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Molecular characterization of Iranian Trichogrammatids (Hymenoptera: Trichogrammatidae) and their *Wolbachia* endosymbiont

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ABSTRACT

During 2009–2010, a field survey of native *Trichogramma* species was carried out in six provinces of Iran, including Khorasan Razavi, Tehran, Mazandaran, Guilan, Golestan, and Qom. In this study, a molecular method for identifying *Trichogramma* and for determining the prevalence of *Wolbachia* in those species was used. Based on ITS2 (internal transcribed spacer 2) sequence, 14 populations were identified as the species *T. embryophagum*, *T. evanescens*, or *T. brassicae*. *Wolbachia* infection in these Trichogrammatids was detected using *wsp* gene sequencing. The highest infection rates in *Trichogramma* were found in Mazandaran and Golestan provinces. There was no evidence of infection in *Trichogramma* species in Guilan and Qom provinces. Of the three infected populations, two populations of *T. evanescens* were infected with only one *Wolbachia* strain from *sib* subgroup and one population was superinfected. Here, we report the first data on molecular characterization of Iranian Trichogrammatids and their *Wolbachia*-endosymbionts.

Introduction

The parasitoid wasp family Trichogrammatidae consists of approximately 80 genera and 620 species worldwide (Pinto and Stouthamer, 1994). In the past, identification of *Trichogramma* was based on morphological parameters or characters such as body color, chaetotaxy, and, more recently, male genitalia (Pinto et al., 1989; Pinto, 1999; Stouthamer et al., 1999b). Modern approaches to identification include molecular tools. Precise identification of *Trichogramma* species is an important step for the use of these parasitoids in biological control programs against several lepidopterous pests. In particular, the correct identification of thelytokous isolates of *Trichogramma* is critical in biocontrol programs which use these agents (Pinto et al., 1997). For morphological identification, males need to be reared but they are often produced only at exceedingly low frequencies. Incorrect identification may lead to release of unsuitable species, resulting in subsequent failure of biocontrol (Stouthamer et al., 2000). In some cases, the release of the wrong species in an area where another closely related species is present, can lead to a long time suppression of both native and introduced species in biological control programs (Stouthamer et al., 2000). Given the economic importance of *Trichogramma* species as biocontrol agents of pests, especially of Lepidoptera (Hassan, 1988), simple, quick and widely applicable identification methods need to be developed. Novel approaches that use the DNA sequence of the internal transcribed spacer 2 (ITS2) have provided a tool (Stouthamer et al., 1999b) that successfully distinguished closely related *Trichogramma* species. DNA sequences of the ITS2 of the ribosomal cistron are very useful for taxonomy (Agudelo-Silva, 1993; Hoy, 1994; Orrego and Pinto et al., 1997). In recent years, several molecular keys have been developed using ITS2 sequences of different *Trichogramma* species. Kumar et al. (2009) produced a molecular key for *Trichogramma* species in India using ITS2 PCR followed by restriction digest of the PCR products. Similarly, molecular keys have been developed using ITS2 sequences of *Trichogramma* species in Brazil (Ciociola et al., 2001) and in the Mediterranean area (Sumer et al., 2009).

Wolbachia is an intracellular bacteria found in many species of arthropods. It manipulates the reproduction of their hosts in several ways, including induction of complete parthenogenesis (thelytoky) in several parasitoid wasp species and nematodes. It is estimated that at least 20% of all insect species are infected with *Wolbachia* (Werren and Windsor, 2000).

The manipulation of the host's reproduction may play a role in host speciation and has potential applications in biological pest control (Stouthamer et al., 1999a). In *Trichogramma*, parthenogenesis-inducing *Wolbachia* occur in ca. 9% of species. This bacterium may have positive, negative, or neutral effects on the biological-specific traits of *Trichogramma* (Stouthamer and Luck, 1993; Stouthamer et al., 1994; Varve et al., 1999; Tagami et al., 2002). According to Stouthamer (1993), potential advantages for biological control of wasps infected with parthenogenesis-inducing *Wolbachia* are: (a) production of no males, which increases population growth rate of the natural enemy, (b) decrease in the cost of mass rearing because only females are

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produced, (c), colonization is faster because thelytokous females do not have to waste time in searching for mates at low wasp population densities, and (d) depression of host populations to low level. These advantages encouraged us to look for *Wolbachia* in *Trichogramma* populations. This information will be useful in biocontrol programs because selection of the best *Trichogramma* species/strain for inundative releases is a key first step in a biological control plan.

