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Genetic structure of Spanish populations of *Ceratitis capitata* revealed by RAPD and ISSR markers: implications for resistance management

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Abstract

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is considered one of the most economically damaging pests of citrus orchards in Spain. The characterization of *C. capitata* population structure, at a large geographical scale, by using a combination of RAPD and ISSR markers can allow analyzing the genetic variability of this species, and provide some insight in decision making for resistance management, recently recorded in Spain. We compared six Spanish populations along the Mediterranean area (Gerona, Amposta, Tortosa, Castellón, Valencia and Málaga) with populations from other geographical areas where this pest is widely distributed (Africa, Middle East, South America and Atlantic Islands) and two laboratory strains. The results obtained with both types of molecular markers were similar. A dendrogram based on Nei genetic distances showed that all Mediterranean Spanish populations, except the population collected in Gerona, were clearly separated from the rest. However, no clear differentiation among Spanish populations was found, probably as a result of the high levels of gene flow (Nm value of 2.8 for RAPD and 3.9 for ISSR). Implications of these findings on resistance management of *C. capitata* are discussed.

Additional key words: gene flow; medfly; molecular markers.

Resumen

Estudio de la estructura genética de poblaciones españolas de *Ceratitis capitata* mediante marcadores RAPD e ISSR: implicaciones en el manejo de la resistencia

La mosca mediterránea de la fruta, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), está considerada como una de las plagas económicamente más perjudiciales de los cultivos de cítricos en España. La caracterización de la estructura poblacional de *C. capitata*, a gran escala, mediante el uso conjunto de marcadores RAPD e ISSR puede permitir analizar la variabilidad genética de esta especie, y proporcionar alguna información en la toma de decisiones del manejo de la resistencia, registrada recientemente en España. Se compararon seis poblaciones españolas del área mediterránea (Gerona, Amposta, Tortosa, Castellón, Valencia y Málaga) con poblaciones de otras zonas geográficas donde esta plaga está ampliamente distribuida (África, Oriente Medio, América del Sur e Islas del Atlántico) y dos líneas de laboratorio. Los resultados obtenidos con ambos tipos de marcadores moleculares fueron similares. Un dendrograma basado en las distancias genéticas de Nei mostró que todas las poblaciones mediterráneas españolas, a excepción de la población recogida en Gerona, estaban claramente separadas del resto. Sin embargo, no se encontró una diferenciación clara entre las poblaciones españolas, probablemente como resultado de los altos niveles de flujo génico (un valor de Nm de 2,8 para RAPD y 3,9 para ISSR). Se discuten las implicaciones de estos resultados en el manejo de la resistencia de *C. capitata*.

Palabras clave adicionales: flujo génico; marcadores moleculares; mosca mediterránea de la fruta.

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Abbreviations used: AMOVA (analysis of molecular variance); DI (diversity index); EMR (effective multiplex ratio); ISSR (inter-simple sequences repeat); MI (marker index); P (polymorphism); PCR (polymerase chain reaction); RAPD (random amplification of polymorphic DNA); RFLP (restriction fragment length polymorphism); UPGMA (unweighted pair group method).

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is among the world's most economically damaging pest species. Medfly infests more than 250 species of fruits and vegetables. It is responsible for direct economic losses in fruit production and quarantine costs, and is the focus of considerable and costly detection, control and/or eradication programs. Over the last two centuries, the medfly has expanded rapidly via its own dispersal capabilities and human-mediated transport from its putative source area in Sub-Saharan Africa to almost all regions with temperate or tropical climates: the Mediterranean region, South Africa, Australia and South and Central America. Medfly invasions have also been detected in California (USA) and its potential to expand further has been recently discussed (De Meyer *et al.,* 2008).

Medfly is considered one of the most damaging pests of citrus orchards in Spain. Control of medfly populations is mainly based on the use of insecticides. However, resistance to malathion has been reported in field populations (Magaña *et al.,* 2007, 2008), showing as well cross-resistance to different insecticides (lambda-cyhalothrin, methyl-chlorpyrifos, and lufenuron) currently approved for the control of *C. capitata* (Couso-Ferrer *et al.,* 2011). In this context, the improvement of diagnostic procedures for the determination of genetic structure of populations, as well as the gene flow among different geographic areas have a practical application in integrated control programs. The knowledge of the colonization pathways will help for the design of balanced quarantine measures, while gene flow estimation among populations will help in designing appropriate resistant management strategies preventing the rapid dispersion of resistance when it is detected in field populations.

