Evidence of two lineages of the symbiont ‘*Candidatus* Erwinia dacicola’ in Italian populations of *Bactrocera oleae* (Rossi) based on 16S rRNA gene sequences

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The close association between the olive fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) and bacteria has been known for more than a century. Recently, the presence of a host-specific, hereditary, unculturable symbiotic bacterium, designated ‘*Candidatus* Erwinia dacicola’, has been described inside the cephalic organ of the fly, called the oesophageal bulb. In the present study, the 16S rRNA gene sequence variability of ‘Ca. E. dacicola’ was examined within and between 26 Italian olive fly populations sampled across areas where olive trees occur in the wild and areas where cultivated olive trees have been introduced through history. The bacterial contents of the oesophageal bulbs of 314 olive flies were analysed and a minimum of 781 bp of the 16S rRNA gene was sequenced. The corresponding host fly genotype was assessed by sequencing a 776 bp portion of the mitochondrial genome. Two ‘Ca. E. dacicola’ haplotypes were found (htA and htB), one being slightly more prevalent than the other (57 %). The two haplotypes did not co-exist in the same individuals, as confirmed by cloning. Interestingly, the olive fly populations of the two main Italian islands, Sicily and Sardinia, appeared to be represented exclusively by the htB and htA haplotypes, respectively, while peninsular populations showed both bacterial haplotypes in different proportions. No significant correlation emerged between the two symbiont haplotypes and the 16 host fly haplotypes observed, suggesting evidence for a mixed model of vertical and horizontal transmission of the symbiont during the fly life cycle.

INTRODUCTION

The microbiota associated with Tephritid flies (Diptera, Tephritidae) has been the subject of many studies (Behar *et al.*, 2009). In particular, the association between the olive fly *Bactrocera oleae*, the most important olive crop pest, and its symbiotic bacteria has been explored for over a century. The presence of bacterial masses inside an intestinal diverticulum located in the fly head, called the oesophageal bulb, and the intestinal lumen of the midgut of *B. oleae* was described for the first time by Petri (1909), based on microscopic observations. The nature of this unculturable and host-specific bacterium was investigated

in a previous study (Capuzzo *et al.*, 2005), using molecular techniques and microbiologically controlled conditions. The bacterium was described as a novel taxon within the family *Enterobacteriaceae* as ‘*Candidatus* Erwinia dacicola’. The olive fly symbiont is considered to be the most common and widespread bacterium within the olive fly population (Ben-Yosef *et al.*, 2010); it has been found in Italy, where it was first described (Capuzzo *et al.*, 2005; Sacchetti *et al.*, 2008), in Spain (Silva *et al.*, 2008), in the south-western USA (Estes *et al.*, 2009) and in Greece (Kounatidis *et al.*, 2009).

The last afore-mentioned study did not detect ‘Ca. E. dacicola’ in their laboratory strains and found that the culturable bacterium *Acetobacter tropicalis* was predominant. The literature reports that symbiont losses are a consequence of rearing larvae on artificial media (Girolami & Cavalloro, 1972; Capuzzo *et al.*, 2005), as well as their substitution by acidophilus bacteria (Hagen, 1966).

Abbreviation: AMOVA, analysis of molecular variance.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and 23S rRNA gene sequences of ‘*Candidatus* Erwinia dacicola’ are HQ667588 to HQ667589 and HQ667590, respectively. Those for the NADH1-tRNA-16S gene sequence of *Bactrocera oleae* are HQ667572 to HQ667587, respectively.

