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Fertility life table parameters, COI sequences and *Wolbachia* infection in populations of *Trichogramma brassicae* collected from *Chilo suppressalis*

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Abstract

Trichogramma wasps are frequently applied in the biological control of *Chilo suppressalis* (Walker), which causes economic damage to rice in Iran. A survey was carried out to collect local *Trichogramma brassicae* Bezdenko populations from *C. suppressalis* eggs in the northern Iran. Fertility life tables were constructed and the mitochondrial cytochrome oxidase subunit I (COI) sequences were determined to estimate the genetic distance between populations. Also, the prevalence of *Wolbachia* and its effect on host fitness was determined. Thirteen populations of *T. brassicae* were sampled in which two were infected by *Wolbachia*. Results revealed biological and molecular differences between populations collected from a single host species in a relatively small geographic region. A significant positive effect of *Wolbachia* infection was found in the host fitness such as net reproductive rate and intrinsic rate of increase. The genetic relationship among populations is discussed in the context of historical wasp releases in the rice fields of northern Iran.

Key words: striped stem borer, host fitness, population dynamics, Rickettsiaceae.

Introduction

The striped stem borer (SSB), *Chilo suppressalis* (Walker) (Lepidoptera Crambidae) is one of the most important pests causing economic damage to rice in Asia and Europe. SSB larvae bore into the rice stem and feed inside. In the infested rice fields, the "dead heart" and "white heads" appear in the vegetative and reproductive stages of rice (Bowling, 1975; Browning *et al.*, 1989; Way *et al.*, 2003). It was introduced to Iran in 1973 and has since been widely distributed in all rice fields in northern Iran (Khanjani, 2004). Chemical control of SSB is difficult because it is protected within the grass stem throughout much of its life cycle and control is only achieved after repeated foliar applications (Bess, 1967; Prakasarao *et al.*, 1970). Development of integrated pest management programs is therefore needed with application of various alternative control methods including biological control as a major component.

Several *Trichogramma* (Hymenoptera Trichogrammatidae) species have been recorded for SSB biological control world-wide (Chen *et al.*, 2010; Yuan *et al.*, 2012; Lou *et al.*, 2013). In Iran, the first use of *Trichogramma* wasps to control SSB dates back to 1974, when an unknown *Trichogramma* species introduced from Germany and they were reared and released without considering indigenous species (Attaran and Dadpour, 2011). In 1980s, several attempts were made to collect and identify the *Trichogramma* fauna from different regions of Iran (Moussavi, 1986; Radjabi, 1986; Shojai *et al.*, 1988; Momeni *et al.*, 1989).

After first report of *Trichogramma brassicae* Bezdenko from North of Iran in 1986 (Shojai, 1986), several field surveys showed that this species has a wide distribution in northern parts of Iran, especially in rice fields (Ebrahimi *et al.*, 1998; Poorjavad, 2011; Poorjavad *et al.*, 2011; Najafi Navaie and Bayat Assady, 1989). Although *Trichogramma japonicum* Ashmead has often been recommended as the most suitable *Trichogramma* species against lepidopteran rice pests (Chen *et al.*, 2010), *Trichogramma chilonis* Ishii, *Trichogramma dendrolimi* Matsumura and *Trichogramma ostriniae* Pang et Chen (Lou *et al.*, 2013; Yuan *et al.*, 2012) have also been widely used for the control of rice pests in China. The natural occurrence of these *Trichogramma* species, except *T. dendrolimi*, has not yet been reported from Iran. Despite of no documented evidence for the successful use of *T. brassicae* in the biological control of SSB in other countries, this species is the most commonly employed agent in the control of SSB in Iran (Attaran and Dadpour, 2011). In 2011, approximately 100,000 hectares of rice field were controlled using *T. brassicae* wasps (Attaran and Dadpour, 2011).