DNA methodologies in general, and those based on the polymerase chain reaction (PCR) in particular, are contributing significantly to our knowledge of population structures and dynamics. Over the last two decades several works have attempted to determine the genetic structure of medfly populations at different geographical scales, as well as to resolve more exactly the possible colonization pathways using these methodologies (*e.g.* Baruffi *et al.,* 1995; Malacrida *et al*., 1998; He & Haymer, 1999; Ochando *et al.,* 2003; Reyes & Ochando, 2004; Barr, 2009; Alaoui *et al.,* 2010). Regarding medfly Spanish populations, our studies revealed high levels of within population variability and low inter-population differentiation. Moreover, the results did not evidence clear latitudinal clines, neither a geographic distribution trend of the genetics variation in Spain.

The random amplification of polymorphic DNA by the PCR (RAPD-PCR), that detects nucleotide sequence polymorphisms using single primers of arbitrary nucleotide sequence, has been used to investigate gene flow and genetic structure in different insect species (see Jain *et al.,* 2010 for a review). This molecular marker is generally faster and less expensive than other methods of detecting DNA sequence variation, no previous knowledge of the DNA sequence is required, the whole genome can be screened and minute quantities of DNA are sufficient (Williams *et al.,* 1990). Problems concerning the reliability and repeatability of RAPD markers have been raised but they can be minimized by optimizing experimental conditions. The inter-simple sequences repeat (ISSR) markers, that permit detection of polymorphisms in inter-microsatellite loci using specific primers designed from dinucelotide or trinucleotide sequence repeats, combines the simplicity and ease of RAPD with the reliability of the more laborious RFLPs. The initial works with ISSR demonstrated the hypervariable nature of ISSR markers (Zietkiewicz *et al.,* 1994). Later, the potential of this method for population studies was proved in plants (*e.g.* Raina *et al.,* 2001; Martins-Lopes *et al.,* 2007) and to a lesser extent in insects (*e.g.* Ochando *et al.,* 2010; Soliani *et al.,* 2010).

In this paper we examine for the first time the genetic variation of *C. capitata* populations, by two molecular approaches, RAPD and ISSR analysis. The aim of the present work was, first, to compare both molecular methodologies to detect geographic patterns of genetic variation in this pest, and second, to get information about the genetic structure of Spanish populations that would assist in designing strategies for medfly control and provide some insights in decision making for resistance management.

Material and methods

Insect populations

In the present work we have analysed two laboratory strains and eleven wild populations of *C. capitata*. Six field populations were collected along the

Spanish Mediterranean area (Gerona, Amposta, Tortosa, Castellón, Valencia and Málaga), two from Atlantic Islands (Tenerife and Madeira) and three from other geographical regions (Morocco, Israel and Brazil). Adult flies were obtained during the period 2004-2005 by using field traps or by harvesting the infested fruits and allowing the larvae to pupate in the laboratory. The laboratory strain LAB-3 was established at Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain) from wild *C. capitata* collected at non-treated experimental fields in 2001 and maintained until the molecular analysis was performed. The LAB-20 has been maintained for more than 20 years under laboratory conditions. Table 1 and Fig. 1 contain the collection sites, the code used and the collection date. In all samples 25 individuals were analysed except for one Spanish sample (S-MON, 20 individuals).

DNA extraction

Genomic DNA was extracted from medflies individuals according to a standard protocol based on Aubert & Lightner (2000). The individuals were homogenized in 400 L of an extraction buffer (100 mM NaCl, 10 mM Tris, 25 mM EDTA, pH 8). The homogenate was treated with proteinase K (1 mg mL^{-1}) and SDS (1%) and incubated at 37 °C overnight. Then, NaCl and CTAB (cetyltrimethylammonium bromide) were added to a final concentration of 0.7 M and 1%, respectively, keeping the solution at 62°C for 10 min. Purification was continued by consecutive passes through phenol and chlorophorm-isoamilic alcohol (25:24:1). Water was added to reduce NaCl concentration to 0.5 M and DNA was precipitated by adding one volume of isopropanol. The resulting pellets were washed in 70% ethanol, dried and resuspended in Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8) and RNAase (0.1 g L^{-1}).

RAPD analysis

A protocol for DNA amplification was followed, modifying the conditions reported by Williams *et al.* (1990). Eight different oligodecamers from two sets of primers (kits A and E, Operon Technologies) were used (OPE-02, OPE-04, OPE-06, OPE-08, OPE-11, OPE-19, OPA-02, and OPA-07).