Table 1. Collection sites of the *B. oleae* populations, 16S rRNA haplotype frequencies of 'Ca. E. dacicola' (htA and htB) and geographical distribution of the 16 *B. oleae* mitochondrial haplotypes

Location	Coordinates	Date of collection	Sample size*	'Ca. E. dacicola' haplotypes		<i>B. oleae</i> haplotypes†															
				htA	htB	h1	h2	h3	h4	h5	h6	h7	h8	h9	h10	h11	h12	h13	h14	h15	h16
1. Campione (Lombardy)	45° 58' N 08° 58' E	10/2009	8	3	5																
2. Bardolino (Veneto)	45° 32' N 10° 43' E	12/2008	8	5	3																
3. Dueville (Veneto)	45° 38' N 11° 32' E	10/2009	14	3	11																
4. Marostica (Veneto)	45° 44' N 11° 39' E	10/2007	13 (10)	0	13	4 _B	3 _B	2 _B											1 _B		
5. Montegrotto Terme (Veneto)	45° 19' N 11° 47' E	12/2007	15 (5)	4	11	2 _B	1 _B		1 _B											1 _B	
6. Diano Marina (Liguria)	43° 54' N 8° 04' E	11/2008	8	4	4																
		12/2009	17	5	12																
		11/2006	8	6	2																
		09/2007	12 (4)	11	1	1 _B ; 1 _A	1 _A	1 _A													
7. Firenze (Tuscany)	43° 46' N 11° 15' E	09/2008	18 (6)	16	2	4 _A								1 _A							1 _A
		12/2007	4	3	1																
8. Capoliveri (Tuscany)	42° 44' N 10° 22' E	08/2009	9 (5)	5	4	1 _B ; 2 _A		1 _B						1 _B							
9. Arezzo (Tuscany)	43° 28' N 11° 51' E	11/2009	10	6	4																
10. Roma (Lazio)	41° 53' N 12° 29' E	12/2009	12 (5)	2	10	1 _A ; 3 _B	1 _B														
11. Ancona (Marche)	43° 37' N 13° 31' E	09/2009	16 (5)	3	13	2 _B	3 _B														
12. Moscufo (Abruzzo)	42° 25' N 14° 03' E	11/2009	11	5	6																
13. Capua (Campania)	41° 06' N 14° 13' E	09/2009	10	1	9																
14. Portici (Campania)	40° 48' N 14° 20' E	09/2009	15 (5)	4	11	1 _B			2 _B				1 _B							1 _B	
15. Rossano Calabro (Calabria)	39° 34' N 16° 38' E	09/2009	9 (4)	2	7	2 _B	1 _A ; 1 _B														
16. Rende (Calabria)	39° 19' N 16° 10' E	10/2009	7	2	5																

Table 1. cont.

Location	Coordinates	Date of collection	Sample size*	'Ca. E. dacicola' haplotypes		<i>B. oleae</i> haplotypes†															
				htA	htB	h1	h2	h3	h4	h5	h6	h7	h8	h9	h10	h11	h12	h13	h14	h15	h16
17. Gioia Tauro (Calabria)	38° 25' N 15° 54' E	11/2009	6 (3)	2	4		1 _B				1 _A							1 _B			
18. Bari (Apulia)	41° 07' N 16° 51' E	11/2002	9	1	8																
19. Castellana Grotte (Apulia)	40° 53' N 17° 09' E	10/2009	9	5	4																
20. Otranto (Apulia)	40° 08' N 18° 29' E	10/2009	10 (5)	9	1	1 _A ; 1 _B	1 _A			1 _A							1 _A				
21. Messina (Sicily)	38° 11' N 15° 33' E	08/2007	6 (5)	0	6			2 _B	1 _B	2 _B											
22. Siracusa (Sicily)	37° 03' N 15° 17' E	11/2008	5	0	5																
23. Marsala (Sicily)	37° 47' N 12° 26' E	07/2008	15 (4)	0	15	2 _B	1 _B	1 _B													
24. Bancali (Sardinia)	40° 44' N 8° 27' E	12/2009	5 (3)	5	0	2 _A											1 _A				
25. Alghero (Sardinia)	40° 33' N 8° 19' E	10/2008	6 (6)	6	0	4 _A	1 _A						1 _A								
26. Cagliari (Sardinia)	39° 12' N 9° 06' E	09/2009	16 (5)	16	0		2 _A				1 _A			1 _A	1 _A						
Total			311 (80)	134	177	15 _A ; 19 _B	6 _A ; 11 _B	1 _A ; 6 _B	4 _B	1 _A ; 2 _B	2 _A	2 _B	2 _A	1 _A	1 _A	1 _A	1 _A	1 _B	1 _B	2 _B	1 _A

*Numbers in parentheses indicate the number of olive flies for which the mitochondrial region was sequenced.

