, assessment of the genetic similarity, performed using three different hierarchical clustering algorithms, showed a relevant similarity of the isolates. With UPGMA analysis no clustering of the isolates was obtained, and their overall similarity was 81%. Single-link and complete-link clustering algorithms confirmed such findings. The topology of the dendrograms was always bush-like rather than tree-like.

The genetic structure of *O. europaea*, inferred by using genetic markers (AFLP, RAPD, SSR), always shows bifurcating (tree-like) dendrograms, regardless of the genetic marker, distance measure and clustering algorithm used, and grouping of cultivars is always possible. Belaj *et al.* (2003) used three different genetic markers to study the relationships among 32 olive cultivars. UPGMA analysis revealed two groups related at 24, 48 and 61% similarity using SSR, RAPD and AFLP, respectively. In addition, the whole data set combined revealed a similarity of 45% between the two groups of cultivars (Fig. 4). Consequently, the genetic structure of the host-plant population does not

reflect that of its pathogen, at least as far as the Italian olive germplasm is concerned.

The occurrence of mirror-image dendrograms (Fahrenholz's rule), obtained on assessment of the genetic structures, suggests that the coadaptation between host and parasite might be a case of coevolution (the reciprocal selective influence between the two parties over a prolonged period) (Ridley, 1996; Page & Holmes, 1998). For example, coevolution has been shown for aphids and their endosymbiotic bacterium *Buchnera aphidicola* which is associated by descent (Moran *et al.*, 1993). The findings obtained in the present study suggest a case of association by colonization for the *P.s.* pv. *savastanoi*-*O. europaea* pathosystem.

Epidemiological studies support such a hypothesis. *Pseudomonas savastanoi* pv. *savastanoi* incites knots in the host plant through scars formed when leaves fall (Hewitt, 1938), or through wounds caused by hail, pruning or harvesting (Smith, 1920). The knots tend to enlarge after their induction, and the bacterium escapes from knot fissures during rain. From there it can colonize other natural or artificial openings on the tree (Horne *et al.*, 1912) or stem and leaves as an epiphytic bacterium (Ercolani, 1978). Systemic spread of the bacterium within the tree, however, appears infrequent and not extensive (Wilson & Magie, 1964), while seed transmission of the pathogen has never been documented. The olive fruit fly can contain the microorganism (Petri, 1909). From these findings, *P.s.*

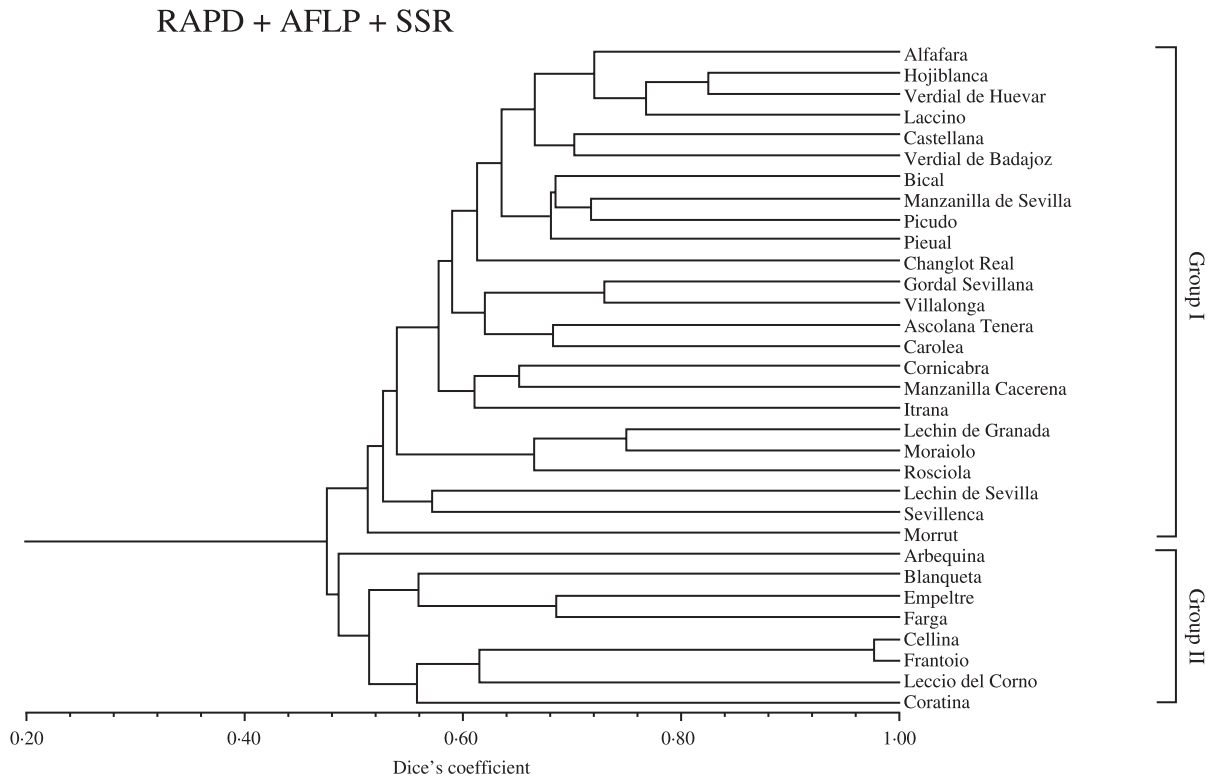


Figure 4 Dendrogram of relationships among 32 olive cultivars obtained using the combined data set of RAPD, AFLP and SSR markers. UPGMA analysis and Dice's coefficient have been used. Two main groups of cultivars are shown. (Reproduced with permission from Belaj *et al.*, 2003.)

pv. savastanoi appears as a typical plant colonizer establishing a pathogenic relationship with the host plant.

The structure of the various *P.s. pv. savastanoi* populations varied insignificantly between spring and autumn and over the 2 years of sampling. The same genomic fingerprints have been found for isolates obtained from the same cultivars and locality during spring and autumn in both years, although different fingerprints of the bacterium occur in the same area or associated with the same cultivar. Similar results were observed in other areas of Italy (Campisano *et al.*, 2001).

Acknowledgements

The authors wish to thank all colleagues who sent olive knot samples for the study.

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