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Presence of the endosymbiont *Wolbachia* among some fruit flies (Diptera: Tephritidae) from Iran: A multilocus sequence typing approach

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ABSTRACT

Wolbachia is a widespread endosymbiont of insects with a diverse range of biological effects on its hosts. We studied the prevalence of *Wolbachia* in some important species of tephritids in Iran. Among different populations of five fruit fly species, *Dacus ciliatus* (cucurbit fly), *Rhagoletis cerasi* (cherry fruit fly), *Ceratitis capitata* (Mediterranean fruit fly), *Myiopardalis pardalina* (melon fly) and *Carypomya vesuviana* (jujube fly), two species, *R. cerasi* and *C. vesuviana*, showed infection with separate *Wolbachia* strains, namely *w*Cer6 and *w*Ves1, respectively. *C. vesuviana* is introduced here as a novel host for *Wolbachia*. Genotyping of *Wolbachia* strains in 12 populations of five fruit fly species, using multilocus sequence typing (MLST) and the *wsp* gene sequence showed the occurrence of two new strains as well as a new strain type (ST) belonging to the A supergroup. On the basis of the results of this study, 12 barcodes under five species of Iranian tephritids have been added to the database of DNA barcodes. Inter- and intra-specific differences among COI sequences showed a clear gap in barcoding among most fruit flies.

Introduction

Fruit flies (Diptera: Tephritidae) are among the most destructive and well-publicized pests of fruits and vegetables worldwide. Most species in this family are phytophagous, including some important agricultural pests (Foote et al., 1993; Merz, 2001).

Area-wide projects have been conducted in some regions, including Mexico, Australia and Chile for the eradication or suppression of *Ceratitis capitata* and in New Zealand for the eradication of painted apple moth, *Teia anartoides*. Furthermore, some other projects are about the control of *Bactrocera dorsalis*, *Bactrocera cucurbitae*, and *Cydia pomonella* (Bourtzis and Miller, 2006; Bourtsiz, 2008; Vreysen et al., 2006). Among several causes of cytoplasmic incompatibility of insects, *Wolbachia pipientis* plays a key role (Stouthamer et al., 1999). This endosymbiont is a maternally inherited intracellular bacterium. The host range of *Wolbachia* is wide within arthropods and nematodes but the effects of *Wolbachia* are specific for different taxonomic groups (Hughes and Rasgon, 2012). *Wolbachia* induces reproductive manipulations of the host, including parthenogenesis, feminization, male killing, and, most commonly, cytoplasmic incompatibility (CI). *Wolbachia*-infected males are released to cause bi- or unidirectional CI in wild-type females in crosses between infected males with females of different infection status. The released individuals have a reduced probability of establishment in the field (Zabalou et al., 2004; Bourtzis, 2008; Sarakatsanou et al., 2011), which ultimately leads to suppression or elimination of the populations.

The use of *Wolbachia* to control insects may have considerable potential for managing pest populations. Nevertheless, a major concern is identifying the correct status of infection type as well as genotype/subgroup of the *Wolbachia* strain. The genus *Wolbachia* is highly diverse and has been subdivided into several phylogenetic units within invertebrates (Bourtzis, 2008; Augustinos et al., 2011). The grouping and detection of lineages have been based on *wsp* sequence data as a taxonomic tool (Zhou et al., 1998). However, there are some major concerns, including recombination in the *wsp* gene (Jiggins, 2002), as well as chimeric structures caused by extensive intragenic recombination and strong diversifying selection (Baldo et al., 2005) for the *wsp* gene. Therefore, another approach that included a multilocus sequence typing (MLST) system (Baldo et al., 2006) was introduced as a novel tool for *Wolbachia* typing. MLST typically involves sequencing internal fragments of five single copy housekeeping genes per strain.

Wolbachia infection and its prevalence have been addressed in several species of fruit flies such as *Rhagoletis pomonella* (O'Neill et al., 1992), *Anastrepha suspensa* (Werren et al., 1995), *Anastrepha fraterculus* (Selivon et al., 2002), *Ceratitis capitata* (Lincoln et al., 2005), *Bactrocera dorsalis* (Sun et al., 2007), and *Rhagoletis cerasi* (Riegler and Stauffer, 2002; Arthofer et al., 2009b).

Zabalou et al. (2004) transferred *Wolbachia* (*w*Cer2 and *w*Cer4) from *R. cerasi* to *C. capitata*. Their results showed that *Wolbachia*-infected *C. capitata* lines had complete cytoplasmic incompatibility (CI) (unidirectional and bidirectional) in single-pair genetic crosses. This result

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showed promise of a new approach for the application of *Wolbachia*-infected individuals in insect pest management programs.