The genus *Trichogramma* has been successfully used to control pests in agriculture, forest areas and stored products. This genus consists of about 210 species (Kot, 1964; Li, 1994). *Trichogramma* wasps are thought to be more habitat- than host-specific (Salt, 1935; Flanders, 1937; Curl and Burbutis, 1978;). Habitat and plant factors can directly or indirectly affect host selection process in these wasps. Hence, preference should always be given to indigenous populations or species which can be collected from the same region (van Lenteren *et al.*, 2003). Furthermore, the success of *Trichogramma* wasps in release programs may depend on their mode of reproduction (Stouthamer *et al.*, 1999a). The most common mode of reproduction in *Trichogramma* wasps is arrhenotoky, but thelytokous reproduction also occurs. Thelytoky is often induced by endosymbiotic bacteria belonging to the family Rickettsiaceae and the genus *Wolbachia* (Louis *et al*., 1993; Stouthamer *et al*., 1993). Considering that the effect of *Wolbachia* on host fitness can be negative (Hoffmann *et al*., 1990; Stouthamer and Luck, 1993; Stouthamer *et al*., 1994; Wu *et al*., 2016), positive (Girin and Bouletreau, 1995; Stolk and Stouthamer, 1996) or neutral (Stouthamer *et al*., 1994; Hoffmann *et al*., 1996), the interaction between *Wolbachia* and *Trichogramma* populations needs to be determined to assure the success of biological control programs.

In the present study, a survey was carried out by sampling local *Trichogramma* populations from SSB eggs in rice fields in northern Iran. Fertility life tables were constructed for each population. Because significant biological differences were found between populations, their genetic relatedness was analysed using the mitochondrial cytochrome oxidase subunit I (COI) marker sequence. The *Wolbachia* infected and uninfected populations of *Trichogramma* were compared by fertility life table parameters and COI sequences.

Materials and methods

Trichogramma populations

Trichogramma parasitized SSB eggs on different host plants in northern Iran were collected from March 2008 to November 2009, as previously described (Poorjavad *et al*., 2012), by inspecting SSB eggs on foliage in regions with no history of *Trichogramma* release programs (table 1). For culture establishment, the wasps emerging from collected eggs were used to establish laboratory populations on the sterile eggs of *Ephestia kuehniella* Zeller (Lepidoptera Pyralidae) at 25 ± 1 °C, 60-70% RH and 16D:8L conditions. A strip of diluted honey (10%) was placed on the side of the vial as food. Populations were defined as the progeny of the emerged wasps from one SSB egg batch. Each parasitoid population was reared in the laboratory for at least six generations before the fertility life table was assessed.

Trichogramma identification and *Wolbachia* detection

Identification of *Trichogramma* species and detection of *Wolbachia* inside wasps were described earlier (Poorjavad *et al*., 2012). Briefly, the wasps were identified based on morphological characters (Pintureau, 2008) and their restriction digestion pattern of the internal transcribed spacer 2 (ITS2) PCR products (Stouthamer *et al*., 1999a). All of the dead individuals who started laboratory cultures were tested for *Wolbachia* infection by PCR amplification with *Wolbachia* surface protein (*wsp*) primers (Braig *et al*., 1998). DNA was extracted from single wasps using the Chelex method (Walsh *et al*., 1991). PCR assays were carried out in 50 µl reactions containing 2 mM MgCL2, 0.2 mMdNTP's (Fermentas), 0.2 lM of each primer 5 µl PCR-buffer (Invitrogen), 5 µl template and 1 U Taq DNA polymerase (Invitrogen). ITS2 and *wsp* primer pairs were selected based on Stouthamer *et al*. (1999b) and Braig *et al*. (1998), respectively. PCR cycling conditions for the ITS2 fragment were: 3 min at 94 °C, 35 cycles of 94 °C for 45 s, 53 °C for 45 s and 72 °C for 45 s; and a final elongation step of 72 °C for 3 min. PCR conditions for *wsp* gene fragment was: 3 min at 94 °C,35 cycles of 94 °C for 20 s, 52 °C for 30 s and 72 °C for 45 s; and a final extension step of 72 °C for 3 min. PCR products were sequenced by LGC Genomics (Berlin, Germany).

