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Molecular Characterization of Iranian *Encarsia formosa* Gahan Populations with Natural Incidence of *Wolbachia* Infection

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ABSTRACT

Encarsia formosa Gahan is a common parasitoid of *Trialeurodes vaporariorum* Westwood and *Bemisia tabasi* Gennadius (Hemiptera: Aleyrodidae) which has extensively been used for biological control programs in greenhouses. Apart from the behavioral and molecular variations among members of *luteola* species-group, it is noticeable that *E. formosa* is the only telytokous species due to prevalence of a maternally inherited parthenogenesis-inducing (PI) bacteria called *Wolbachia* whereas males are common in other species of *luteola* group. In this study, the validity of COI and D2-28S rRNA genes to characterize Iranian *E. formosa* in correct species-group was addressed based on parsimonious analysis. The variation of *Wolbachia* endosymbiont of *E. formosa* populations corresponding to other hosts has also been carried out. Furthermore, the characterization of the *Wolbachia* supergroup, subgroup and strain were studied based on *wsp* gene and HVRs. In COI-based phylogeny of *Encarsia* the positions of Iranian populations were not determined in correct grouping near GenBank *E. formosa* and *E. luteola* in *luteola* species-group but D2-28S rRNA could differentiate all populations with high accuracy in *luteola* group. The phylogenetic relationship among strains of *Wolbachia* indicated that all of them were belonged to supergroup B, strain *w*For and subgroup For, based on *wsp* gene through Neighbor-Joining analysis. While *wsp* gene sequence alone was sufficient to characterize *Wolbachia* in our populations but studies on MLST comprising genes (*CoxA*, *gatB*, *fbpA*, *fcpA* and *ftsZ*) is undergoing.

Key words: Encarsia, COI, D2-28S rRNA, *wsp*, *Wolbachia.*

INTRODUCTION

Aphelinids of the genus *Encarsia* Förster, are the common parasitoid of the aleyrodid pests, of which specially *E. formosa* Gahan has a great impact in population of *Trialeurodes vaporariorum* Westwood and *Bemisia tabasi* Gennadius (Hemiptera:

Aleyrodidae) (Pedata *et al*., 2002; Giorgini and Baldanza, 2004). This species is recorded from the entire six zoogeographical regions of the world and frequently used for biological control programs in greenhouses (Van Lenteren *et al*., 1997; Begum *et al*., 2011). *Encarsia* is the largest genus within Aphelinidae, with 343 nominal species (Noyes, 1982; Heraty *et al*., 2008) but the systematic status of many species that already used in biological control of whiteflies is still unresolved. This situation is due to their small size, diversity and existence of morphologically indistinguishable species resulted in having the complexes of cryptic species (Heraty and Polaszek, 2000; Giorgini, 2001; Manzari *et al*., 2002). This problem has made systematic scientists to use the so-called species-group to study *Encarsia* species easier (Heraty and Polaszek, 2000). Although some researchers provided some species-group placement for different species of *Encarsia*, but the most impressive work was conducted by Abd-Rabou and Ghahari (2007) which all the valid species until that time were classified in 21 taxonomic groups. However, even now few of these groups can be recognized by discrete morphological characters and some species have been included in different groups by different authors (Hayat, 1989; Polaszek *et al*., 1992; Heraty and Polaszek, 2000; Abd-Rabou and Ghahari, 2007; Ghahari *et al*., 2011). Sometimes, there are species whose placement in these groups are questionable, because they may not share all characters in the group or the species description and/or illustrations do not include sufficient details of characters needed to place the species in the group (Evans and Polaszek, 1997). In spite of problematic systematic study of *Encarsia* species, the taxonomy and classification of *Encarsia* species is now undergoing rapid changes using both morphological and molecular techniques (Heraty *et al*., 2008). Closely related species are much more readily distinguished by the insights from the sequence of ITS2, COI or COII and 28S rRNA rather than the morphological differences (Stouthamer *et al*., 1999; Giorgini and Monti, 2003). Differences in the D2-28S rRNA were used to differentiate two closely related species, *E. formosa* and *E. luteola* Howard (Babcock and Heraty, 2000). These species belong to the *luteola* group as well as eight other *Encarsia* species (Babcock and Heraty, 2000) based largely on having a four rather than five segmented midtarsus, number of multi-porous plate sensilla on the antennae, color of the occipital region, the number of cells along the diagonal axis of the axilla and degree of surface sculptures on the mesosoma. These characters required laborious slide mounting techniques and are difficult to discern in slide mounted preparations and variable within each species collected from different regions or host plants. Polaszek *et al*. (1992) acknowledged they have faced certain individuals that cannot confidently identify as either *luteola* or *formosa* species but the D2 expansion region of 28S rRNA provides sufficient genetic variation to characterize and unambiguously distinguish these species (Babcock and Heraty, 2000). Furthermore, *E. estrellae* Manzari and Polaszek and *E. inaron* Walker from *inaron* species-group could also be easily distinguished by Manzari *et al*. (2002) through the expansion of D2 region of 28S rRNA.