†A and B indicate symbiont haplotype.

Olive fly larvae feed upon the pulp of fruits of the genus *Olea*, both wild (oleaster) and domesticated forms (Tzanakakis, 2006). Botanically, the oleaster and the cultivated form of the olive tree correspond, respectively, to *Olea europaea* subsp. *europaea* var. *sylvestris* and *Olea europaea* subsp. *europaea* var. *europaea* (Breton *et al.*, 2006).

At present, the olive fly is found in East and South Africa, the Mediterranean basin and Pakistan (Tzanakakis, 2006). Recently, the fly has been accidentally introduced into California, USA (Rice, 1999). In Italy, as in other Mediterranean countries where the pest is recorded, the range of the olive fly traditionally matches with that of the olive tree, including both the oleaster and cultivated forms.

On the basis of these statements, the distribution of the olive fly in Italy can be divided according to: a) areas where olive trees are grown extensively and the presence of oleaster is diffused (these areas overlap with historic olive cultivating regions and with the Italian phytoclimatic subzone of warm *Lauretum* according to Pavari, 1916); and b) areas where olive trees are marginally cultivated and oleaster is absent. It is well-known that in the former of these two zones, populations of *B. oleae* breed all-year round and also exploit residual fruits that are present on wild olive trees during spring time (Blando & Mineo, 2006).

To date, the genetic diversity present within the olive fly symbiont has not been investigated. In this study, the 16S rRNA gene sequence diversity of the olive fly symbiont '*Ca. E. dadicola*' was examined by focusing on Italian olive fly populations. The study had the following objectives: (i) to verify the 16S rRNA gene sequence diversity of '*Ca. E. dadicola*' from across Italian territory and (ii) to assess possible correlations between the genetic variability of '*Ca. E. dadicola*' and the mitochondrial haplotypes of the host fly.

METHODS

Olive fly sampling. Infested olives were collected from 26 different locations throughout Italy from 2002 to 2009 in areas where olive trees were regularly cultivated. The sampling sites were chosen in order to represent wild olive tree areas according to Pavari (1916) and Carrión *et al.* (2010) (Liguria coast, south Italy, Sardinia and Sicily, locations 6 and 13 to 26) and areas where olive cultivation was introduced and spread historically through human activities and where the wild olive (oleaster) was absent (North and Central Italy, locations 1 to 5 and 7 to 12) (Table 1 and Fig. 1). In addition, two populations from both areas were collected over three consecutive years and during the same season in order to detect possible variations in the distribution of different '*Ca. E. dadicola*' haplotypes over time (location 5, from 2007 to 2009 and location 6, from 2006 to 2008) (Table 1).

Fig. 1. Geographical distribution and proportion of the two '*Ca. E. dadicola*' haplotypes among the 26 *B. oleae* populations sampled in Italy. The first number indicates the location code, reported in Table 1; n, sample size. For locations 5 and 6, the three different years of sampling are reported in detail. The distribution area of oleaster is from Carrión *et al.* (2010).

The olives were kept in transparent plastic boxes (20 × 15 × 8 cm) topped with a net until the adult flies emerged. Insects were then transferred into net rearing cages (10 × 10 × 10 cm) and fed with a sugar diet (50 % w/v glucose solution) and water *ad libitum*.