Reaction volumes of 25 µL contained 100 ng of genomic DNA, 1X Stoffel buffer, 3 mM $MgCl_2$, 0.4 M primer, 200 M of each dNTP and 2 U of Stoffel Fragment Amplitaq DNA Polymerase (Applied Biosystems).

Amplifications were performed in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems). The reaction mixture was preheated for an initial hold of 6 min at 94 °C followed by 45 cycles of amplification (1 min at 94 °C, 1 min at 36 °C, and 4 min at 72 °C) and a final step at 72 °C for 6 min. A negative control was added to each amplification run. With the RAPD-PCR method we followed a strict protocol with standardised conditions, repeating the amplification reactions twice in order to score clearly reproducible bands.

Table 1. Code, location and collection date of the *Ceratitis capitata* populations analysed

Code	Population, Location	Collection date	Latitude	Longitude
LAB-3	Laboratory strain-3 years old, Spain			
LAB-20	Laboratory strain-20 years old, Spain			
S-GER	La Tallada d'Empordà, Gerona, Spain	Oct. 2004	42°04'N	03°03'E
S-AMP	Amposta, Tarragona, Spain	Oct. 2003	40°42'N	00°34'E
S-TOR	Tortosa, Tarragona, Spain	Oct. 2003	40°48'N	00°31'E
S-COV	Les Coves, Castellón, Spain	July 2003	40°18'N	00°07'E
S-MON	Moncada, Valencia, Spain	June 2003	39°32'N	00°23'W
S-MAG	Torre de Benagalbón, Málaga, Spain	Oct. 2003	36°42'N	04°15'W
S-TEN	Valle de Guerra, La Laguna, Tenerife, Canary Islands, Spain	May 2004	28°31'N	16°23'W
MAD	Madeira Island, Portugal	Mar. 2004	32°45'N	16°57'W
AGA	Agadir, Morocco	Dec. 2003	30°23'N	09°36'W
ISR	Ma'agan Michael, Haifa, Israel	June 2004	32°33'N	34°55'E
BRA	Sao Paulo de Sapucai, Brazil	Oct. 2004	22°19'S	46°41'W

Figure 1. Map showing the location of medfly *Ceratitis capitata* populations sampled in this study.

ISSR analysis

Amplification reactions for ISSR analysis were performed following a modified method from Zietkiewicz *et al.* (1994). Eight different primers from British Columbia University, UBC Primer Set #9 were used (808, 810, 814, 846, 849, 853, 855, 886).

Reaction volumes of 10 µL PCR contained 5 ng of template DNA, 5 pM of primer, Qiagen PCR buffer 1X, 1.5 mM $MgCl_2$, 200 M of each dNTP and 2.5 U of *Taq* DNA polymerase (Qiagen).

Amplifications reactions were performed in a MJ Research PT-100 thermal cycler programmed for an initial hold of 5 min at 94 °C followed by 45 cycles of amplification (1 min at 94 °C; annealing 45 sec at 42 °C, 45 °C, 50 °C or 52 °C depending on the primer; and 2 min at 72 °C) and a final step at 72 °C for 6 min. Each amplification reaction was performed at least twice. A negative control was added to each amplification run.

Data analysis

Amplified products were separated in 2% agarose gels with TAE buffer (40 mM Tris-Acetate, 1 mM EDTA pH 8.0) containing ethidium bromide and visualized using a UV transilluminator. A 100 bp ladder marker (Roche) was used as a molecular size standard.

Amplified bands were scored for their presence (1) or absence (0) to create a matrix dataset with all individuals. Mean band-sharing similarity indices (S_{AB}) were calculated according to Nei & Li (1979): $S_{AB}= 2N_{AB}/(N_A+N_B)$ where N_{AB} is the number of bands shared by both individuals and N_A and N_B are the total number of bands present in individuals A and B respectively. The programme employed in both cases (RAPD and ISSR) was RAPDplot from the RAPD-PCR software package (Black IV, 1995).

Genetic differentiation among samples was examined by clustering and multivariate analysis. Lynch

& Milligan's (1994) corrections for dominant marker loci were applied. Nei's (1972) genetic distances were calculated from marker frequencies using the RAPDdist programme (RAPD-PCR software package) and these values were used to construct dendrograms with the unweighted pair-group (UPGMA) method of clustering (NTSYSpc software v. 2.01b; Rohlf, 1997). The reliability of the trees was evaluated using 1000 bootstrap replicates (RAPDdist programme). Correlations between genetic and geographic distances among populations were calculated using a randomised test for matrix correspondence: the Mantel test. Gene flow (Nm) was estimated from F_{ST} (Wright, 1951) using the RAPDF_{ST} programme (RAPD-PCR software; Black IV, 1995).