Apart from behavioral and molecular variations among members of *luteola* species-group, it is noticeable that *E. formosa* is the only telytokous species among

luteola group due to the prevalence of a maternally inherited parthenogenesis-inducing (PI) bacteria called *Wolbachia* (Hertig, 1936), whereas males are common in other species of *luteola* group (Babcock and Heraty, 2000). *Wolbachia* has been classified into 13 supergroups and identified strains in different hosts (A to M, although the validity of supergroup G is disputed (Baldo and Werren, 2007)) but strains related to Hymenopterans are from A and B supergroups (Lo *et al*., 2002; Casiraghi *et al*., 2005). On the basis of *wsp*, 12 subgroups of *Wolbachia* were distinguished within the A and B supergroups (Zhou *et al*., 1998; Copeland *et al*., 2008). Additional subgroups have subsequently been recognized; Van Meer *et al*. (1999) added seventh and Ruang-Areerate *et al*. (2003) assigned another eighth subgroup. However, though its fast rate of mutation has made it useful for fine discrimination between subgroups. Recent discoveries of a high recombination propensity may compromise the value of the *wsp* gene as a tool for larger scale phylogenies (Baldo *et al*., 2006; Copeland *et al*., 2008).

In this study, we addressed multiple purposes; first to study the molecular identification of Iranian *E. formosa* populations based on D2-28S rRNA region and their status in *luteola* group. Second to study whether COI gene can be a suitable marker for *Encarsia* identification as a mitochondrial gene less used for aphelinid Hymenoptera. Third to detect the diversity of *Wolbachia* in Iranian *E. formosa* corresponding to other hosts and to characterize the *Wolbachia* supergroup, sub group and strain based on *wsp* gene and HVR regions.

MATERIALS AND METHODS

Collection of specimens

Eight populations of *E. formosa* were reared from parasitized pupae of *B. tabaci* and *T. vaporariorum* that collected from different host plants in Khorasan-Razavi Province (Mashhad, Iran) (59º 34’ 0’’ E-36º 16’ 0’’ N), during 2010-2011 (Table 1). Samples were kept until the emergence of adult wasps from pupae and were preserved in 96% ethanol at -20ºC until use. A series of the adult specimens were then slide mounted as described by Noyes (1982) and initially confirmed as *luteola* species-group through their 4-segmented midtarsus as a reliable morphological character.

DNA Extraction, amplification and sequencing

Total genomic DNA of each individual wasp was extracted while the whole wasp body was ground by micro pestle in liquid nitrogen. 30 µl of 5% Chelex®-100 and 2µl of Proteinase K (20 mg.ml) were added and then incubated for 4h at 60°C followed by 10 min at 95°C. The mixture was spun at 13000 g for 3 min. The supernatant was extracted and stored at -20ºC. PCR were carried out in a Biometra thermal cycler (Biometra, Tpersonal combi) in standard 25µl reactions containing 3µl DNA template, 3 µl PCR buffer (10X), 1µl MgCl2, 0.5µl dNTPs, 1µl of each forward and reverse primers (10 picomoles), 0.3 µl *Taq* polymerase 5U.µl and 15.2µl ddH_2O for both COI and 28S genes. Primers used for COI and 28S are presented in Table 2. The PCR temperature

profile for COI gene was as follow: one cycle as initial denaturation step at 94ºC for 60s, followed by 30 cycles at 94ºC denaturation for 60s, 53ºC annealing for 90s and 72ºC elongation for 90s and a final elongation at 72ºC for 8 min. For 28S gene, the reaction condition was one cycle initial denaturation step at 94ºC for 3min, followed by 30 cycles at 94ºC denaturation for 45s, 55ºC annealing for 30s and 72ºC elongation for 90s and one cycle at 72ºC final elongation for 30 min according to Campbell *et al*. (2000). All PCR products were gel-purified in a 1% agarose gel and visualized by 5µl DNA green viewer in 0.5gr agarose, 2.5ml TBE (10X) and 50ml dH_2O. PCR-amplified products were sequenced in 3730XLDNA analyzer by Macrogen Co. after purification (Seoul, Korea) (http://www.dna.macrogen.com).