Insect dissection and symbiont analysis. In newly emerged adults, the presence of endosymbionts is sometimes too low to be detected (Girolami, 1973; Estes *et al.*, 2009) and so only 2–3 day-old flies were processed. This ensured that a sufficient number of bacteria was present in the oesophageal bulbs.

Flies were dissected aseptically (Capuzzo *et al.*, 2005) under a laminar flow hood in physiological saline solution [0.9 % NaCl (w/v)] under a stereomicroscope, extracting the oesophageal bulbs. Dissecting tools (forceps and tweezers) were sterilized before processing each individual insect. Oesophageal bulbs were gently transferred into Eppendorf tubes and kept at –20 °C until further processing.

A protocol originally developed for actinomycetes was used (Palmano *et al.*, 2000) to extract the microbial DNA content of the insect oesophageal bulbs. The bacterial 16S rRNA gene was partially amplified by PCR using two pairs of universal bacterial primers: fd1 and rp1 (Weisburg *et al.*, 1991) or 63f and 1389r (Osborn *et al.*, 2000). The reaction mixture contained, in a total volume of 20 µl, 1 × PCR GoTaq Flexi Buffer (Promega), 2.5 mM MgCl₂, 0.1 mM dNTPs, 0.5 µM each primer, 1 U GoTaq Flexi DNA polymerase (Promega) and a 1 µl sample of a 1:30 dilution of the DNA extract. All reaction mixtures were prepared at 4 °C in 0.2 ml reaction tubes to avoid non-specific priming. The standard thermal profile for the amplification of the 16S rRNA genes was as follows: initial denaturation at 95 °C, 2 min; 27 cycles of: denaturation at 95 °C 30 s; annealing at 56 °C 30 s and extension at 72 °C 90 s; final extension at 72 °C, 5 min.

The 23S rRNA gene region was also tested for some samples using the universal primer pair 1623f and 2490r (Hunt *et al.*, 2006) under the following conditions: initial denaturation at 94 °C, 3 min, 30 cycles of: denaturation at 94 °C 60 s, annealing at 51 °C 60s and extension at 72 °C 90 s; final extension at 72 °C, 5 min.

PCR products were checked by running them on a 1.0 % agarose gel stained with SYBR Safe (Invitrogen) and purified with the ExoSAP-IT kit (Amersham Biosciences) before sequencing.

The contents of eight oesophageal bulbs after PCR were cloned into JM109 competent cells using the P-GEM-T Easy vector (Promega), following the manufacturer's recommendations. Transformation was verified using PCR assays with the M13-T7 universal primer pair.

Insect host analysis. A portion of the mitochondrial DNA of the olive fly corresponding to part of the NADH dehydrogenase subunit 1, the leucine tRNA and the 16S mitochondrial region, was amplified and sequenced using the universal primer pair N1-J12261m and LRN13398 (Simon *et al.*, 1994, 2006). This analysis was performed on the same flies in which the contents of the oesophageal bulbs were sequenced. A total of 15 olive fly populations out of 26, representative of the main Italian regions, was processed for this task. Amplifications were performed in 20 µl reaction volumes [1 × PCR GoTaq Flexi buffer (Promega), 2.5 mM MgCl₂, 0.1 mM dNTPS, 0.2 µM each primer, 0.5 U of Taq polymerase (Promega), 2 µl template DNA]. Thermal cycling conditions were 5 min at 96 °C followed by 35 cycles of 96 °C for 50 s, 56 °C for 50 s and 72 °C for 2 min, with a final extension step of 72 °C for 5 min.

Sequencing and data analysis. Sequencing was performed at the BMR Genomics service (Padova, Italy) on automated DNA sequencers employing the primers used for PCR amplification and, in the case of '*Ca. E. dadicola*', two additional primers for the 16S rRNA gene: fL2 (Capuzzo *et al.*, 2005) and fL4 (Mazzon *et al.*, 2008).