An analysis of molecular variance (AMOVA; Excoffier *et al.,* 1992) was carried out to estimate variance components for RAPD and ISSR phenotypes in order to partition the total genetic variation into that occurring within populations, among populations (within regions) and among regions. Significance levels for variance component estimates were calculated by permutational procedures. Variance components were tested against their being zero (Arlequin 2000 package; Schneider *et al.,* 2000).

In order to measure the efficiency of each marker systems, diversity index (DI), effective multiplex ratio (EMR) and marker index (MI = DI × EMR) were calculated according to Powell *et al.* (1996). Correlation and regression analysis were obtained using SPSS 17.0 (SPSS, 2009).

Results

Results showed that both RAPD and ISSR markers were consistently reproducible when amplification reactions were performed at least twice. Only unambiguous markers were considered (electrophoresis bands from 305 to 2375 bp). A total of 137 and 100 bands were scored for RAPD and ISSR analysis, respectively. The number of bands per primer obtained ranged from 13 (OPE-08) to 21 (OPE-19) in the RAPD analysis, and from 11 (primers 853) to 14 (primer 849) in the ISSR analysis.

The levels of polymorphism measured by mean DI were equal for both techniques (0.32) and the mean of EMR obtained using RAPD was only slightly higher (5.20) than that obtained using ISSR markers (4.73). The efficiency of each marker system was then measured through the mean MI, resulting very similar for RAPD (1.69) and ISSR (1.52) analyses. Pearson's correlation coefficient (0.98) was used to rank the similarities generated using the two different marker assays and linear regression analysis was used to compare individual similarity coefficients from the two markers, being the value near one (0.96).

RAPD polymorphism ranged between 66% for LAB-3 population to 97% for AGA population, being 76% the mean polymorphism for the 13 populations analysed (Table 2, last row). Results obtained after ISSR analysis were similar, exhibiting the laboratory strains (LAB-3, LAB-20) and the BRA population the lowest levels of polymorphism (76-81%) and the Mo-

Table 2. Nei's genetic distances (below diagonal), interpopulation (above diagonal) and intrapopulation simple matching similarity indices (on diagonal) for the studied populations using RAPD. Polymorphism values (P) are provided in the last row

	LAB-3	**LAB-20**	**GER**	**AMP**	**TOR**	**COV**	**MON**	**MAG**	**TEN**	**MAD**	**AGA**	**ISR**	**BRA**
LAB-3	**0.6792**	0.7086	0.6670	0.6985	0.7175	0.7101	0.7094	0.7128	0.6401	0.6355	0.5984	0.6722	0.6559
LAB-20	0.1057	**0.7019**	0.6917	0.6837	0.6996	0.6776	0.7061	0.6837	0.6676	0.6906	0.6418	0.7009	0.7069
S-GER	0.1660	0.1334	**0.7693**	0.6948	0.6774	0.6974	0.6991	0.6421	0.7497	0.6883	0.6655	0.7667	0.7556
S-AMP	0.1424	0.1283	0.1370	**0.6834**	0.7510	0.7300	0.7728	0.7322	0.6787	0.6522	0.6328	0.6940	0.6789
S-TOR	0.1291	0.1280	0.1760	0.0849	**0.6826**	0.7407	0.7697	0.7445	0.6697	0.6483	0.6167	0.6714	0.6609
S-COV	0.1274	0.1549	0.1362	0.1047	0.0903	**0.6927**	0.7416	0.7000	0.6900	0.6430	0.6148	0.6826	0.6652
S-MON	0.1350	0.1228	0.1530	0.0544	0.0787	0.0930	**0.7053**	0.7135	0.6827	0.6591	0.6309	0.6929	0.6890
S-MAG	0.1324	0.1428	0.1969	0.0899	0.0952	0.1276	0.0901	**0.6838**	0.6470	0.6391	0.6162	0.6408	0.6383
S-TEN	0.2115	0.1627	0.0584	0.1477	0.1852	0.1526	0.1656	0.1946	**0.7443**	0.6736	0.6733	0.7374	0.7186
MAD	0.1880	0.1076	0.1032	0.1384	0.1734	0.1609	0.1644	0.1829	0.1199	**0.6893**	0.6316	0.6758	0.6844
AGA	0.2403	0.1657	0.1080	0.1792	0.2101	0.2106	0.1977	0.2039	0.0946	0.1338	**0.6570**	0.6568	0.6425
ISR	0.1791	0.1500	0.0704	0.1698	0.2162	0.1729	0.1958	0.2327	0.1040	0.1496	0.1532	**0.8346**	0.7510
BRA	0.1980	0.1272	0.0785	0.1642	0.2088	0.1929	0.1810	0.2158	0.1074	0.1417	0.1540	0.0972	**0.7723**
P	0.66	0.75	0.85	0.71	0.71	0.70	0.67	0.71	0.85	0.81	0.97	0.73	0.69