Table 1. *Encarsia formosa* specimens collected from different host plants with accession numbers for mtCOI, 28S rRNA and wsp partial genes.

Specimen name	Host name	Host plant name	Accession number (28S)	Accession number (COI)	Accession number (*wsp*)
UTef1	*Trialeurodes vaporariorum*	*Nicotiana tabacum*	KF017879	KC870907	KC870915
UTef2	*T. vaporariorum*	*Agreatum houstonisum*	KF017880	KC870908	KC870916
UTef3	*T. vaporariorum*	*Solanum lycopersicum*	KF017881	KC870909	KF017873
UTef4	*T. vaporariorum*	*S. lycopersicum*	KF017882	KC870910	KF017874
UTef5	*Bemisia tabaci*	*Cestrum nocturnum*	KF017883	KC870911	KF017875
UTef6	*B. tabaci*	*Rosa* sp.	KF017884	KC870912	KF017876
UTef7	*B. tabaci*	*Morus alba*	KF017885	KC870913	KF017877
UTef8	*B. tabaci*	*C. nocturnum*	KF017886	KC870914	KF017878

Detection of *Wolbachia*

Presence of *Wolbachia* in different populations of *E. formosa* screened using *wsp* gene. The primers used were highly specific for *Wolbachia* (Table 2) which amplified an approximately 580 bp fragment of the *wsp* gene. The PCR reaction to amplify *wsp* gene was performed in a 25 ml volume containing 1µl DNA template, 2.5µl PCR buffer (10X), 0.75µl $MgCl_2$ 10mM, 0.5 µl of dNTPs 25mM, 0.5µl of each forward and reverse primers (10 picomoles), 0.3 µl of *Taq* Polymerase 5U µl and 18.95 ddH_2O. The PCR temperature profile were as follow: one cycle of initial denaturation step at 94ºC for 30s, followed by 36 cycles of denaturation at 94ºC for 30s, 50ºC annealing for 45s and 72ºC elongation for 60s and one cycle at 72ºC final elongation for 5 min. The amplified products were sequenced with adequate sample as described for COI and 28S rRNA. DNA was sequenced with the BigDye Terminator Kit (Applied Biosystem Inc.) with adequate samples.

Statistical Analysis

All chromatograms were checked then edited visually using BioEdit software (7.0.5.3) (Hall, 1999), prepared and finally submitted in NCBI using BankIt (http://www.ncbi.nlm.nih.gov.WebSub) under the accession numbers given in Table 1. The consensus sequences of COI and D2-28S rRNA and *wsp* genes were assembled

using DNA Baser software. Those sequences together with some valid and verified sequences which retrieved from GenBank (EMBL.NCBI) were aligned using CLUSTAL W (Thompson *et al*., 1994). Sequences were compared within GenBank database using nBLAST approach (Altschul *et al*., 1997) through National Center of Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov) with default parameters to identify the similarities between our sequences and those deposited in GenBank. BOLD system was also used to identify species based on COI gene (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). MEGA5 (M5b6.1) program (Tamura *et al*., 2011) was used to check protein translation.

Table 2. The list of primers and their sequences used in the current study.

Target gene	Primer name	Primer sequence 5′- 3′	Reference
COI	LCO1490 F	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*. (1994)
	HCO2198 R	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al*. (1994)
28S rRNA(D2)	D2-3665 F	AGAGAGAGTTCAAGAGTACGTG	Belshaw and Quicke (1997)
	D2-4068 R	TTGGTCCGTGTTTCAAGACGGG	Campbell, Steffen-Campbell and Werren (1993)
wsp	*wsp* 81F	TGGTCCAATAAGTGATGAAGAAAC	Braig *et al*. (1998), Zhou *et al*. (1998)
	wsp 691R	AAAAATTAAACGCTACTCCA	Braig *et al*. (1998) , Zhou *et al*. (1998)