There is little information about *Wolbachia* and its prevalence in parasitic wasps like *Trichogramma* in Iran. Recently, Farrokhi et al. (2010) reported on the performance of PI-*Wolbachia* infected and uninfected *T. brassicae* collected from Northern Iran. All other studies on Iranian *Wolbachia* are limited to medically important insects, such as *Phlebotomus papatasi* and *Culex pipiens* (Parvizi et al., 2010). The purposes of our study were: identification of Iranian *Trichogramma* species from different climate zones of Iran using ITS2 sequence and determination of *Wolbachia* prevalence and phylogenetic status associated with the collected *Trichogramma* species.

Material and method

Sample collection

Trichogramma were collected from parasitized Lepidopteran eggs in Khorasan Razavi, Tehran, Mazandaran, Golestan, Guilan, and Qom provinces of Iran during 2009 and 2010 (Fig. 1). The samples were from different hosts. For each parasitized egg, a single (mated or virgin) female was allowed to establish an isofemale line. These isofemale lines were used in our study. Specimens from these isofemale lines were used for morphological identification and voucher specimens were placed in the insect collection of Ferdowsi University of Mashhad, Iran.

Identification of Trichogramma using sequencing of ITS2

DNA was extracted using a Bioneer kit (Bioneer Co. Daejeon, Korea). For each isofemale line, a sample of ten wasps kept at −20 °C for at least 12 h, then crushed using a micropestle in 200 µl lysis buffer and 20 µl proteinase K. The homogenate was incubated at 60 °C for 4 h, followed by 10 min at 95 °C. The supernatant was stored at −20 °C until use. The PCR reaction was performed using an Eppendorf thermocycler in 50 µl reaction volumes containing 2 µl DNA template, 5 µl *Taq* assay buffer, 1 µl dNTP's (each in 10 mM concentration), 1 µl forward and reverse primers (10 picomoles/µl), and 0.25 µl *Taq* polymerase (1 U). The primers used to amplify the ITS-2 region were 5′-TGTGAACTG CAGGACACATG-3′ (forward) and 5′-GTCTTGCCTGCTCTGAG-3′ (reverse). The ITS2 spacer was then amplified using PCR primers and the conditions described in Stouthamer et al. (1999b). PCR product was electrophoresed on 0.8% agarose gels along with a size ladder. Gels were stained using ethidium bromide.

Fig. 1. Geographical distribution of sampling locations in different provinces of Iran.

Wolbachia detection

We screened for the presence and identity of *Wolbachia* in different *Trichogramma* populations using the *wsp* gene. In addition, each of the isofemale lines sequenced for ITS2 was also screened for *Wolbachia*. The PCR reaction was performed in 25 μl reaction volumes using an Eppendorf thermocycler. Each reaction mixture contained 1 μl DNA template, 2.5 μl (10×) buffer, 0.75 μl MgCl2, 0.5 μl dNTPs, 0.5 μl forward and reverse primer (10 picomoles/μl), and 0.3 μl *Taq* polymerase (5 U). We used the *wsp*-forward (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and *wsp*-reverse primer (5′-AAAAATTAAA CGCTACTCCA-3′), as previously reported (Braig et al., 1998). The temperature profile consisted of an initial denaturation step at 94 °C for 30 s followed by 36 cycles (denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, and extension at 72 °C for 60 s), with a final extension at 72 °C for 5 min. For each DNA extraction, three control extractions were performed using a *Drosophila melanogaster Wolbachia*-positive line, a *Trichogramma Wolbachia*-negative line, and a non-DNA sample. After amplification, PCR products were purified and sequenced directly using standard Fluorescent cycle-sequencing by MilleGen Co. (France). Chromatograms were checked visually and sequences were aligned manually using BioEdit software (Hall, 1999). The resulting sense and antisense sequences were edited and used in alignment. Representative sequences for all known *Wolbachia* groups were retrieved from GenBank and included in phylogeny reconstruction. Sequences were aligned using the default settings of Clustal W (Thompson et al., 1994). Unweighted parsimony analysis of the alignments was conducted with PAUP*4.0b2 (Swofford, 1999). Gaps were treated as missing characters for the analyses and the reliability of trees was tested with a bootstrap test (Felsenstein, 1985). Parsimony bootstrap analysis included 1000 resamplings using the Branch and Bound algorithm. The most appropriate model of sequence evolution was determined using Hierarchical Likelihood Ratio Tests (hLRTs) in the program jModelTest 0.1.1 (Posada, 2008). Number of trees held at each step during stepwise addition was 1 and branch-swapping algorithm was tree-bisection-reconnection (TBR). Analysis was implemented into PAUP* for a 1000 replicate random addition heuristic search.