Additionally, some control strategies, such as the use of sterile insect technique, are strongly dependent on the release of appropriate strains. Consequently, careful identification of the target pests is required (Han and Ro, 2005). Immature stages of tephritid flies are almost identical and very difficult to identify using morphological features alone. Since some species of fruit flies are important quarantine pests, a rapid and reliable method for species delimitation, such as the DNA barcoding method, would be highly useful. In recent years, many molecular studies have been performed using tephritid flies (e.g., Smith et al., 2002; Feder et al., 2003; Jamnongluk et al., 2003; An et al., 2004; Gilchrist et al., 2004; Armstrong and Ball, 2005; Augustinos et al., 2005; Han and Ro, 2005; Barr and McPheron, 2006; Velez et al., 2006). Taxonomic studies on Iranian tephritid flies have been performed by Parchami-Araghi (1995), Gharali et al. (2006), Karimpour and Merz (2006), Gilasian and Merz (2008), Mohammadzade-Namin et al. (2010), and Zarghani et al. (2010). However, there is no molecular data of these flies in the country.

In this study, we attempted the following: (1) to detect *Wolbachia* infection in some species of Iranian tephritid fruit flies; (2) to identify *Wolbachia* strain types within some tephritid fruit flies using the MLST approach and the *wsp* gene; and (3) to determine inter-intra specific COI divergence among some populations of certain fruit flies originating from different regions of Northeastern Iran.

Materials and Methods

Insects

Fruit flies were collected from Razavi, North and South Khorasan provinces in Northeastern Iran. Fruits infested with tephritid larvae were collected during the spring and summer of 2011 and reared in plastic vessels containing a 5-cm sand layer. The samples were collected from Mashhad, Shirvan, Bojnourd, Torbat Heydariyeh, Kashmar, Birjand, Sarakhs Road, Khaf, and Taibad. From each location, several populations of different tephritids were collected. The rearing vessels were maintained in a controlled growth chamber (25 ± 1 °C) for 3 weeks until the emergence of adult flies. The emerged flies were harvested daily using an aspirator and stored in absolute ethanol at −80 °C for further examination. The taxonomic position of the fruit flies was confirmed by Professor Mehdi Modarres Awal, from Ferdowsi University of Mashhad, Iran. The list of species with different populations is presented in Table 1.

DNA extraction

The thorax and abdomen of liquid nitrogen-frozen (to prevent hemolymph contamination) individual fruit flies were ground with 50 μl of 5% Chelex-100 and 2 μl of proteinase K (20 mg/ml) and incubated 3 h at 60 °C followed by 10 min at 95 °C. The mixture was spun at 13,000 ×*g* for 3 min. The supernatant was extracted and stored at −20 °C. For each species, five populations were selected. Ten specimens from each population were used for DNA extraction and molecular analyses.

Wolbachia detection

wsp gene-based detection

Amplification of the *wsp* gene using the *Wolbachia* primers was taken as evidence of the presence of *Wolbachia*. For PCR, the primer set of Briag et al. (1998) was used to amplify a 560–632-bp segment of the *wsp* gene. PCRs were performed in 25-μl reaction volumes containing 3 μl of DNA, 3 μl of 10× PCR buffer, 0.75 μl $MgCl_2$, 0.5 μl dNTPs, 0.6 μl forward and reverse primers, and 0.3 μl *Taq* polymerase. The PCR amplification of the *wsp* gene included an initial denaturation at 94 °C for 30 min, followed by 36 cycles with a denaturation step at 94 °C for 30 s, annealing at 46 °C for 45 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. For each population, 10 individuals were selected. Samples of *Wolbachia*-infected *Drosophila melanogaster* were used as a positive control for the PCR. Negative controls consisted of samples lacking the DNA template from insects and treated *D. melanogaster* DNA. The mitochondrial COI gene was used as the positive control for DNA quality and amplifiability (Folmer et al., 1994). DNA was sequenced with the BigDye Terminator Kit (Macrogen Inc., Republic of Korea). Both DNA chains of each sample were sequenced separately with the corresponding primers. All sequence chromatograms were checked for any ambiguous peaks in the sequences. We done so to avoid any false data due to superinfection or multiple infections. DNA templates that could not be amplified with *Wolbachia*-specific primers were used in the PCR using the control COI primers.

The forward and reverse sequences were used to assemble the consensus sequence. MEGA 5 (Tamura et al., 2011) software was used for conceptual translation of the *wsp* sequences into protein. The sequences were edited and aligned with BioEdit software (Hall, 1999). The nucleotide sequences were aligned manually by comparing the alignment of proteins. This alignment was used in the phylogenetic analysis. Distance between the *wsp* consensus sequences was calculated on nucleotide and amino acid levels using MEGA5.

Genotyping of Wolbachia strains and their phylogeny

All available *wsp* gene sequences of fruit fly species from GenBank, together with the data of the current study, were aligned. In the case of two *Wolbachia* strains (wCer4 and wCer5) as endosymbionts of *Ceratitic capitata*, we did not find their corresponding *wsp* sequences in the GenBank. Therefore, we requested these from Wolfgang Arthofer. The multiple aligned file was analyzed using maximum parsimony

Table 1
List of fruit fly species, infection status to *Wolbachia*, and accession numbers of *wsp*, MLST, and COI gene sequences.