The fragments with length of 438, 422 and 404 bp were selected for the phylogenetic analysis based on D2-28S rRNA, COI and *wsp* sequences respectively. Phylogenetic relationships were determined based on maximum parsimony (MP), Neighbor joining (NJ) and maximum likelihood (ML) for both 28S and COI genes using PAUP*4.0b10 (Swofford, 2001). The TVM+G sequence evolution were chosen via the Akaike Information Criterion using Modeltest v3.06 (Posada and Crandall, 1998). Pairwise distances estimated based on the Kimura two-parameter (K2P) model using MEGA5 (M5b6.1) program (Tamura *et al*., 2011). Gaps were treated as missing characters for the analyses and a single most parsimonious tree was constructed using the heuristic search method, tree-bisection-reconnection (TBR), and random branch-swapping algorithm. The reliability of trees was tested for 1000 bootstrap replicates (Felsenstein, 1985).

wsp Gene

The whole sequences (Table 4) were all used to construct a Neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) for *wsp* gene based on K2P model using MEGA5 (M5b6.1) program (Tamura *et al*., 2011). GenBank *wsp* queries were from A, B, C, D, F and G *Wolbachia* supergroups (Table 4) with the final 450bp nucleotide characters. The sequences were preliminarily aligned in CLUSTAL W (Thompson *et al*., 1994). A second alignment was conducted using the software MUSCLE 3.6 (Edgar, 2004). The resulted alignment was used for the phylogenetic analysis. Based on the *wsp* gene, protein sequences were obtained by conceptual translation, and sequences were reconstructed and aligned with the software BioEdit software (7.0.5.3) (Hall, 1999).

The nucleotide sequences were aligned manually by comparing the alignment of proteins. This alignment was used in the phylogenetic analysis. Each WSP amino acid sequence (corresponded to amino acid sequence of *w*Mel strain between 52 to 222) is partitioned into four consecutive sections whose breakpoints fall within conserved regions between the hyper variable regions: HVR1 (amino acid range 52-84), HVR2 (85-134), HVR3 (135-185), HVR4 (186-222) (Baldo *et al*., 2005). The HVRs of the WSP protein were employed as an additional, optional marker to assess strain diversity of *Wolbachia* based on Baldo *et al*. (2005, 2006). These four hyper variable regions (HVRs) of corresponded WSP sequence were used to further characterization of *E. formosa* populations based on Baldo *et al*. (2005, 2006) through WSP database (http://pubmlst.org/wolbachia/wsp).

RESULTS

Phylogenetic Parsimony Analysis of COI Gene and D2-28S rRNA Region

Unweighted parsimony analysis of the COI sequences alignments for 18 taxa of 422 total characters and bootstrap method with heuristic search indicated that 146 sites were conserved, 22 variable sites were parsimony uninformative and 254 variable sites were parsimony-informative characters. In verifying identification, our samples were successfully identified with 100% similarities to *E. formosa* through BOLD system for COI gene. Also, Nblast analysis showed 100% max ident and 100% query cover to *E.formosa* sequenes for 28S rRNA gene. Similarly, the resulted COI sequences had 100% max ident and 71% query cover to COI sequences of *E.formosa*. Little data on *Encarsia* species are available in GenBank for COI gene unlike 28S rRNA, maybe that is why nuclear DNA as a strong marker is much more prevalent in Hymenopteran studies (Gillespie *et al*., 2005). This lack is also can be detected in COI-based phylogeny of *Encarsia* as shown in Figure 1 because the positions of our sequences were not determined in correct grouping near *E. formosa* and *E. luteola* in *luteola* species group. The two latter cladograms (NJ and ML) were not shown because the results were in agreement with MP method. Phylogenetic analysis based on the COI sequence, using the maximum parsimony method, revealed four clades exclude the outgroup: the first one contained the outgroup; *Coccophagoides moeris* Walker (AY264342), the second clade included *E. formosa* (AY264337), *E. hispida* and *E. luteola* (*luteola* species-group), the third one comprised of *E. sophia* and *E. protransvena* (*strenua* species-group), the fourth, included *E. inaron* (*E. inaron* species-group) and the fifth one contained *E. formosa* populations of the current study. Surprisingly, in another comparison carried out based on COI sequences of *E. formosa* populations, we observed that COI could successfully separate different subfamilies of Aphelinidae. Also, Monti *et al*. (2005) acknowledged that COI could successfully place *E. formosa* in *luteola* group near *E. luteola* and differentiate it from other species-groups. Their result have verified in our *Encarsia* COI cladogram (Fig. 1).