Result and discussion

The complete ITS2 gene sequence with portions of the flanking 5.8S and 28S rDNA genes was successfully sequenced for 14 isolates. The boundaries of the ITS2 were determined using the conserved sequence of the flanking regions by comparison with the sequence of *Trichogramma*. Three species of *Trichogramma* were identified based on the ITS2 sequences. ITS2 gene sequences were deposited in GenBank (Table 1).

T. brassicae was the dominant species in our collection and 9 isofemale lines were identified as this species. Four and two isofemale lines of *Trichogramma* were identified as *T. brassicae* from Tehran and Qom provinces, respectively. Moreover, *T. brassicae* was commonly found in the Northern part of Iran, South of the Caspian Sea (Table 1). This species is commonly used as biocontrol agent against some key insect pests, including the European corn borer, *Ostrinia nubilalis*, the carob moth, *Ectomyelois ceratoniae*, and the rice stem borer, *Chilo suppressalis* (Ebrahimi et al., 1998). Ebrahimi et al. (1998) reported that *T. brassicae* as the most widespread *Trichogramma* species in Iran.

The Iran 13 sample was deposited with accession number HM063427. It is typical for the *T. embryophagum* that is found in Iran. *T. embryophagum* was the least common species found in our study. This species is reared in an insectary located in the agricultural research center of Khorasan Razavi province (Mashhad, Torogh) in Northeastern Iran. Its sequence is identical to the Genebank accession number EU547670. Here, this species was named *T. embryophagum* and not *T. cacoeciae* because males are present in the culture. *T. cacoeciae* consists of only females and males are extremely rare (Pinto, 1999), while *T. embryophagum* may have males. The thelytokous status of a species carrying the *Wolbachia* symbiont can make identification impossible because males are lacking. In such cases, antibiotic and heating treatments can eliminate the symbiont which may result in the production of males. Based on cytogenetic mechanisms, thelytoky in *Trichogramma cacoeciae* is different from other species such as *T. embryophagum* (Stouthamer et al., 1990; Vavre et al., 2004). *T. cacoeciae* exhibits complete parthenogenesis and males are absent in natural populations. Pinto (1999) observed only five males out of approximately 15,000 individuals under laboratory conditions. Males cannot be induced by antibiotic or heat treatment, and neither *Wolbachia* nor other symbionts have ever been found (Vavre et al., 2004).

Table 1
Trichogramma samples used in this study, their geographic origin, GenBank accession numbers for their ITS2 sequences, and presence of *Wolbachia*.

Assign	Source	Province	Species	Accession no.	Isolate	*Wolbachia*
G3	Amol	Mazandaran	*T. brassicae*	HQ343301	Iran10	–
G7	Langerud	Guilan	*T. brassicae*	HQ332598	Iran15	–
G11	Amol	Mazandaran	*T. brassicae*	HQ335390	Iran7	+FUM5
M2	Qom	Qom	*T. brassicae*	HQ143679	Iran25	–
M3	Qom	Qom	*T. brassicae*	HQ143680	Iran23	–
T2	Tehran	Tehran	*T. brassicae*	HQ143675	Iran2	–
T3	Tehran	Tehran	*T. brassicae*	HQ143676	Iran3	–
T4	Tehran	Tehran	*T. brassicae*	HQ143678	Iran4	+FUM2
T5	Tehran	Tehran	*T. brassicae*	HQ143677	Iran5	–
T.e	Mashhad	Khorasan	*T. embryophagum*	HM063427	Iran13	+FUM7
G5	Behshahr	Mazandaran	*T. evanescens*	HQ335391	Iran8	+FUM1
G8	Gorgan	Golestan	*T. evanescens*	HQ332599	Iran6	+FUM3
G10	Gorgan	Golestan	*T. evanescens*	HM214958	Iran20	+FUM4 and +FUM6
M1	Qom	Qom	*T. evanescens*	HQ162663	Iran1	–