			Wolbachia		Accession no.					
Species	Location	Isolate	*wsp*	COI	*wsp*	*cox*A	*gat*B	*fts*Z	*fbp*A	*hcp*A
R. ceraci	Mashhad	FUM 31	(+)	JQ668126	JQ668139	JQ668141	JQ668143	JQ668145	JQ668147	JQ668149
C. vesuviana	Birjand	FUM 32	(+)	JQ668127	JQ668140	JQ668142	JQ668144	JQ668146	JQ668148	JQ668150
C. capitata	Guilan	FUM 33	(−)	JQ668128						
D. ciliatus	Torbat H.	FUM 34	(−)	JQ668129						
D. ciliatus	Golmakan	FUM 35	(−)	JQ668130						
D. ciliatus	Bojnourd	FUM 36	(−)	JQ668131						
D. ciliatus	Kashmar	FUM 37	(−)	JQ668132						
D. ciliatus	Mashhad S	FUM 38	(−)	JQ668133						
D. ciliatus	Shirvan	FUM 39	(−)	JQ668134						
M. pardalina	Jim Abad	FUM 40	(−)	JQ668135						
M. pardalina	Mashhad	FUM 41	(−)	JQ668136						
M. pardalina	Sarakhs Road	FUM 42	(−)	JQ668137						
M. pardalina	Khaf	Khaf 1	(−)	HM070409						
M. pardalina	Khaf	Khaf 2	(−)	HM063426						

through PAUP* 4.0b (Swofford, 2002) and the neighbor-joining (NJ) method using MEGA5 software (Tamura et al., 2011). In the absence of a suitable outgroup for rooting the trees, the evolutionary rate was assumed uniform for all branches, and trees were midpoint rooted. Recombination analyses were carried out on single locus and concatenated gene sequence data files using the MaxChi method implemented in RDP3 program (Martin et al., 2005).

MLST system for Wolbachia screening

Five housekeeping genes (*gat*B, *cox*A, *hcp*A, *fts*Z, and *fbp*A) were amplified with standard primers (Baldo et al., 2006). The reactions were carried out in a 25-μl reaction volume containing approximately 3 μl template DNA, 1 μl forward and reverse primers (10 pmol/μl), 0.5 μl deoxynucleotide triphosphate (dNTPs), 1 μl $MgCl_2$, 3 μl of 10× PCR buffer and 0.3 μl *Taq* polymerase (5 U) (Bioneer Inc., Republic of Korea). The reactions were performed under the conditions specified by Baldo et al. (2006) for MLST genes. All PCR products were purified in a 1% agarose gel using a Bioneer gel band purification kit (Bioneer Inc., Republic of Korea).

MLST analysis

The MLST sequences were concatenated into a supergene alignment with 2079 nucleotides. Related sequences from Baldo et al. (2006), considered as valid and verified data, were retrieved from GenBank and used to construct a multiple alignment file using Clustal X (Larkin et al., 2007). Phylogenetic analyses were performed using maximum likelihood (ML) and NJ in MEGA 5 (Tamura et al., 2011) with 1000 bootstrap replications (Felsenstein, 1985).

Mitochondrial COI as DNA barcode for Iranian fruit flies

In order to determine inter-intraspecific divergence among COI sequences of fruit fly species, the primer set of Folmer et al. (1994) (LCO1490 and HCO2198) was used for amplification of this gene. Ten individuals from each population were used for the PCR. PCRs were carried out in an Eppendorf Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) in standard 25-μl reactions containing 2 μl DNA template, 3 μl (10×) buffer, 1 μl $MgCl_2$, 0.5 μl dNTPs, 1 μl of both forward and reverse primers (10 pmol/ml), and 0.3 μl *Taq* polymerase (5 U). Thermal conditions for PCR were denaturation at 94 °C for 60 s; annealing at 55 °C for 90 s, and extension at 72 °C for 90 s (30 cycles, plus an initial denaturation at 94 °C for 1 min and a final extension at 72 °C for 8 min). PCR products were sequenced directly as mentioned earlier. To remove the possible effect of nuclear mitochondrial pseudogenes (numts) on DNA barcoding, we followed the Song et al. (2008) method. Numts are non-functional copies of mtDNA in the nucleus that have been found in major clades of eukaryotic organisms.

NBLAST was conducted to compare our sequences with similar sequences archived in the NCBI database. For phylogenetic analysis, 28 valid sequences were retrieved from the GenBank and Boldsystem (www.boldsystems.org). Sequences were aligned using Clustal X (Larkin et al., 2007) with default settings. Phylogenetic analyses were performed using ML and NJ methods (Saitou and Nei, 1987) in MEGA 5 (Tamura et al., 2011) with 1000 replicates of bootstrapping (Felsenstein, 1985). Nucleotide diversity was calculated using the K2P model (Kimura, 1980).