D2 region of 28S rRNA gene not only successfully identified and characterized the Iranian *E. formosa* populations with 99% bootstrap from two other *E. formosa* from GenBank, but also verifies our populations are all from this species and could separate them from *E. luteola* with 88% bootstrap accuracy. Unweighted parsimony analysis of the alignments for 26 taxa of 438 total characters and bootstrap method with heuristic search indicated that 271 sites were conserved, 33 variable sites were parsimony uninformative and 134 variable sites were parsimony-informative characters. Phylogenetic analysis of 28S rRNA sequences could successfully separate the *luteola* group with 100% bootstrap accuracy from other *Encarsia* species-groups. All other *luteola* species group including, *E. luteola* Howard *E. meritoria* Gahan, *E. haitiensis* Dozier, *E*. *dispersa* Polaszek, *E*. *hispida* DeSantis and *E*. *quadeloupae* Viggiani were properly grouped too. Likewise, *E. inaron*, *E.* near *inaron* and *E. azimi* Hayat were also grouped in *inaron* species-group correctly (Fig. 2). The result was in accordance with those achieved by Babcock and Heraty (2000), Manzari *et al*. (2002) and Heraty *et al*. (2008).

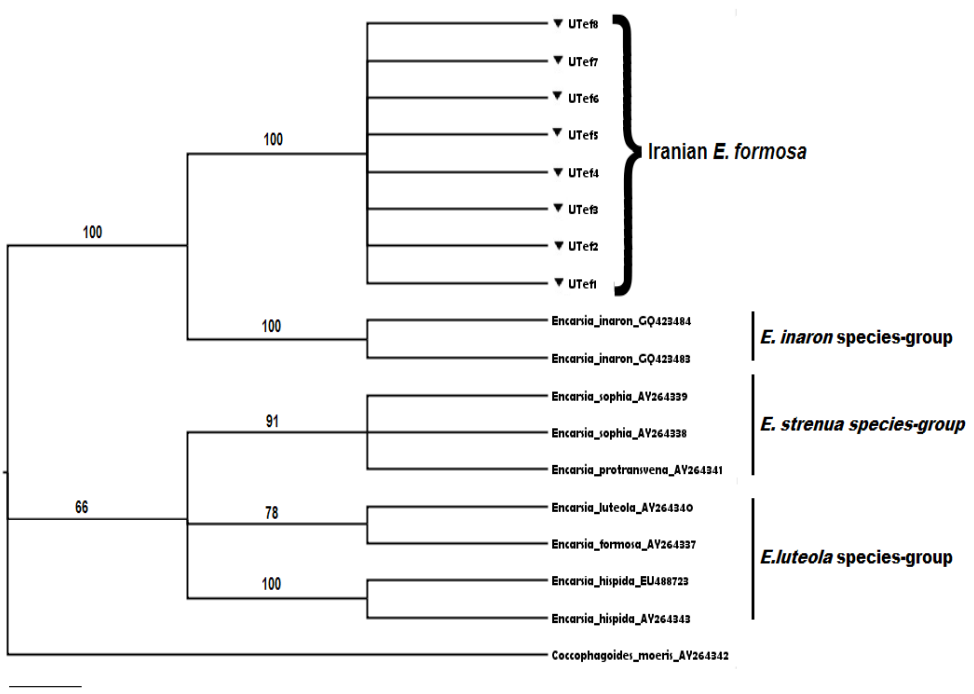

Fig. 1. The phylogenetic maximum parsimony cladogram of Iranian *E. formosa* used in the current study as well as those species retrieved from GenBank based on COI gene and their classification based on species-group using PAUP*. *Coccophagoides moeris* (AY264342) was used as the outgroup. Bootstrap probabilities (>50%) are indicated above mid-branches.