Sequences were edited and aligned using MEGA 4.0.2 (Tamura *et al.*, 2007) and chromatograms were visually inspected. A BLASTN GenBank analysis of the sequences obtained was run through the NCBI website (www.ncbi.nlm.nih.gov) to assess the identity of the sequences obtained. The Arlequin Version 3.1 software package (Excoffier *et al.*, 2007) was used to perform the following genetic analyses on the olive fly symbiont: (i) analysis of population differentiation was accomplished by conducting exact tests of population differentiation with 100 000 steps in Markov chain, with 10 000 dememorization steps. (ii) An analysis of molecular variance (AMOVA) was performed to define the partition of genetic variability among populations and among groups of populations. (iii) The correlation between geographical and genetic distances (F_{ST} values) among populations was assessed by the non-parametric Mantel test using 1000 permutations. For this analysis, the pairwise F_{ST} values were transformed as $\log [F_{ST}/(1 - F_{ST})]$ (Rousset, 1997).

A nested clade phylogeographic analysis (NCPA) was implemented by the ANeCa ver. 1.2 program package (Panchal, 2007), which included TCS 1.21 (Clement *et al.*, 2000) and GeoDis 2.5 (Posada *et al.*, 2000). The haplotype network for the mitochondrial olive fly region was constructed using the statistical parsimony algorithm (Templeton *et al.*, 1992) which output the 95 % plausible set of most parsimonious linkages among haplotypes. The NCPA first tests the null hypotheses of no association between geography and the haplotype network. Only when this null hypothesis is rejected at the 5 % level of significance is it possible to infer likely historical and geographical events (Templeton *et al.*, 1995). Fisher's exact test was applied to observe associations between the haplotype of '*Ca. E. dadicola*' and the haplotype of the insect host.

RESULTS AND DISCUSSION

'*Ca. Erwinia dadicola*' diversity

A total of 314 olive fly oesophageal bulbs from 26 Italian populations were analysed with an average of 11.5 individuals per population. A GenBank similarity search (BLASTN) showed that the 781 bp 16S rRNA gene fragment matched with 16S rRNA gene of '*Ca. E. dadicola*'. Other bacteria were predominant in only three olive flies (data not shown). When aligned by MEGA, '*Ca. E. dadicola*' sequences showed the presence of three associated point mutations. Two '*Ca. E. dadicola*' haplotypes were thus identified, hereafter referred to as haplotype A (htA) and haplotype B (htB). Sequences were deposited under GenBank accession nos HQ667588 and HQ667589, respectively. In particular, three transitions were identified. At positions 11 and 282, htA showed a C and htB showed a T. At position 657, htA showed a G and htB showed an A. In total, htA was present in 43 % of samples (134/311) and htB was present in the remaining 57 % (177/311).

Moreover, the 16S rRNA gene sequences of 43 '*Ca. E. dadicola*' samples derived from nine localities, 21 of which belonged to htA and 22 to htB, were extended in the 3' direction up to 1280 bp using the fL2 primer. Two further mutations were identified: at position 943, htA showed a C and htB showed an A, and at position 945 bp, htA showed a T and htB showed an A.

The presence of these point mutations allowed a restriction enzyme (*Apa*I) to be used in order to distinguish the two

different bacterial haplotypes more quickly and with good accuracy. The digestion technique was used in addition to standard sequencing.

To date, ten sequences attributed to the '*Ca. E. dadicola*' 16S rRNA gene have been deposited in GenBank (five from the USA; four from Spain and one from Italy). Eight of these sequences provided full-length coverage with the sequences of our two haplotypes, hence only these sequences were compared with our results. All the US sequences (GenBank accession nos GQ478373, GQ478377 and GQ478378), two from Valencia, Spain (FM958429 and FM958431) and the sequence from Italy (AJ586620) matched with htB, while the remaining two sequences (FM958428 and FM958430), both from Valencia, Spain, were identical to htA.