For determination of gap barcoding between inter- and intraspecific COI divergence, 405 sequences of the family Tephritidae were retrieved from the GenBank and the BOLD system and analyzed by constructing a histogram based on Kimura 2-parameter distances using TaxonDNA software (Meier et al., 2006). For mitochondrial haplotype determination, we used sequences of the COI gene of fruit flies. The haplotype network was estimated using TCS1.21 software (Clement et al., 2000).

Nucleotide sequence accession numbers

All *wsp*, MLST, and COI gene sequences generated in this study have been deposited in GenBank under accession numbers JQ668126 to JQ668150. A single sequence for the COI gene from each population of the fruit flies and a representative sequence of *wsp* and MLST genes of the *Wolbachia* strain were selected for submission.

Results

Wolbachia detection

We positively diagnosed *Wolbachia* infection for populations of two tephritid species through the amplification of a fragment of the *wsp* gene. Among five populations of *R. cerasi*, all populations were infected with the endosymbiont. All positive individuals harbored a strain of *Wolbachia*. A threshold of 2.5% sequence divergence in the *wsp* gene as the grouping criterion within *Wolbachia* supergroups has been suggested by Zhou et al. (1998). We used these criteria to assign a new subgroup of *Wolbachia* related with *R. cerasi* as wCer6. The highest infection rates were found in the Bojnourd population of *R. cerasi* (95%). For *C. vesuviana*, we checked 10 individuals from five different populations from the Birjand region. The results showed occurrence of infection among some individuals. The maximum level of *Wolbachia* infection among the populations of *C. vesuviana* was 25%. In the case of *D. ciliatus*, we tested six populations. All assayed individuals of these populations were negative for *Wolbachia* (0 for 50 individuals). The melon fly and Mediterranean fruit fly populations had similar statuses of *D. ciliatus*. We observed no *Wolbachia* infection among any of the populations of *M. pardalina* and *C. capitata*. Among the native populations of fruit fly species of Iran, only *R. cerasi* and *C. vesuviana* were infected with *Wolbachia*. Diagnosis of *Wolbachia* infection was negative for *D. ciliatus*, *C. capitata*, and *M. pardalina* (i.e., no amplification of the *wsp* gene). *R. cerasi* and *C. vesuviana* showed two new *Wolbachia* strains, named *w*Cer6 and *w*Ves1, respectively (Table 1).

The infection rates of *Wolbachia* in different populations of these species collected from various geographical areas varied, ranging from 25% to 95%. The highest infection rates were found in the *R. cerasi* Bojnourd population (95%), while the lowest infection rates were recorded in the Birjand population of *C. vesuviana* (25%).

The prevalence of *Wolbachia* infection in the cherry fruit fly was fixed. However, in the case of *C. vesuviana*, the prevalence of infection rate was variable. The screening results for different individuals of each population also confirmed this infection status of all tephritids. DNA templates that could not be amplified with *Wolbachia*-specific primers were successfully amplified in a PCR using the control COI primers. This shows that failure to amplify with the *Wolbachia*-specific primers was not caused by poor DNA quality.

Analysis of the *wsp* sequences of two *Wolbachia* strains (JQ668139 for *R. cerasi* and JQ668140 for *C. vesuviana*) indicated that these strains belong to supergroup A.

The multiple alignment of the *wsp* region for 49 taxa indicated that 238 sites were conserved, 259 sites were variable, and 210 sites were parsimony informative. Analysis of partial sequences of *wsp* gene segments generated from *R. cerasi* and *C. vesuviana*, together with sequences of *Wolbachia* strains from some other insect species, resulted in a well-structured tree. Fig. 1 shows this tree with the corresponding bootstrap values. The two new strains of *Wolbachia*, which were detected and identified in *R. cerasi* and *C. vesuviana*, have been clustered with other strains of supergroup A (Fig. 1). The mean pairwise distance of *wsp* sequences within the A and B supergroups was 0.144% and 0.166%, respectively. In addition, the distance between the species that belong to supergroups A and B was 0.232%.

The genotyping of new *Wolbachia* strains showed that the *Mel* subgroup is nearest to the *w*Cer 6 strain, but that it is distantly isolated from all members of this group distantly. The clade containing *w*Ves1