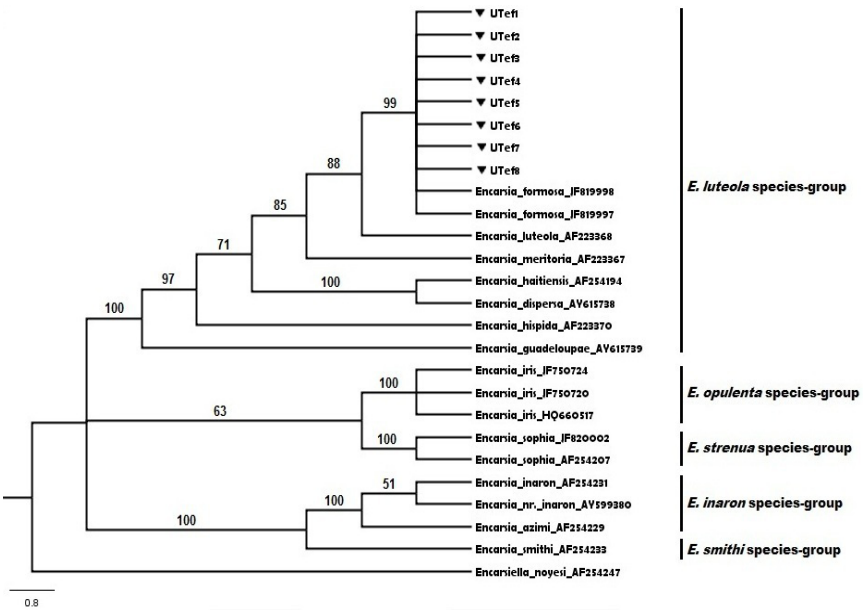

Fig. 2. Phylogeny of Iranian *E. formosa* populations and other species of the genus retrieved from GenBank based on DNA sequences of D2-28S gene as calculated in maximum parsimony analysis using PAUP*. *Encarsiella noyesi* (AF254247) was used as the outgroup. Bootstrap probabilities (>50%) are indicated above mid-branches.

For the high accuracy of D2-28S rRNA to differentiate species in the current study, we presented phylogenetics of *Encarsia* species based on these sequences to estimate the relationships and divergence times among taxa to infer the systematic status of species-groups. Based on K2P model with1000 bootstrap replicates, in five *Encarsia* species-groups comprised of *E. luteola*, *E. strenua* Silvestri, *E. inaron*, *E. opulenta* Silvestri, and *E. smithi* Silvestri, the mean sequence divergence for overall populations and interpopulations were 0.13% and 2.15%, respectively. Intraspecies-group variations between *E. luteola* populations were 0.046% (Between 0-0.11%) and interspecies-group differences between *E. luteola* and *E. strenua*, *E. inaron*, *E. opulenta* and *E. smithi* were 0.08%, 0.1%, 0.08% and 0.06%, respectively (Table 3).

Genetic Diversity of *Wolbachia* Endosymbiont of *Encarsia* Genus

All specimens of *E. formosa* screened for *Wolbachia* infection were positive to *wsp* gene amplification. Single infection was verified and there was no evidence for double or multiple infections. The phylogenetic relationship of *Wolbachia* from different supergroups (A, B, C, D, F and G) and sub groups was analyzed based on *wsp* gene sequence (Table 4, Fig. 3). Analyses indicated that all *Wolbachia* strains in *E. formosa* populations, were belonged to supergroup B, strain *w*For and sub group For, based on *wsp* gene (Fig. 3). Information about all *wsp* gene sequences is shown in Table 5 with details.

Table 3. Sequence diversity of D2-28S gene within and between species of Encarsia measured as heterozygosity per nucleotide site in percent. Average heterozygosity measures within species are given along the diagonal in bold type. Average heterozygosity measures between species, are given below and overall mean diversity/distance are given above the diagonal respectively. -Data unavailable.

Species-groups	*E. luteola*	*E. strenua*	*E. inaron*	*E.opulenta*	*E. smithi*
E.luteola	**0.05**	0.08	0.1	0.08	0.06
E.strenua	0.194	**0.01**	0.14	0.08	0.12
E.inaron	0.22	0.22	**0.04**	0.1	0.07
E.opulenta	0.17	0.13	0.16	**0**	0.07
E.smithi	0.192	0.18	0.11	0.13	-