rocco population (AGA) the highest one (96%), and being the mean polymorphism for all populations (85%) slightly higher than that in RAPD analysis (Table 3, last row).

The intrapopulation similarity indices ranged from 65 to 83% in RAPD assays (Table 2, diagonal) and from 67 to 79% in ISSR analysis (Table 3, diagonal). The highest intrapopulation similarity indices were observed in the Israel population, ISR, for both markers and the lowest was in the Moroccan population, AGA. Interpopulation similarity indices ranged from 59 to 77% in RAPD (Table 2, above diagonal) and from 62 to 80% in ISSR analysis (Table 3, above diagonal). The mean similarity interpopulation values for all *C. capitata* samples were similar in both markers, 68% for RAPD and 69% for ISSR.

Nei's genetic distances between all pairwise comparisons were, in general, low. The values ranged from 0.05 to 0.24 with RAPD markers (Table 2, below diagonal), and between 0.02 and 0.19 for ISSR markers (Table 3, below diagonal).

Based on Nei's genetic distances, three phylogenetic trees by the UPGMA method were obtained using three different data sets: RAPD data, ISSR data, and the compilation of RAPD and ISSR data. The three dendrograms showed the same general topology, but the bootstrap values were slightly higher in the compilation of RAPD and ISSR data than those obtained from RAPD or ISSR data sets alone. Fig. 2 therefore only shows the UPGMA dendrogram from the compilation of RAPD and ISSR data. Two significant clusters are identified in the tree. One includes all Spanish Mediterranean field populations —except S-GER— and laboratory strains LAB-20 and LAB-3. In the second cluster, the Brazilian population is placed in a separate branch; at the next level Israel (ISR) forms a separate group. The Atlantic populations from Madeira (MAD), Morocco (AGA) and Tenerife (S-TEN) group together and include the population collected in Gerona (S-GER).

Gene flow (Nm) values among the populations, indirectly estimated from F_{ST}, were quite high. Nm values considering all the population tested were 1.3 and 1.6 for RAPD and ISSR analysis, respectively. When only Spanish population were taking into account, gene flow increased to 2.7 and 2.5 (RAPD and ISSR, respectively), and even higher when considering only wild Spanish peninsular populations (2.8 and 3.9).

The Mantel test revealed a low correlation (r = 0.48, p [random Z ≥ observed Z] = 0.20) between the genetic and linear geographic (km) distances for Spanish peninsular populations, not statistically significant.

The AMOVA analyses performed (considering all populations and only Mediterranean Spanish populations) indicated that the higher percentage of variance corresponded to the genetic variation among individuals within populations (Table 4). In all cases variation within populations was twice to three times higher than variation among populations (61.93% *vs*. 38.07% for RAPD, and 72.52% *vs*. 27.48% for ISSR when the thirteen populations were consider in the same group). A random permutational test revealed that all the variance components were statistically significant ($p < 0.001$).

Table 3. Nei's genetic distances (below diagonal), interpopulation (above diagonal) and intrapopulation simple matching similarity indices (on diagonal) for the studied populations using ISSR. Polymorphism values (P) are provided in the last row