The *wsp* sequences can be accessed on GenBank under the numbers shown in Table 1. Amplification of *wsp* gene showed that seven *Trichogramma* populations (two populations of *T. brassicae*, four populations of *T. evanescens*, and one population of *T. embryophagum*) were infected with *Wolbachia*. The *Wolbachia* were characterized and named FUM1 to FUM7. Four *Wolbachia* strains, FUM1, FUM3, FUM5, and FUM7, belonged to the supergroup B and subgroup *Sib* (van Meer et al., 1999). The other strains, FUM2, FUM 4 and FUM6, belonged to supergroup A. Supergroup A and supergroup B of *Wolbachia* were found in different isofemale lines of *T. evanescens*. In addition, supergroup A *Wolbachia* were found in two different isofemale lines of *T. brassicae*. Both single infection and superinfection existed within *Trichogramma*. The strong similarity between the three FUM strains and *Wolbachia* in other hosts suggests that these strains have been transmitted horizontally between hosts.

We aligned our *wsp* sequences with 45 *Wolbachia wsp* sequences in the GenBank. Fig. 2 shows the tree after bootstrapping 1000 times.

The molecular methods applied in this study give quick results of *Trichogramma* identifications and can be used to survey laboratory colonies for contamination.

The phylogeny of *Wolbachia* has been studied using number of different genes, i.e. 16S, 23S, *fts*Z, SR2, and the *wsp* gene (Braig et al., 1998). Phylogenetic studies have led to the subdivision of the *Wolbachia* clade into 11 supergroups (Ros et al., 2008). *Wolbachia* from *Trichogramma* that induce parthenogenesis belong to supergroup B. Thelytokous populations of *Trichogramma* resulting from *Wolbachia* infection have different life-history characters than uninfected conspecifics. Consequently, finding *Wolbachia* infections and determining its effect on the host behavior could be useful.

Characterization of the *sib* subgroup of *Wolbachia* was reported by van Meer et al. (1999) and Pintureau et al. (2002). It was later confirmed by de Almeida (2004). Supergroup B *Wolbachia* infection is the cause of thelytoky in at least 17 out of 190 described species of *Trichogramma* (de Almeida, 2004).