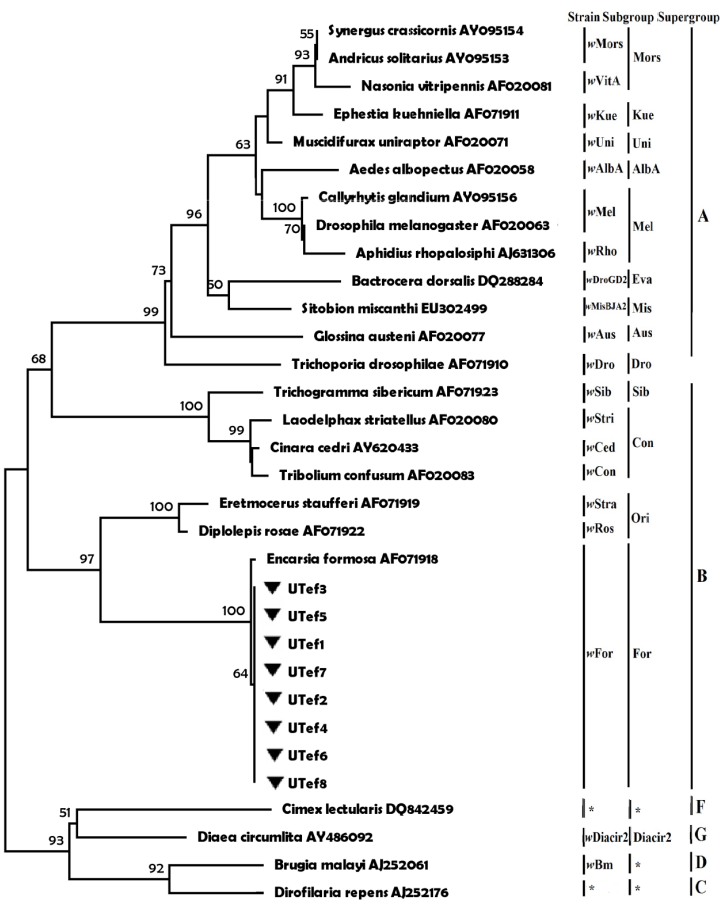

Fig. 3. Phylogenetic cladogram of *Wolbachia* indicating strains, subgroups and supergroups based on Neighbor-Joining algorithm for wsp sequences. Undetermined *Wolbachia* strains and subgroups are shown with * mark. Bootstrap probabilities (>50%) are indicated above nodes.

Table 4. *Wolbachia* sequences used in this study with accession numbers, Strains, sub and super groups based on wsp gene. Undetermined *Wolbachia* strains and subgroups are shown with * mark.

Host isolate species	Accession numbers	Host orders- Families	*Wolbachia* strains	(Sub)groups	Supergroups
UTef1	KF870915	Hymenoptera-Aphelinidae	*w*For	For	B
UTef2	KF870916	Hym.-Aphelinidae	*w*For	For	B
UTef3	KF017873	Hym.-Aphelinidae	*w*For	For	B
UTef4	KF017874	Hym.-Aphelinidae	*w*For	For	B
UTef5	KF017875	Hym.-Aphelinidae	*w*For	For	B
UTef6	KF017876	Hym.-Aphelinidae	*w*For	For	B
UTef7	KF017877	Hym.-Aphelinidae	*w*For	For	B
UTef8	KF017878	Hym.-Aphelinidae	*w*For	For	B
En. formosa	AF071918	Hym.-Aphelinidae	*w*For	For	B
Trichogramma sibericum	AF071923	Hym.-Trichogrammatidae	*w*Sib	Sib	B
Laodelphax striatellus	AF020080	Hemiptera-Delphacidae	*w*Stri	Con	B
Cinara cedri	AY620433	Hem.-Aphididae	*w*Ced	Con	B
Tribolium confusum	AF020083	Coleoptera-Tenebrionidae	*w*Con	Con	B
Eretmocerus staufferi	AF071919	Hym.-Aphelinidae	*w*Sta	Ori	B
Diplolepis rosae	AF071922	Hym.-Cynipidae	*w*Ros	Ori	B
Synergus crassicornis	AY095154	Hym.-Cynipidae	*w*Mors	Mors	A
Andricus solitarius	AY095153	Hym.-Cynipidae	*w*Mors	Mors	A
Nasonia vitripennis	AF020081	Hym.-Pteromalidae	*w*VitA	Mors	A
Ephestia kuehniella	AF071911	Lepidoptera-Pyralidae	*w*Kue	Kue	A
Muscidifurax uniraptor	AF020071	Hym.-Pteromalidae	*w*Uni	Uni	A
Aedes albopectus	AF020058	Diptera-Culicidae	*w*AlbA	AlbA	A
Callyrhytis glandium	AY095156	Hym.-Cynipidae	*w*Mel	Mel	A
Drosophila melanogaster	AF020063	Dip.-Drosophilidae	*w*Mel	Mel	A
Aphidius rhopalosiphi	AJ631306	Hym.-Braconidae	*w*Rho	Mel	A
Bacterocera doralis	DQ288284	Dip.-Tephritidae	*w*DroGD2	Eva	A
Sitobion miscanthi	EU302499	Hem.-Aphididae	*w*MisBJA2	Mis	A
Glossina austeni	AF020077	Dip.-Glossinidae	*w*Aus	Aus	A
Trichoporia drosophilae	AF071910	Hym.-Diapriidae	*w*Dro	Dro	A
Cimex lectularis	DQ842459	Hem.- Cimicidae	*	*	F
Diaea circumlita	AY486092	Araneae-Thomisidae	*w*Diacir2	Diacir2	G
Brugia malayi	AJ252061	Spirurida- Onchocercidae	*w*Bm	*	D
Dirofilaria repens	AJ252176	Spirurida- Onchocercidae	*	*	C