	LAB-3	**LAB-20**	**GER**	**AMP**	**TOR**	**COV**	**MON**	**MAG**	**TEN**	**MAD**	**AGA**	**ISR**	**BRA**
LAB-3	**0.6736**	0.6969	0.6644	0.6735	0.6634	0.6729	0.6771	0.6900	0.6604	0.6827	0.6380	0.6757	0.6484
LAB-20	0.1810	**0.7112**	0.7088	0.7026	0.6979	0.7000	0.7164	0.6951	0.6812	0.7150	0.6761	0.6983	0.7064
S-GER	0.1187	0.0854	**0.7120**	0.6764	0.6760	0.6985	0.7011	0.7001	0.7031	0.7025	0.6852	0.7120	0.6947
S-AMP	0.1180	0.0823	0.0787	**0.6830**	0.7060	0.7316	0.7176	0.7028	0.6757	0.7028	0.6718	0.6592	0.6574
S-TOR	0.1207	0.0945	0.0830	0.0524	**0.6812**	0.7144	0.7132	0.6997	0.6761	0.6941	0.6705	0.6637	0.6281
S-COV	0.1163	0.0926	0.0661	0.0291	0.0529	**0.7023**	0.7309	0.7304	0.7044	0.7044	0.6700	0.6830	0.6603
S-MON	0.1048	0.0695	0.0497	0.0319	0.0535	0.0317	**0.6992**	0.7084	0.6807	0.7005	0.6782	0.6872	0.6765
S-MAG	0.1043	0.1033	0.0537	0.0614	0.0680	0.0493	0.0542	**0.6988**	0.7120	0.6935	0.6808	0.7008	0.6502
S-TEN	0.1518	0.1482	0.0793	0.1140	0.1190	0.0908	0.1051	0.0671	**0.6909**	0.6892	0.6925	0.6804	0.6440
MAD	0.1035	0.0947	0.0617	0.0701	0.0812	0.0604	0.0595	0.0604	0.0904	**0.7006**	0.7089	0.6738	0.7018
AGA	0.1542	0.1255	0.0698	0.0877	0.0981	0.0878	0.0790	0.0723	0.0798	0.0555	**0.6837**	0.6616	0.6561
ISR	0.1401	0.1374	0.1021	0.1416	0.1548	0.1197	0.1104	0.1073	0.1405	0.1451	0.1467	**0.7970**	0.8053
BRA	0.1739	0.1292	0.1053	0.1344	0.1606	0.1332	0.1194	0.1429	0.1760	0.1033	0.1357	0.1961	**0.7111**
P	0.81	0.81	0.90	0.87	0.86	0.85	0.85	0.89	0.87	0.89	0.96	0.76	0.76

Table 4. Analysis of the molecular variance (AMOVA) of the 320 individuals from all 13 populations of *C. capitata* using RAPD and ISSR bands. The data show the degrees of freedom (d.f.), variance component estimates, percentage of total variance contributed by each component, and the probability (*p*) of obtaining a more extreme component estimate by chance alone. For each analysis 1000 permutations were used

Source of variation	d.f.	Variance component	% total variance	*p*-value
All populations: 1 group (RAPD)				
Among populations	12	8.22	38.07	< 0.001
Within populations	307	13.38	61.93	< 0.001
Spanish wild populations[a]: 1 group (RAPD)				
Among populations	6	6.76	34.23	< 0.001
Within populations	163	12.99	65.77	< 0.001
Mediterranean populations[b]: 1 group (RAPD)				
Among populations	6	7.12	36.28	< 0.001
Within populations	163	12.52	63.72	< 0.001
All populations: 1 group (ISSR)				
Among populations	12	4.27	27.48	< 0.001
Within populations	307	11.28	72.52	< 0.001
Spanish wild populations[a]: 1 group (ISSR)				
Among populations	6	2.88	19.41	< 0.001
Within populations	163	11.98	80.59	< 0.001
Mediterranean populations[b]: 1 group (ISSR)				
Among populations	6	3.13	20.92	< 0.001
Within populations	163	11.84	79.08	< 0.001

[a] Spanish wild populations: S-MAG, S-COV, S-MON, S-TOR, S-AMP, S-TEN, S-GER. [b] Mediterranean populations: S-MAG, S-COV, S-MON, S-TOR, S-AMP, S-GER.