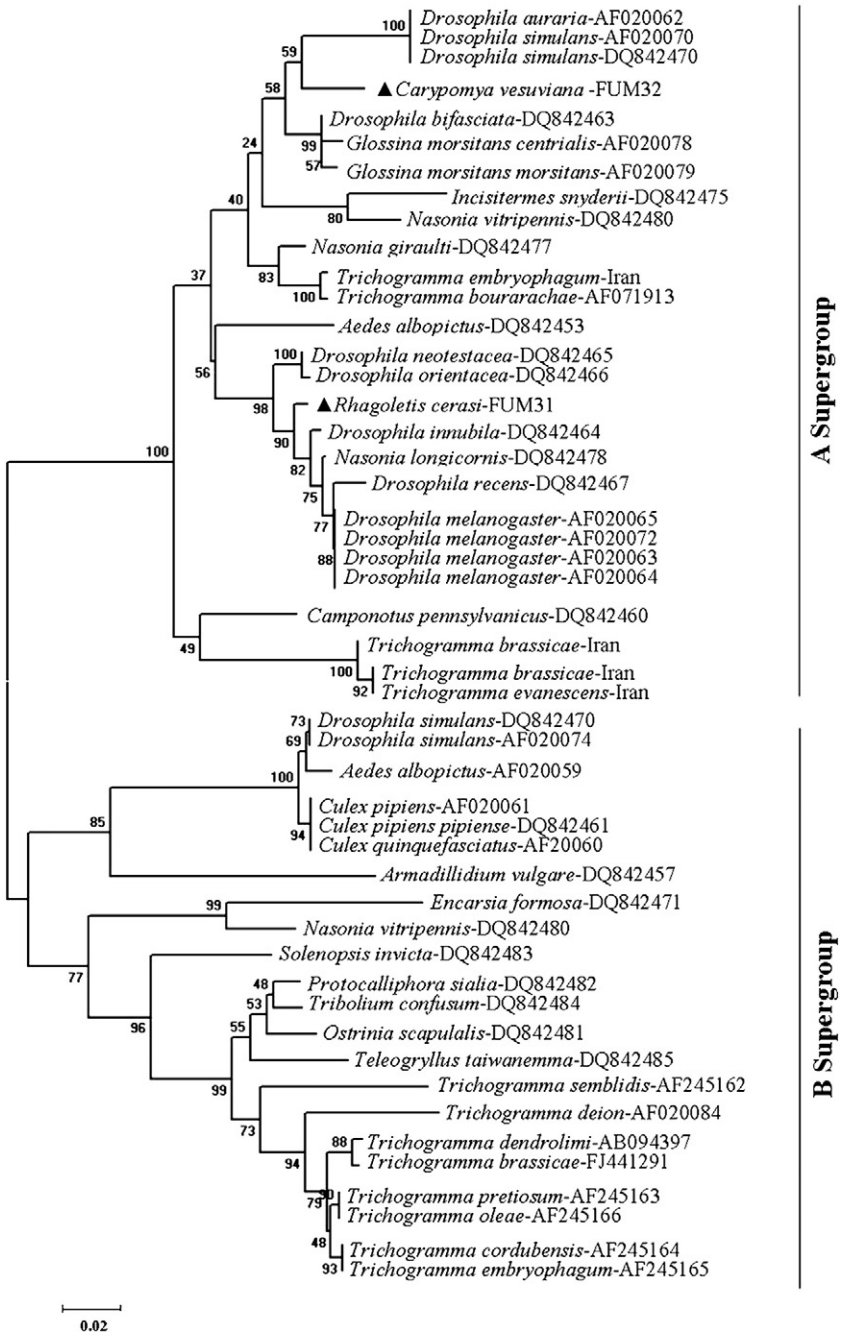

Fig. 1. Phylogenetic relationships among *Wolbachia* strains, endosymbiont of fruit flies, based on *wsp* gene sequence using the neighbor-joining method (NJ) and K2P model with 1000 bootstrap replications.

is between two clades, *Mors* and *RIV*. Here, we suggest a new subgroup for *Wolbachia* strain associated with *C. vesuviana* as *w*Ves1, due to the sequence distance threshold proposed by Zhou et al. (1998).

MLST analysis, allelic variation, and strain diversity

The phylogenetic analyses for concatenated MLST loci revealed that the two *Wolbachia* strains detected in *R. cerasi* and *C. vesuviana* belonged to supergroup A (Fig. 2). Allelic profiles for the five housekeeping genes of *w*Ves1 were 159 (*cox*A), 36 (*fbp*A), 75 (*fts*Z), 53 (*gat*B), and 186 (*hcp*A). Comparison of sequences from the five MLST genes with those in the databases suggested that the *Wolbachia* strain found in *C. vesuviana* represents a new sequence type (ST), hereafter *w*Ves1 (ST 277). For *w*Cer 6, four allelic profiles were determined. These are 91 (*cox*A), 160 (*fbp*A), 79 (*fts*Z), and 8 (*gat*B). Determination of the allelic profile for the *hcp*A gene failed. MaxChi analyses showed no recombination in *Wolbachia* strains based on either single gene or concatenated MLST data set alignments.

COI sequences and divergence

The COI gene was amplified successfully in all populations of *D. ciliatus*, *R. cerasi*, *C. capitata*, *M. pardalina*, and *C. vesuviana*. The sequenced fragments were 648 bp long and have been submitted to GenBank (Table 1). Sequence comparison against the BOLD system confirmed the classical identification results.