The 23S rRNA region of the contents of ten oesophageal bulbs was also tested. These flies were chosen from different populations (locations 2, 3, 6, 10, 12, 13, 17, 20, 22 and 24). A 761 bp fragment was obtained from DNA amplification and sequencing with the universal bacterial primers (GenBank accession no. HQ667590). Upon alignment of these sequences, no nucleotide differences were observed between them. 16S rRNA gene sequencing of the same samples showed that six of them corresponded to htA and four of them corresponded to htB.

In order to check for the presence of both bacterial haplotypes in a single oesophageal bulb, the contents of eight *B. oleae* bulbs were cloned. Flies whose oesophageal bulbs were cloned were chosen mainly from populations that exhibited both bacterial haplotypes (locations 3, 5, 6, 9, 10, 16, 17 and 24, Table 1). Seven to 12 amplicons for each individual were sequenced or analysed with a restriction enzyme (*ApaI*). The results confirmed the presence of a unique '*Ca. E. dadicola*' haplotype in every olive fly oesophageal bulb, suggesting that htA and htB do not appear to coexist inside the same olive fly. Intermediate haplotypes between htA and htB were never found, thus implying an ancient separation of the two bacterial variants which probably originated after a prolonged period of isolation in different geographical areas. This isolation could correspond with the Pleistocene fragmentation of different glacial refugia of the oleaster and therefore of olive fly populations (Nardi *et al.*, 2010).

'*Ca. E. dadicola*' haplotype distribution

Both htA and htB were present at different rate frequencies in all the peninsular populations, with the exception of location 4 (Table 1). Surprisingly, the olive fly populations of the two main Italian islands (Sicily and Sardinia) harboured a single symbiont haplotype: in Sardinia only htA was found, while htB was the only one recorded in Sicily. The geographical distribution of the two '*Ca. E. dadicola*' haplotypes is shown in Fig. 1. Geographical isolation could be the main explanation for this evident homogeneous distribution within the islands, as opposed to the haplotype mix observed in the peninsular populations. However, it is

unlikely that accidental introduction of olive flies carrying the other symbiont haplotype to the islands has never happened, especially for a species such as *B. oleae* that is tightly connected with commercial cultivation and human-mediated exchanges.

The presence of population differentiation was confirmed by the tests of population differentiation ($P < 0.001$). When analysing the '*Ca. E. dadicola*' haplotype distribution among the 26 Italian olive fly populations by AMOVA, a clear geographical pattern did not appear. AMOVA was performed to analyse the origin of molecular variability in the different hierarchical levels and groups (Table 2). In the first analysis, all 26 populations analysed were considered to form a single group. In this case, AMOVA showed that about 39 % of the variation was explained by differences among populations, although the highest variation occurred within the populations themselves (60.83 %). When only the peninsular populations were considered, the percentage of variance among the populations decreased to 26.25%. In the successive AMOVA tests, the populations were grouped according to the Italian phytoclimatic zones proposed by Pavari (1916) or according to the presence of geographical isolation. A variance value of 38 % was observed when considering the sea as a geographical barrier and thus combining the populations into three groups (peninsular populations vs. Sicily populations vs. Sardinia populations) (Table 2). Moreover, in order to test the additional influence of the orographic barrier of the Apennine mountain range, the populations were clustered into eight groups: north-east vs. north-west vs. central-west vs. central-east vs. south-west vs. south-east vs. Sicily island vs. Sardinia island (Table 2). This grouping explained a lower, but significant, proportion of variation (34.53 %), whereas it decreased (19.42 %) upon removing the two island groups (Table 2). Finally, the populations belonging to the phytoclimatic subzones of the warm *Lauretum* (supposedly the refugial areas of olive tree during cold periods) were grouped into four clusters that currently appear geographically isolated from each other (Liguria region vs. south Italy vs. Sicily island vs. Sardinia island). In this case, the AMOVA test showed a very high and significant percentage of variation (51.15 %) (Table 2).