Life table experiments

Fertility life table parameters of the *T. brassicae* populations were determined at 25 °C, 70% RH and 16D:8L. The fertility life table for each *Trichogramma* population was constructed by following cohorts of 35 newly emerged female parasitoids as described by Poorjavad *et al*. (2011; 2014). Briefly, emerged females (<24 h old) were transferred individually to glass vials (1 × 10 cm) containing a streak of honey and small piece of cardboard with approximately 100 eggs of *E. kuehniella*. The cardboard pieces were replaced daily until death of the female parasitoid. Cardboards with parasitized eggs were held individually in glass vials and inspected daily until

Table 1. Populations of *T. brassicae* collected on SSB eggs and relative host plants.

Number*	Geographic origin (latitude, longitude)	Host	Accession number in NCBI
1	Hoseinabad (36.51957N, 52.26059E)	*Oryza sativa*	MG850865
2	Talebamoli (36.619386N, 52.265396E)	*O. sativa*	MG850875
3	Varazdeh (36.452218N, 52.2000165E)	*O. sativa*	MG850863
4	Chaboksar (36.95291N, 50.541573E)	*O. sativa*	MG850868
5	Sharam kala (36.536123N, 52.441177E)	*O. sativa*	MG850864
6	Tonekabon (36.815881N, 50.873566E)	*O. sativa*	MG850869
7	Rasht (37.259572N, 49.536324E)	*O. sativa*	MG850870
8	Kasgarmahal (36.533916N, 51.933746E)	*O. sativa*	MG850871
9	Lekode (36.521777N, 52.277069E)	*Xanthium* sp.	MG850872
10	Velisde (36.458983N, 52.271404E)	*O. sativa*	MG850866
11	Sote (36.64611N, 52.540741E)	weed	MG850867
12	Gorgan (36.82234N, 54.425583E)	*O. sativa*	MG850873
13	Keteshest (37.213925N, 49.850464E)	*O. sativa*	MG850874

*Numbers refer to populations in text, table and figures.

all F1 adults emerged. Adult longevity, the number of parasitized eggs per female, emergence percentage, the number of F1 female progeny produced, and development time were recorded for each population.

COI sequencing of *T. brassicae* populations and phylogenetic analysis

Genetic variability comparisons between populations of *T. brassicae* were conducted using the sequences of COI mitochondrial DNA. Total DNA was extracted from single ethanol preserved wasps using the Chelex method (Walsh *et al.*, 1991). PCR assays were carried out in 50 µl reactions containing 2 mM MgCl2, 0.2 mMdNTP's (Fermentas), 0.2 µM of primers LCOI490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAAT CA-3′) (Simon *et al.*, 1994), 5 µl 10× PCR-buffer (Invitrogen), 5 µl template DNA and 1 U Taq DNA polymerase (Invitrogen). PCR cycling conditions for the COI fragment were: 2 min at 94 °C, 37 cycles of 94 °C for 30 s, 54 °C for 45 s and 72 °C for 1 min and 30 s; and a final elongation step of 72 °C for 10 min. PCR-products were separated on a 1% agarose gel in 0.5× TAE-buffer, visualized under UV-light and purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek). PCR products were sequenced by LGC Genomics (Berlin, Germany).

The COI sequences obtained in the current study and those retrieved homologs of different *T. brassicae* populations from the National Centre for Biotechnology Information (NCBI) were aligned by CLUSTALW as implemented in BIOEDIT (Hall, 1999) and primer sequences were removed. The retrieved sequences were included two Iranian *T. brassicae* populations (JX131627 and JX442923) and three European populations (FM210196, FM210197 and FM210198) and their information was already presented in Nazeri *et al.* (2015) and Pasquer *et al.* (2009), respectively.

The neighbour joining NJ tree (Saitou and Nei, 1987) was obtained by MEGA version 4.0 (Tamura *et al.*, 2007) with pairwise deletion treatment of gaps and Maximum Composite Likelihood model, bootstrap support derived from 1,000 replicates, and values are shown in the tree.