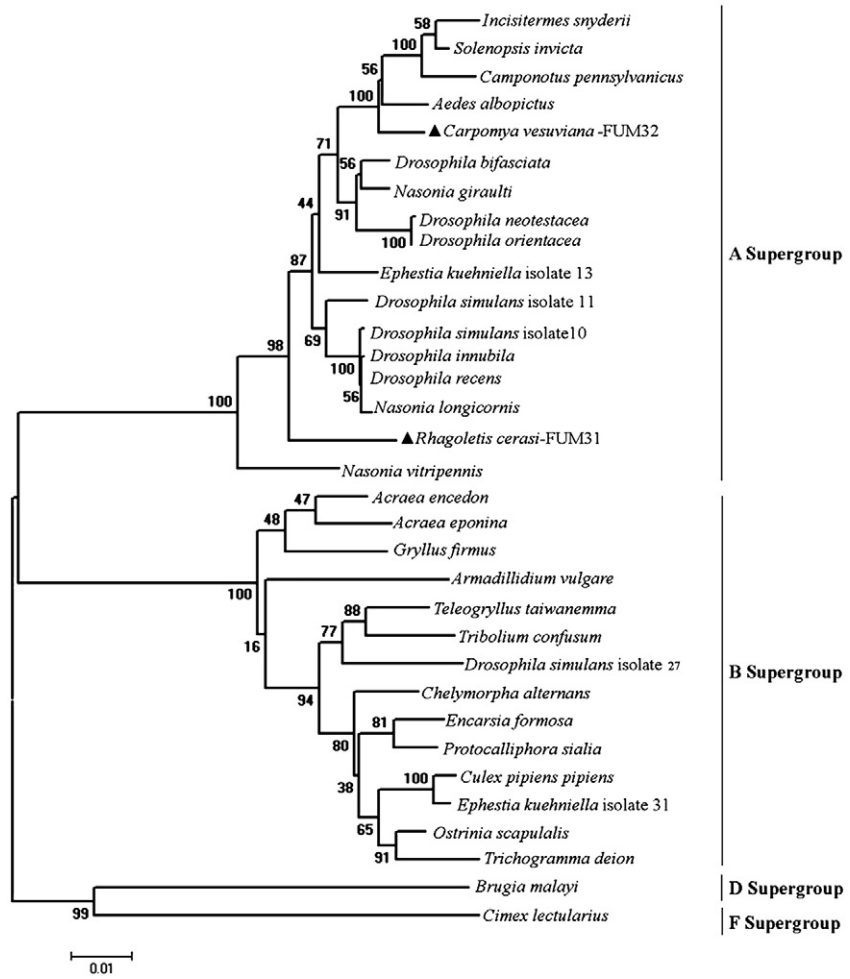

Fig. 2. Phylogenetic relations of *Wolbachia* strains, endosymbiont of certain fruit flies, based on concatenated sequences of MLST gene sequences using neighbor joining in MEGA 5 with 1000 bootstrap replications.

The multiple alignment of partial COI region for 49 taxa indicated that 322 sites were conserved, 225 sites were variable, and 197 sites were parsimony informative. The mean pairwise distance of COI sequences of the Tephritidae family was 0.163%, which was calculated using the K2P model. Additionally, intraspecificities among *D. ciliatus*, *R. cerasi*, and *C. capitata* were 0.009, 0, and 0.004, respectively. COI sequence differences between the species were almost 32.6 times higher than the average differences within species. We did not find any

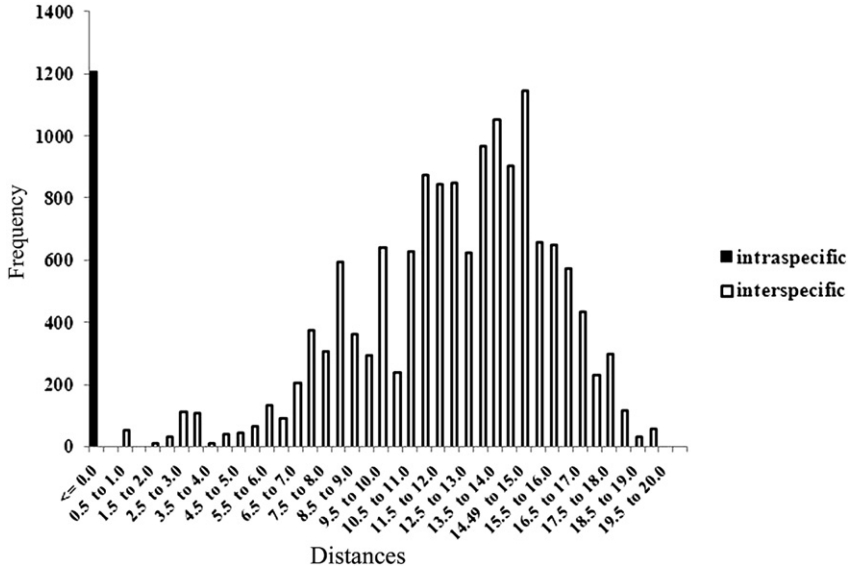

Fig. 3. Intraspecific (solid) and interspecific (black) divergence among COI gene sequences of 405 taxa of the Tephritidae family.

evidence of numts. These pseudogenes accumulate in-frame stop codons because they become non-functional after nuclear integration and are no longer under selective pressure to conserve an ORF.