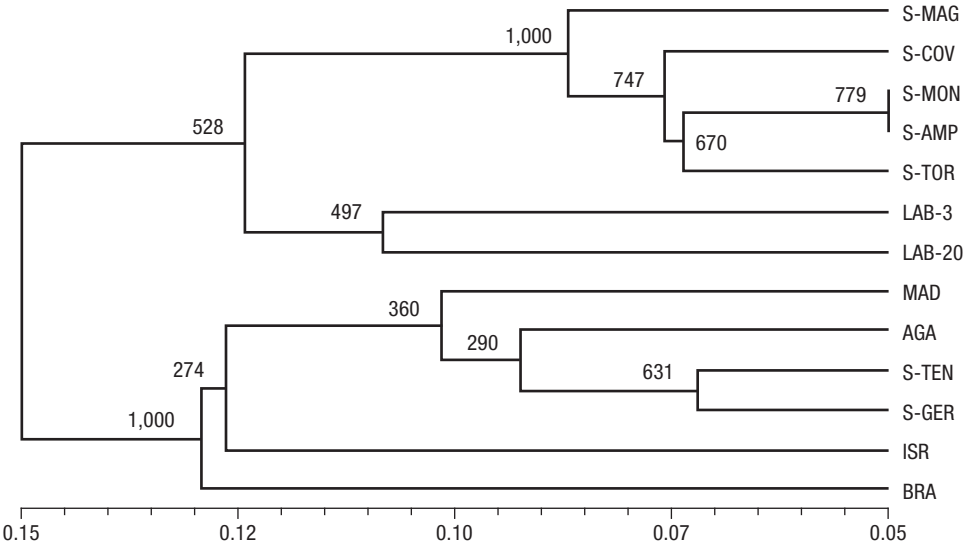

Figure 2. UPGMA dendrogram of populations of *C. capitata* based on Nei's genetic distances obtained from RAPD and ISSR markers. Bootstrap values, based on 1,000 replications, are shown near the corresponding branches.

Discussion

Comparative analysis of RAPD and ISSR markers

The choice of a particular molecular marker for population genetic structure studies depends on its reproducibility, its simplicity and, certainly, its sensitivity to detect variation in the species of interest. The analytical sensitivity and the results obtained with both types of molecular markers used in this study were similar, confirming the utility of ISSR markers to detect geographic patterns of genetic variation in *C. capitata*.

The total number of amplification products detected was higher in the case of RAPD (137 bands) than in the case of ISSR (100 bands), and, similarly, the average number of products per assay (17.1 RAPD *vs.* 12.5 ISSR). Notwithstanding, the level of polymorphism detected were higher for ISSR (85%) than in RAPD (76%) analysis. Similar results have been reported in studies with plant species involving both types of markers (Raina *et al.,* 2001; Martins-Lopes *et al.,* 2007). This finding could be related to the high mutation rate in the tandem highly repetitive microsatellites sequences in the case of ISSR, and to the higher number of arbitrary sequences targeted for RAPD. In general, the detected polymorphism in *C. capitata* with these two methodologies (mean: 76% for RAPD and 85% for ISSR) was within the range observed in other RAPD studies [Baruffi *et al.* (1995): P = 45-85%, mean = 66%; Fernández (2002): P = 29-75%, mean = 49%; Alaoui *et al.* (2010): P = 81% in Moroccan medfly populations and P = 89% in Kenyan population]. Studies performed using other markers showed lower polymorphisms (Baruffi *et al.,* 1995; Ochando *et al.,* 2003).

To measure, as an overall, the efficiency of both types of markers we used the "marker index", MI, introduced by Powell *et al.* (1996). As average, both techniques were equally efficient in the variability detection (1.69 for RAPD and 1.52 for ISSR analyses). Moreover, values were very similar with both kind of markers for all the populations, corresponding the highest (AGA population: 3.05 with RAPD and 1.88 with ISSR) and the lowest (LAB-20: 1.06 with RAPD and 1.14 with ISSR) values to the same populations. In fact, correlations obtained between the genetic similarities calculated using RAPD and ISSR similarity matrices give a value of 0.98 for Pearson correlation and of $R^2 = 0.96$ for regression analysis (highly significant statistically), indicating a high correspondence between both types of markers.

We have substantiated that RAPD and ISSR markers could get reliable results on the genetic structure of the populations of this pest, but the simultaneous use of both techniques could give a more robust analyses. Thus, the analysis of our results was done taking into account both markers simultaneously or summing up data of both.

Patterns of distribution of the genetic variability

The second aim of this work was to study the genetic variability and structure of Mediterranean medfly Spanish populations in comparison with populations from other geographical areas where this pest is distributed (Africa, Middle East, South America and Atlantic Islands). The polymorphism, considering both markers simultaneously, varied between the 0.96 for the population from Morocco to the 0.72 for the population from Brazil, which reflects the African origin of this species and the recent colonization of South America (Malacrida *et al.,* 1998; Barr, 2009). Mediterranean Spanish populations showed intermediate levels of polymorphism, as reported in other studies including Mediterranean basin populations (Malacrida *et al.,* 1998; Reyes & Ochando, 1998, 2004). When a dendrogram based on Nei genetic distances was constructed using simultaneously both kinds of markers, all Mediterranean Spanish populations, except the population collected in Gerona, were clearly differentiated from the rest. In the second cluster, the population from Brazil (BRA) is placed in a separate branch, whereas the Atlantic samples are clustered together, although the bootstrap values were low.