Statistical analysis

Age-specific survival rates (*lx*) and average number of female offspring (*mx*) for each age interval (*x*) were used to construct age-specific fertility life tables. Replications for the main fertility life table parameters (r_m, R_0, T) were made using the bootstrap technique (these replications are called "pseudo values", Meyer *et al.*, 1986). Bootstrap pseudo values for net reproductive rate (R_0), intrinsic rate of increase (r_m) and generation time (T) as well as the values for preimaginal development time, preimaginal mortality, adult longevity and sex ratio (expressed as female percentage) for each population were subjected to analysis of variance (ANOVA) followed by least significant difference (LSD) test, when the effects were statistically significant (SPSS, 2006). All percentage values were subjected to arcsine transformation before analysis. The precise values of the main fertility life table parameters and their standard errors were calculated using the r_m program developed by Naveh *et al.* (2004).

Results

Trichogramma populations

Thirteen cultures of *Trichogramma* wasps were established from SSB eggs and all identified as *T. brassicae* morphologically as well as by their ITS2 restriction digestion pattern (table 1). Because two cultures were lost during experiments, eleven populations were subjected to life table analysis. Two laboratory *T. brassicae* populations, population four and five, were one-hundred percent *Wolbachia* infected and they were thelytokous. No infection was detected from other *T. brassicae* populations and they had arrhenotokous reproduction.

Life table parameters of *T. brassicae* populations

Preimaginal development time and mortality, adult longevity and sex ratio of the *Trichogramma* populations are shown in table 2. Preimaginal development times were found to vary significantly among populations, but their range was narrow (from 9 to 10 days). The mean number of parasitized eggs per female and

Table 2. Fertility life table parameters of *T. brassicae* populations.

Number of population	Preimaginal development time (days)	Preimaginal mortality (%)	Adult longevity (days)	Sex ratio (female %)	r_m (day^{-1})	R_0 (eggs)	T (days)	Parasitized eggs/female
1	9.00±0.52^{c}	25.73±2.44^{a}	7.36±0.88^{cd}	68.13±2.10^{ef}	0.3236±0.0337^{a}	29.64±0.50^{d}	10.42±0.02^{g}	42.86±8.11^{a}
2	9.50±0.51^{b}	25.07±3.00^{a}	7.63±0.96^{bc}	67.74±2.12^{ef}	0.2876±0.0949^{b}	26.58±0.94^{g}	11.28±0.02^{b}	37.86±6.57^{ab}
3	9.50±0.51^{b}	20.71±1.58^{cd}	7.43±0.97^{bcd}	72.19±0.11^{d}	0.3034±0.0377^{b}	27.61±0.37^{f}	10.97±0.02^{d}	40.76±5.47^{ab}
4	10.00±0.00^{a}	13.65±2.42^{e}	6.80±0.92^{d}	100^{a}	0.3179±0.0316^{a}	36.13±0.62^{b}	11.28±0.02^{b}	37.10±5.83^{bc}
5	10.00±0.00^{a}	18.95±1.84^{d}	7.06±0.52^{cd}	100^{a}	0.3272±0.0303^{a}	41.14±0.71^{a}	11.36±0.02^{a}	42.66±5.51^{a}
6	10.00±0.00^{a}	13.41±1.74^{e}	6.83±0.61^{d}	84.10±3.47^{c}	0.3022±0.0347^{b}	30.22±0.51^{c}	11.29±0.02^{b}	37.43±5.59^{b}
7	9.00±0.52^{c}	4.07±0.34^{g}	9.2±0.48^{a}	93.75±1.50^{b}	0.2979±0.0256^{b}	24.70±0.24^{h}	10.77±0.01^{e}	21.26±3.52^{d}
8	9.50±0.51^{b}	22.75±2.81^{b}	8.16±0.74^{b}	67.00±4.30^{ef}	0.3030±0.0337^{b}	27.64±0.50^{f}	10.96±0.02^{d}	41.36±6.66^{ab}
9	9.50±0.51^{b}	9.21±1.44^{f}	8.21±0.59^{b}	68.17±1.08^{e}	0.2988±0.0387^{b}	26.73±0.37^{g}	11.00±0.02^{c}	41.10±5.57^{ab}
10	9.50±0.51^{b}	22.57±2.87^{bc}	7.20±0.01^{cd}	73.22±1.81^{d}	0.2986±0.0326^{b}	24.62±0.39^{h}	10.74±0.02^{f}	32.30±7.15^{c}
11	9.50±0.51^{b}	25.18±2.51^{a}	8.20±0.61^{b}	66.36±1.77^{f}	0.3062±0.0334^{ab}	28.61±0.51^{e}	10.96±0.02^{d}	42.86±6.00^{a}
	$F_{10,329}$=19.25 P=0.000	$F_{10,329}$=316.82 P=0.000	$F_{10,329}$=18.36 P=0.000	$F_{10,329}$=1220.471 P=0.000	$F_{10,329}$=2.35 P=0.011	$F_{10,329}$=2502.34 P=0.000	$F_{10,329}$=5744.78 P=0.000	$F_{10,329}$=33.22 P=0.000