Gap barcoding and haplotype

Our results confirmed the existence of a clear gap between inter- and intraspecific divergences for the COI gene without any overlap (Fig. 3). The existence of this gap between the inter- and intraspecific variations in COI sequences enables these specimens to be distinguished by DNA barcoding. Here, a surprisingly minimum level of genetic variability was found among different populations of *D. ciliatus*. All populations of *D. ciliatus* showed a similar haplotype.

Phylogenetic relationships analysis, based on the COI sequence, using both ML and NJ methods for the *Tephritidae* family, indicated well-supported clades. While the topology of the trees constructed using ML and NJ were similar, we decided to present the resultant data obtained using the ML method. In the phylogenetic tree, all species of each genus were placed in one clade (Fig. 4). The monophyly of each genus had strong quantitative support as measured by bootstrapping.

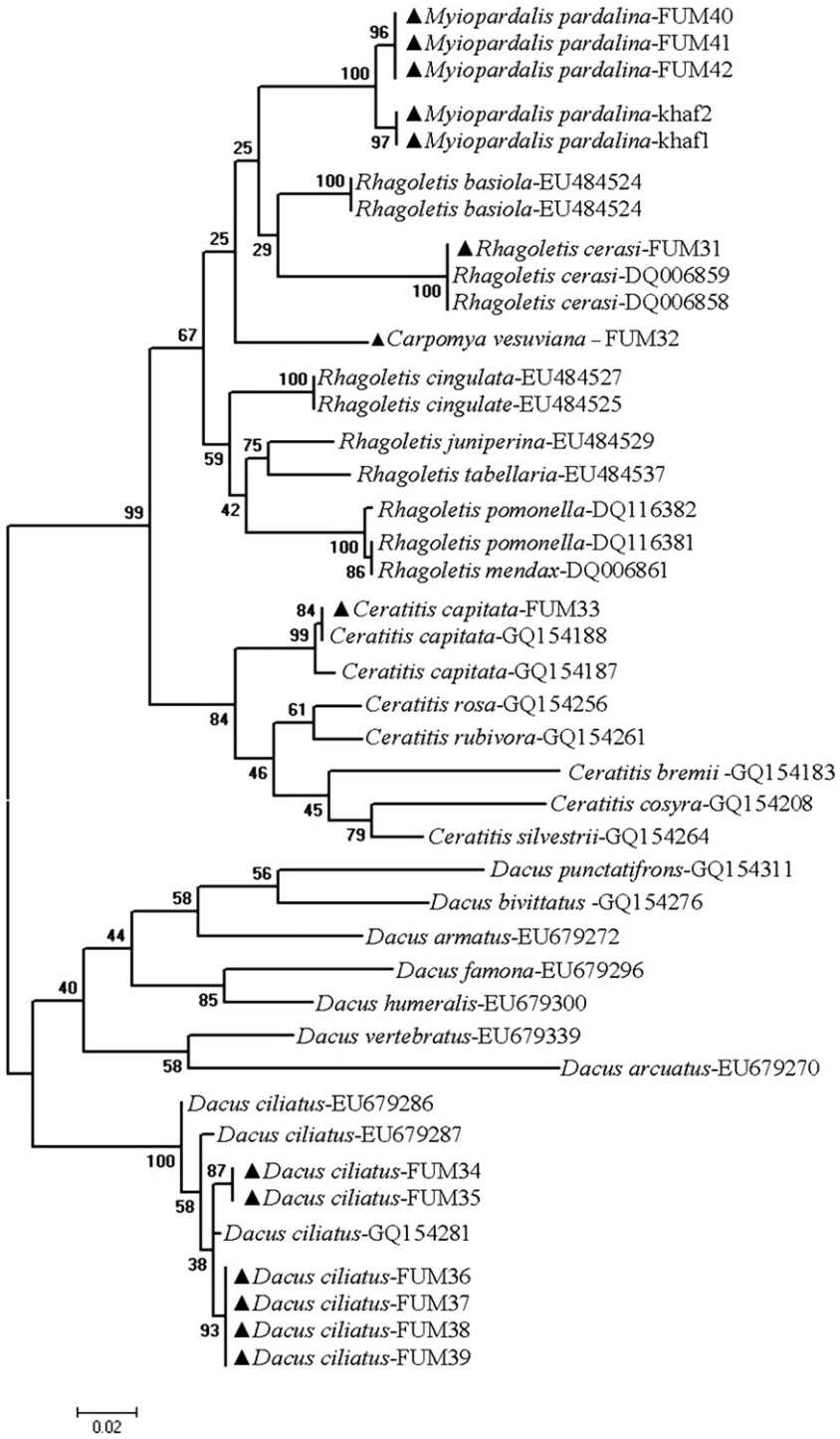

Fig. 4. Phylogenetic relationship among Tephritidae species based on the COI gene sequences using the maximum likelihood method and K2P model with 1000 bootstrap replications.