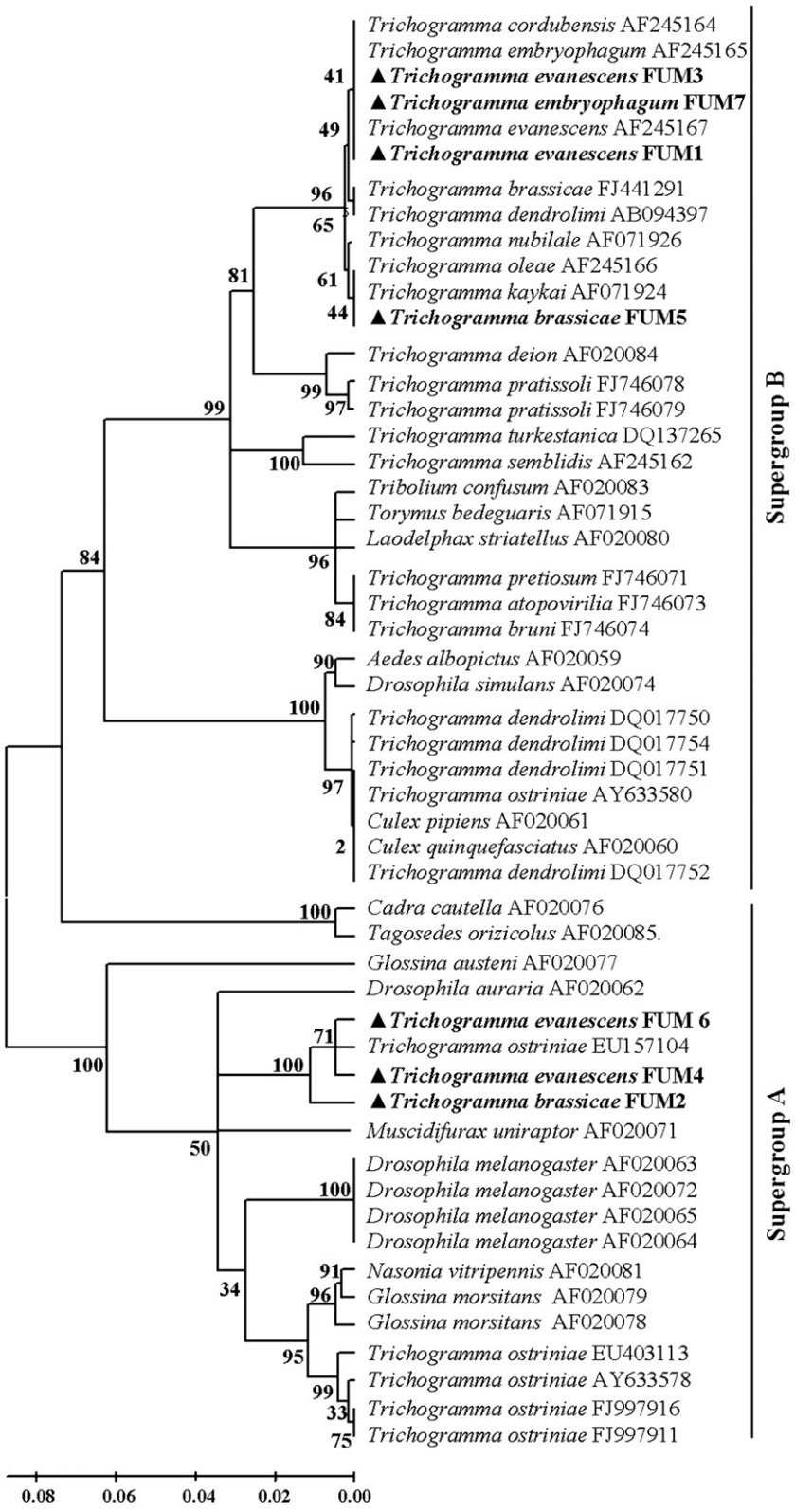

Fig. 2. Phylogenetic tree of *wsp* sequences of the *Wolbachia* strains from *Trichogramma* and closely related *Wolbachia* strains from other insects. Bootstrap values are given as percentage 1000 replicates.

In this work, we attempted to detect *Wolbachia* strains in native *Trichogramma* populations in Iran. The phylogenetic relationship among all published strains of *Wolbachia* related to *Trichogramma* and some other insect groups throughout the world is analyzed based on *wsp* gene sequence. This data about *Wolbachia* and the ITS2-based identification of related *Trichogramma* are new for Iran. More analysis is necessary due to concerns about relationships of *Wolbachia* strains based on *wsp* gene. The *wsp* sequence is a common marker for *Wolbachia* detection and provides many more informative characters with which to determine evolutionary relationships between strains (Braig et al., 1998; Zhou et al., 1998). Screening of *Wolbachia* using the *wsp* gene is a preliminary approach. However, single-locus phylogeny of *Wolbachia* may be

questionable because strains with similar *wsp* sequences often have different allelic profiles (Baldo et al., 2006). The *wsp* gene is divided into four hypervariable regions (HVRs) (Werren et al., 2008). They have high polymorphism (Baldo et al., 2006) and high levels of recombination have been observed in WSP and throughout *Wolbachia* genomes (Werren and Bartos, 2001; Baldo et al., 2006). These characters may not correctly show the true evolutionary and demographic histories of *Wolbachia* strains (Baldo et al., 2006). A multilocus sequence typing (MLST) is a new method which had recently been proposed for *Wolbachia* characterization. This approach may overcome the recombination concern and offer more data for comprehensive analyses (Baldo et al., 2006). Characterization of five housekeeping genes, *gat*B, *cox*A, *hcp*A, *fts*Z and *fbp*A, is the principle data for MLST system. Therefore, the next step toward understanding the phylogeny of *Wolbachia* strains should be the analysis across a broader range of hosts, using sensitive methods for strain typing such as MLST, to determine precise relationships and endosymbionts.

Understanding *Wolbachia* biodiversity could be of major importance in improving biological control using *Trichogramma*. For example, *T. brassicae*, which was released against the European corn borer, *O. nubilalis*, normally has a sexual mode of reproduction. A *Wolbachia* transfer inducing thelytokous reproduction in *T. brassicae* could increase the performance of parasitoids by producing only females (Pintureau et al., 2002).

Low prevalence of *Wolbachia* may have been missed given the number of samples analyzed. Therefore, the probability of finding *Wolbachia* infection depends on the number of samples analyzed.

Generally, information about *Wolbachia* infection rate may be questionable because: (1) not all populations of a species may be infected, and (2) infected and uninfected individuals usually coexist in a single population (Cook and Butcher, 1999). Data analyzed here are restricted to one part of the country. By increasing the number of samples when looking for *Wolbachia*, we can get a much better understanding of infection frequency in different populations.

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