According to the Mantel test based on 26 populations, the genetic and geographical distances were significantly correlated ($r = 0.13$, $P = 0.026$). If the peninsular populations were split into two groups, separated by the orographic barrier of the Apennine mountains, the Mantel test revealed evidence for isolation by distance when considering the olive fly populations located along the Tyrrhenian coast (locations 6, 7, 8, 9, 10, 13, 14, 16 and 17) ($r = 0.35$, $P = 0.03$), showing an increasing proportion of htB going south, but this became non-significant for locations close to the Adriatic coast (locations 11, 12, 18, 19 and 20) ($r = -0.18$, $P = 0.61$).

In order to assess variations in the distribution of the two haplotypes over time, two olive fly populations, each composed of both '*Ca. E. dadicola*' haplotypes, were monitored for three consecutive years (locality 5 from 2007 to 2009

Table 2. AMOVA of the symbiont '*Ca. E. dacicola*' based on 16S rRNA gene sequence data among populations of *Bactrocera oleae* divided according to the phylogeographic hypotheses discussed in the text

Structure	Source of variation	d.f.	Variance (%)	Fixation indices	P value
All populations	Among populations	25	39.17	$F_{ST}=0.39168$	<0.001
	Within populations	285	60.83		
Only peninsular populations	Among populations	19	26.25	$F_{ST}=0.26251$	<0.001
	Within populations	238	73.75		
Grouping by geographical region I*	Among groups	2	38.01	$F_{CT}=0.38011$	<0.001
	Among populations within groups	23	16.98	$F_{SC}=0.27397$	<0.001
	Within populations	285	45.01	$F_{ST}=0.54994$	<0.001
Grouping by geographical region II†	Among groups	7	34.53	$F_{CT}=0.34529$	<0.001
	Among populations within groups	18	6.86	$F_{SC}=0.10484$	<0.001
	Within populations	285	58.61	$F_{ST}=0.41393$	<0.001
Grouping by geographical region III‡	Among groups	5	19.42	$F_{CT}=0.19417$	<0.05
	Among populations within groups	14	8.89	$F_{SC}=0.11035$	<0.01
	Within populations	238	71.69	$F_{ST}=0.28309$	<0.001
Grouping by wild olive areas§	Among groups	3	51.15	$F_{CT}=0.51155$	<0.01
	Among populations within groups	11	12.61	$F_{SC}=0.25820$	<0.01
	Within populations	165	36.23	$F_{ST}=0.63767$	<0.001

*Group 1, peninsular (locations 1–20); group 2, Sicily (locations 21–23); group 3, Sardinia (locations 24–26).

†Group 1, north-east (locations 1–5); group 2, north-west (locality 6); group 3, central-west (locations 7–10); group 4, central-east (locations 11–12); group 5, south-west (locations 13–17); group 6, south-east (locations 18–20); group 7, Sicily (locations 21–23); group 8, Sardinia (locations 24–26).

‡Group 1, north-east (locations 1–5); group 2, north-west (locality 6); group 3, central-west (locations 7–10); group 4, central-east (locations 11–12); group 5, south-west (locations 13–17); group 6, south-east (locations 18–20).

§Group 1, Liguria region (locality 6); group 2, South (locations 13–20); group 3, Sicily (locations 21–23); group 4, Sardinia (locations 24–26).

and locality 6 from 2006 to 2008) (Table 1). The proportions of the two bacterial haplotypes in each population appeared not to be random as Fisher's exact test showed no significant differences in the htA–htB distributions over the years for both populations (locality 5, Fisher's exact test, d.f.=2, $P=0.56$; locality 6, Fisher's exact test, d.f.=2, $P=0.98$). This result suggested that the distribution of the two haplotypes seemed to be constant over time and was specific to the population.