WSP Characterization

Using WSP database, *wsp* allele number 17 was identified for all eight *E. formosa* specimens *wsp* HVR profiles identified and are given in Table 5. besides the status of four other *E. formosa* populations retrieved from GenBank. All populations exhibited high levels of similarity in their WSP profiles as well as *wsp* allele. In comparison, two of four additional sequences gained from GenBank were very variable (AF071918 and AB037897) (Table 5).

Table 5. *Wolbachia* HVR profiles based on *wsp* gene for *E. formosa* populations

Accession number	*wsp* allele	*wsp* profile			
		HVR1	HVR2	HVR3	HVR4
UTef1 (KC870915)	**17**	**12**	**14**	**16**	**15**
UTef2 (KC870916)	**17**	**12**	**14**	**16**	**15**
UTef3 (KF017873)	**17**	**12**	**14**	**16**	**15**
UTef4 (KF017874)	**17**	**12**	**14**	**16**	**15**
UTef5 (KF017875)	**17**	**12**	**14**	**16**	**15**
UTef6 (KF017876)	**17**	**12**	**14**	**16**	**15**
UTef7 (KF017877)	**17**	**12**	**14**	**16**	**15**
UTef8 (KF017878)	**17**	**12**	**14**	**16**	**15**
FJ222455	17	12	14	16	15
DQ842471	17	12	14	16	15
AF071918	510	12	14	211	15
AB037897	199	108	40	59	144

HVR Numbers refer to peptide haplotypes of the four consecutive sections of WSP, each including a hypervariable region (HVR)

Searching all loci together have been displayed an exact match in bold if one exists. If an exact match was not found, the nearest allele or variant were chosen.

As *Wolbachia* is a widespread endosymbiont of arthropodes with diverse range of biological effects on its hosts (Zchori-Fein *et al*., 2001; Varaldi *et al*., 2003), there is an increasing trend towards tracking this endosymbiont and its significant effects on performance of insect host species (Stouthamer and Mak, 2002). In conclusion, Iranian *E. formosa* populations were not exceptional harboring *Wolbachia* causing thelytoky and inducing parthenogenesis as it is recorded for this species prior to this study by other researchers; Some of them are as follows: Stouthamer *et al*. (1990); Zchori-Fein *et al*. (1992); Van Meer *et al*. (1995); Hunter (1999), Stouthamer and Mak (2002). Furthermore, *Wolbachia* super group and sub group for Iranian *E. formosa* isolates was in agreement with previous studies reported by Van Meer *et al*. (1999) and Baldo *et al*. (2006). The comprehensive data of *Wolbachia* infections is a crucial step for obtaining complete knowledge of interactions between *E. formosa* and *Wolbachia*. This is an essential issue toward the development of *Wolbachia*-based biological control approaches and application of this potential tool to management insect pests with agricultural importance as well insect vectors. Therefore, biological

control practitioners should be aware of *Wolbachia* infection and how it effects on parasitoid populations.

This study was the first research on screening of *Wolbachia* in native *E. formosa* populations in Iran, with information on its four HVRs and *wsp* alleles to determine *Wolbachia* strains. This survey extended the *Wolbachia* database of *E. formosa* through a regional and native glance and showed the evolutionary relationship between some other *Wolbachia* arthropod hosts. Hence, there is now data known regarding infection status of this species in Iran. It must be noted that *Wolbachia* surface protein (WSP) is used as a useful marker for strain variability by its four hyper variable regions (HVRs) but since the four hyper variable regions of the protein are subject to extensive recombination and likely are involved in the host-symbiont interaction (Baldo *et al*., 2005), use of this gene as an additional optional strain marker is proposed. Moreover, future studies clarify the specific role of *wsp* in host-parasite interactions. So, information on amino acid motifs in HVRs may prove to be useful besides but not in place of MLST scheme (Baldo *et al*., 2006). Nevertheless, neither multiple peak nor recombination was detected in *E. formosa* populations. This verifies that using *wsp* gene alone were sufficient to characterize *Wolbachia* in our populations but studies on MLST comprising genes (*CoxA*, *gatB*, *fbpA*, *fcpA* and *ftsZ*) studies is undergoing.

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