Some authors have demonstrated the existence of latitudinal clines of genetic diversity from the origin of this species through their routes of expansion at the macro-geographical level (Gomulski *et al.,* 1998; Malacrida *et al.,* 1998). These data are consistent with the colonization of the Mediterranean basin from North Africa through southern Spain (Malaga) in the first half of the 19th century (De Breme, 1842), followed by a spread to other northern and eastern Mediterranean locations, which would mean a colonization of the Iberian Peninsula from the south to the north. However, when analyzing the Mediterranean Spanish populations, no clear relationships can be detected between geographic and genetic distances (Mantel test) or between latitude and degree of polymorphism (data not shown). Previous work with Spanish *C. capitata*

populations failed also to detect geographic (Reyes & Ochando, 1998; Fernández, 2002) and/or host-dependent genetic differentiation (Reyes & Ochando, 1994; Ochando *et al.,* 2003). We must take into account that over the historical aspect of the colonization, other processes can influence the population structure. For one side, the adaptation to local environmental conditions that can differentiate populations and, for the other, the gene flow among populations that tends to reduce differences in their genetic structure. The gene flow estimated (Nm value) for all populations of *C. capitata* studied was 1.3 (RAPD) and 1.6 (ISSR), and even higher for the Mediterranean Spanish populations (2.8 and 3.9, respectively). According to Wright (1951), a value of gene flow higher than one is enough to avoid population differentiation. As a matter of fact, the AMOVA analysis of our results show that more than twice of the variation is explained by within population variability, as already reported for this species using different types of markers (He & Haymer, 1999; Fernández, 2002). Thus, our results suggest that gene flow would definitely play some role in the distribution of variability of Mediterranean populations especially in its geographic uniformity. Gene flow could be masking the "historic pathway", and despite possible adaptation/selection to local conditions, the high mobility of medflies and passive transportation by trade between regions surpass this effect.

Our results revealed that West African and Atlantic Islands populations (Agadir, Madeira and Tenerife) were clustered together. This could be the consequence of the colonization of the Atlantic Islands from West Africa although the bootstrap values were not statistically significant. Likewise the Atlantic Island populations presented similar (ISSR) or even higher (RAPD) polymorphism values than the Spanish peninsular populations analyzed. This may result from earlier colonization events (medfly was detected in Madeira in 1829; Fimiani, 1989), and/or very well adapted island populations in a climate and crop conditions probably optimal for this species and maintained all around the year. Moreover, trade between both islands and the Iberian Peninsula is intensive and re-introduction can not be neglected.

Regarding to the Brazilian population, the genetic similarity between this population and the others seems to indicate a recent introduction event and the time elapsed since then would be not enough to allow its differentiation. With respect to the two laboratory strains studied, both were established from wild *C. capitata* collected in the Spanish Mediterranean area. Their level of polymorphism was within the range of field Spanish populations, and higher for LAB-3 (kept in captivity for three years) than for LAB-20 (maintained more than 20 years in laboratory conditions). It is well known that *C. capitata* go through a narrow bottleneck when initially adapted to laboratory conditions (Haymer & McInnis, 1994; Baruffi *et al.,* 1995; Reyes & Ochando, 1998). However, after several generations laboratory strains recover and the polymorphism levels can increase (Ochando *et al.,* 2003).

Consequences for medfly control and resistance management strategies

The high polymorphism found for medfly populations corresponded to a highly polyphagous species that, in our agroclimatic conditions, seems to maintain high effective number of individuals in populations throughout the year, particularly from March to November, due to a wide range of fruits available. This fact is of relevance for the control of this pest, since area-wide pest management approaches need to be considered for species with nearly panmictic population structure (Solano *et al.,* 2009; Weeks *et al.,* 2010). In addition, knowledge of the genetic relationships among populations of an insect pest is essential for resistance management programs. Gene flow between populations reduces the probability of resistance development within a population but enhances the spread of resistance alleles to other populations once resistance has developed at a certain site. Thus, the high level of gene flow among Spanish medfly populations may help to explain why wild resistance was not reported, despite the intense use of insecticides for the control of this pest in Spain during the 80s and 90s (Viñuela, 1998). However, the rapid dispersal of malathion resistant alleles from areas with high selection pressure resulted in the detection of resistant populations at different geographical areas at the same time (Magaña *et al.,* 2007). Resistance management strategies may therefore focus in proactive resistance-monitoring plans that will enable early detection of resistance alleles in field populations.

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