Discussion

Wolbachia is known to infect members of the genus *Rhagoletis* (Riegler and Stauffer, 2002; Schuler et al., 2011), but previously had not been detected in members of the genus *Carpomya* (Kittayapong et al., 2000; Kohnen et al., 2009). We found *C. vesuviana* infected with a *Wolbachia* strain belonging to supergroup A. All populations of *R. cerasi* were also infected with *Wolbachia* belonging to supergroup A, as was shown previously by Riegler and Stauffer (2002) and Arthofer et al. (2009b). The high frequency of infection by supergroup A of *Wolbachia* strains agreed with a similar high prevalence previously reported for other species within the phylum Arthropoda (Werren et al., 1995; West et al., 1998; Shoemaker et al., 2002). The result obtained indicating the fixed status of infection in an *R. cerasi* population is consistent with earlier reports on fixed infection in this fly by the *w*Cer1 strain of *Wolbachia*, whereas *w*Cer2 was detected only in restricted populations (Riegler and Stauffer, 2002; Arthofer et al., 2009b).

The phylogenetic analyses indicated the occurrence of distantly related *Wolbachia* strains in tephritid fruit flies and supported the hypothesis that horizontal transmission of *Wolbachia* strains occurs between different groups of insects even across distantly related species. This phenomenon has been reported in tsetse flies (*Glossina*), the spider genus *Agelenopsis*, *Nasonia* wasps, termites of the genus *Odontotermes*, and the *Bryobia* genus of Acari (Raychoudhury et al., 2008; Ros et al., 2009; Salunke and Salunkhe, 2010; Doudoumis et al., 2012).

On the basis of the different methods of analyses using single-locus and MLST data, both *w*Cer 6 and *w*Ves1 strains were assigned to supergroup A. Several studies have showed that the *wsp* gene has a high recombination rate. Therefore, the results of phylogenetic analyses based on *wsp* may not correctly reflect the true evolutionary and demographic histories of *Wolbachia* isolates. However, the recombination analysis based on the MLST concatenated data did not show a case of recombination occurrence within all five genes of the MLST nor in the *wsp* sequences.

We suggest two scenarios for the negative result of diagnostic *Wolbachia* infection in several populations of three fruit fly species, *M. pardalina*, *D. ciliatus*, and *C. capitata*. Firstly, misidentification of infection status could be attributed to a rare titer of genomic DNA. Even for positively determined samples, we observed some less intense bands of the *wsp* gene using PCR. This means that *Wolbachia* is present in the populations of tephritids, but at frequencies too low to be detected. Arthofer et al. (2009b) reported that five *Wolbachia* strains were found in the cherry fruit fly, *Rhagoletis cerasi*, in different populations. However, the available documents about PCR results of the *wsp* gene do not support this hypothesis. It was found that even with a minimal DNA concentration, it is possible to detect *Wolbachia* infection. Nevertheless, nested PCR or qPCR may be useful in future studies. Secondly, *Wolbachia* may actually be absent in collected and tested populations of fruit flies.

Our finding is in accordance with that of Arthofer et al. (2009a) who found a diverse status in *Wolbachia* strains associated with *R. cerasi*. *Wolbachia* diversity is expected to affect *Wolbachia* population dynamics and *Wolbachia*-based insect pest control strategies. In other words, this may lead to invasion barriers for expanding and artificially introduced *Wolbachia* strains. Thus, in the case of the cherry fruit fly, this phenomenon could be a concern that requires detailed study on *Wolbachia* diversity in host species.

In terms of DNA sequences for species delimitation of fruit flies, our results are in agreement with previous studies, including those of Armstrong and Ball (2005), Han and Ro (2005), and Barr and McPheron (2006), which showed that molecular data are useful for further molecular phylogenetic study of tephritids. Most molecular work on Tephritidae has considered certain genera, including *Anastrepha*, *Bactrocera*, *Ceratitis*, and *Rhagoletis* (Silva and Barr, 2006). Previous work has also shown a general congruence between molecular results and those based on morphology (Smith et al., 2002). This data revealed that the DNA barcode is a highly important tool for identifying some important pest species of fruit flies. This is promising for wider aspects such as quarantine issues, relating to concerns about the necessity for rapid species identification of non-native species of pests.

The comprehensive data on *Wolbachia* infections is a crucial step in obtaining complete knowledge of fruit fly-*Wolbachia* interactions. This is an essential issue in the development of *Wolbachia*-based biological control approaches. The application of this potential tool to manage insect pests has great agricultural importance. Recent data showed successful transfer of *Wolbachia* into novel hosts and opened a window for *Wolbachi*a-based strategies (Xi et al., 2005; McMeniman et al., 2009). Successful transinfection of the mosquito *Aedes aegypti* with a *Wolbachia* type from *Aedes albopictus* (Xi et al., 2005) shows promise for similar programs to control important arthropod borne-disease vectors and plant pests.

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