The means followed by different letters in each column are significantly different ($P < 0.05$, LSD test).

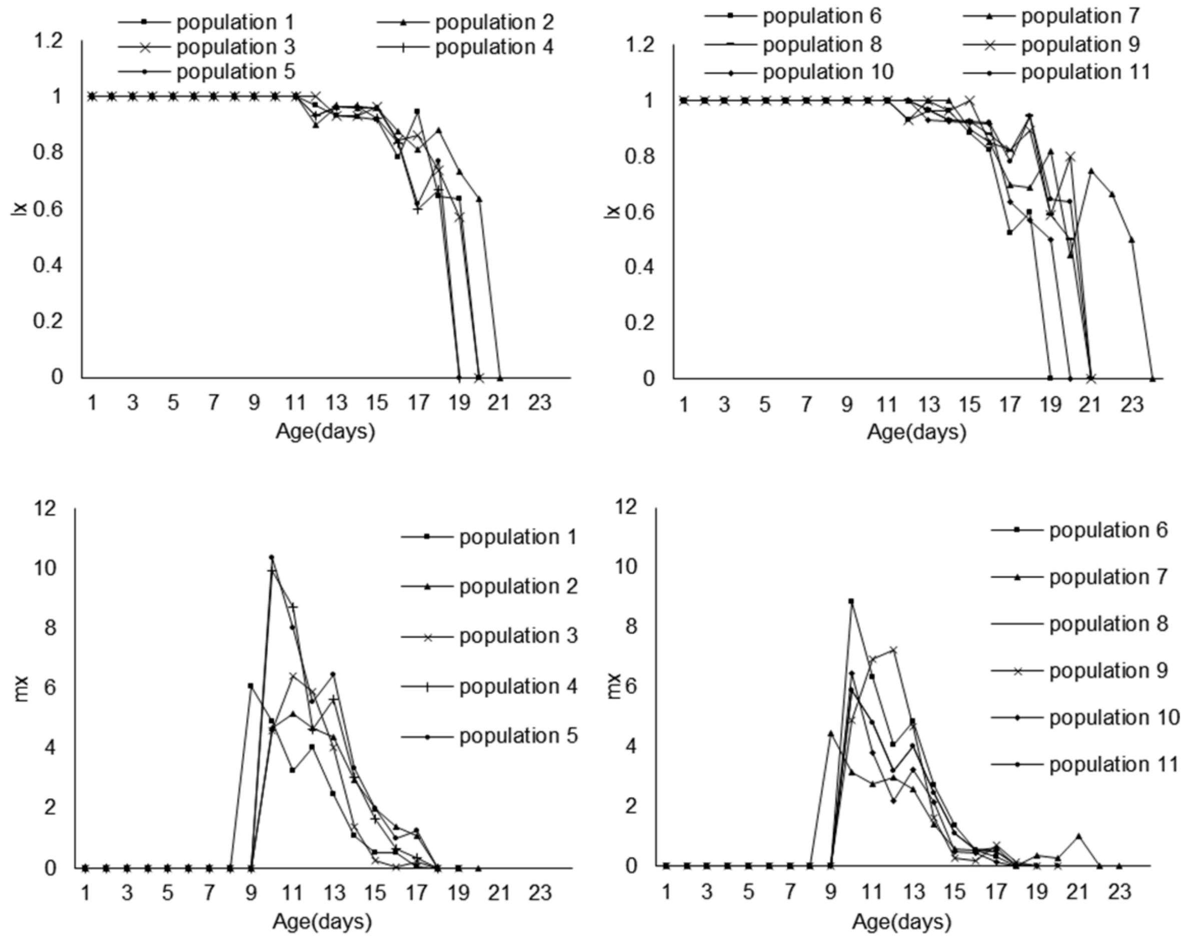

Figure 1. Age-specific survival rate (*lx*) and age-specific numbers of female progeny per day (*mx*) of *T. brassicae* populations.

preimaginal mortality percentage differed significantly among populations with notable lowest values in population seven, with 21.3 eggs/female and 4.1% mortality. There were significant differences in adult longevity and sex ratio among populations. Sex ratio was female-biased in all of the populations and it ranged from 67% to 93%. The highest sex ratio was observed in population seven (94%) as well as in two thelytokous populations (population four and five) that were *Wolbachia* infected.

The intrinsic rate of natural increase (r_m) and the net reproductive rates (R_0) showed significant differences among populations. The values of r_m ranged from 0.2876 to 0.3236 (day^{-1}). The highest and lowest R_0 were observed in population six (30.2 eggs), seven (24.7 eggs), and ten (24.6 eggs). The overall mean generation time (*T*) was significantly different among the populations with the lowest and highest values estimated at 10.4 days for population one and 11.4 days for population five.

The course of the age-specific survival rate (*lx*) and the age specific number of female progeny per day (m_x) are presented in figure 1. For all populations except population seven, the start point of female mortality was recorded on the second day after emergence after which it gradually increased until the fourth day, followed by a sharp increase. In population seven, female mortality started on the sixth day after emergence (figure 1). Maximum daily female offspring production (*mx*) of populations was generally observed in the first three days after female emergence and then gradually decreased (figure 1).

Effect of *Wolbachia* infection on life table parameters

Population four and five were one-hundred percent *Wolbachia* infected and their progeny were all females. Although populations three, six, seven and ten had sex ratios higher than 70%, they were not *Wolbachia*-infected as determined by PCR. Preimaginal development time, mortality percentage and adult longevity of infected populations were estimated in the same ranges as the uninfected populations. However, the three main fertility life table parameters, r_m, R_0 and *T* of *Wolbachia*-infected population five were significantly higher than that of uninfected populations. Population four placed in the second order and it also had significantly higher R_0 and *T* values than that of uninfected populations. No significant difference in r_m was found between *Wolbachia*-infected population four and uninfected populations (table 2). The course of the age-specific survival rate and the age specific number of female progeny per day were not affected by *Wolbachia* infection in these populations (figure 1).