Matching the extent of symbiont and host haplotypes

A total of 776 bases corresponding to part of the NADH dehydrogenase subunit 1, the leucine tRNA and the 16S rRNA mitochondrial region, was sequenced for 80 olive flies representing 15 populations in Italy (Table 1). DNA sequences were aligned to identify polymorphisms. A total of 16 variant sequence forms were identified (no gaps were found) and were given the haplotype designations h1 to h16. These are shown in Table 1, which also provides details of their distribution by locality. The most common and widespread haplotypes (h1 and h2) were shared by 12 and 11 populations, respectively. Seven haplotypes were unique. Each of the Italian olive fly populations analysed harboured 3.2 haplotypes on average. The sequences were deposited in GenBank under accession nos HQ667572 to HQ667587, inclusive. A network of the haplotypes was

constructed (Fig. 2) using the TCS program. The analysis showed that 15 of the 16 haplotypes identified were directly connected and that only two haplotypes were missing (Fig. 2). NCPA performed with ANeCA showed that the null hypothesis of no association between the network structure and geography could not be rejected.

The overlay of the two '*Ca. E. dacicola*' haplotypes over the host mtDNA data did not show any evident association (Fig. 2). In all, the interior haplotypes (h1, h2, h3 and h5) tended to be older and more frequent than the tip haplotypes (Posada *et al.*, 2006), both '*Ca. E. dacicola*' lineages were present and their proportions did not differ significantly (Fisher's exact test, d.f.=3, $P=0.577$). The external mitochondrial haplotypes were not considered in this analysis because they are rare and thus strongly correlated with either one of the two '*Ca. E. dacicola*' haplotypes. The lack of an association between the symbiont and host lineages could be at least partly explained assuming the horizontal transfer of the symbiont. Even though Estes *et al.* (2009) have recently hypothesized an intracellular existence for '*Ca. E. dacicola*' at the larval stage, the olive fly symbiont can, at present, according to Petri (1909), be considered to be extracellular. The extracellular condition offers more potential opportunity for contacts compared with the lifestyle of endocellular symbionts. Moreover, the olive fly lifestyle, especially during the larval stage, offers opportunities for symbiont loss and reacquisition to occur,

Fig. 2. Haplotype network of *Bactrocera oleae* realized by TCS 1.21 (Clement *et al.*, 2000). Each haplotype is represented by a circle, with the area of the circle proportional to its frequency. The two tiny empty circles represent intermediate missing haplotypes. The proportion of the 'Ca. *E. dadicola*' lineage inside each circle is reported: haplotype A (black) and haplotype B (grey).

making it possible for the haplotype of 'Ca. *E. dadicola*' to be replaced.

Like the majority of the fruit flies, *B. oleae* is tightly associated with its host plant and it spends its larval life feeding on the olive fruit pulp (Mazzon *et al.*, 2010). It is common to observe the presence of more than one larva inside the same olive; this could offer the opportunity for possible replacement by a different haplotype of 'Ca. *E. dadicola*', bypassing the model of strict vertical transmission. In such a scenario, the co-existence of both 'Ca. *E. dadicola*' lineages inside the same insect host could be postulated, but in our analysis, even though it was carried out solely on adults, this has never been observed (as confirmed by cloning). Therefore, the combined occurrence of both of the 'Ca. *E. dadicola*' variants in a single fly seems to be extremely rare or not possible at all due to an unknown intimate mechanism that may occur at the larval stage.

There are no clear indications about the colonization history of the olive fly and its geographical origin as they have been obscured by the long history of olive cultivation in the Mediterranean area (Daane & Johnson, 2010), but there are data about its African origin (Nardi *et al.*, 2005, 2010; White, 2006). We think that this report could represent the first standpoint in the understanding of the

genetic variability of the olive fly in relation to that of its symbiont. Further and more comprehensive studies could address the situation that occurs over the entire colonization area of *B. oleae* by extending the research over the whole Mediterranean basin. This line of research, besides being a useful tool to fill in the details of the colonization route of the olive fly, could be a model for many investigations studying the evolutionary interdependency between insects and their associated bacteria.

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