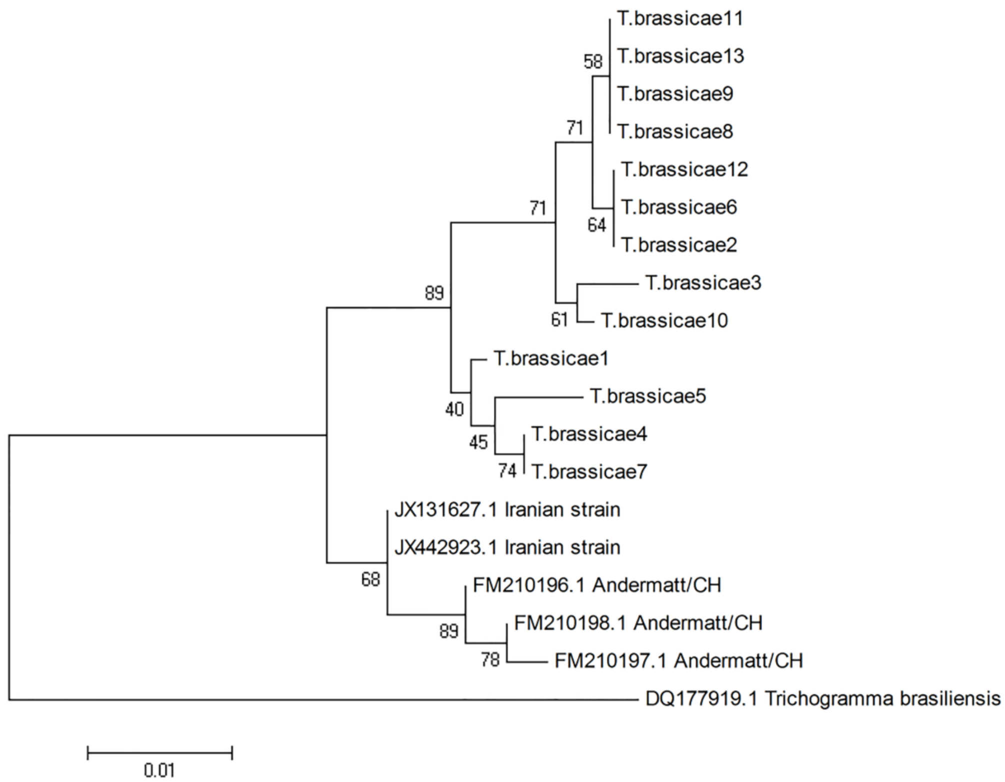

Figure 2. Neighbour joining (with pairwise deletion treatment of gaps and Maximum Composite Likelihood model, bootstrap derived from 1000 replicates and values are shown) tree based on the mitochondrial cytochrome oxidase subunit I (COI) sequences of *T. brassicae* populations. Andermatt/CH (Switzerland) refers to European populations of *T. brassicae*.

COI sequences of *T. brassicae* populations

COI sequences of 13 populations of *T. brassicae* used in the phylogenetic analysis classified into seven groups (figure 2). These groups were phylogenetically separated from other Iranian *T. brassicae* populations and European populations of which COI sequences were obtained from NCBI. These sequences showed 89.03% identity at the DNA level. The sequences of COI from our populations have been deposited in Gen Bank under the accession numbers MG850863-MG850875 (table 1).

Discussion

In present study, all *Trichogramma* populations collected from SSB eggs in rice fields in northern Iran were identified as *T. brassicae*. Our results demonstrate some biological and molecular differences between populations of *T. brassicae* collected from single host species in a relatively small geographic region. Interspecific differences between *Trichogramma* species have been frequently reported (Pak and Oatman, 1982; Schöller and Hassan, 2001; Kalyebi *et al*., 2005a; 2005b) but the intraspecific biological and molecular differences among populations of a single species has been less considered (Smith and Hubbes, 1986; Ram *et al*., 1995; Samara *et al*., 2008). Samara *et al*. (2008) reported that *Trichogramma aurosum* Sugonjaev et Sorokina populations from seven European countries differed in their longevity, fertility, net reproductive rate, cohort generation time and sex ratio, while their cumulative fertility and emergence rate were similar. Differences in the biological characters such as life table parameters among populations of *Trichogramma principium* Sugonyaev and Sorokina and *Trichogramma embryophagum* Hartig were also reported (Poorjavad *et al*., 2011). Our results showed that the degree of variation among *T. brassicae* populations in three life table parameters, r_m, R_0 and T, were not as great as interspecific differences reported for *Trichogramma* species. Although Poorjavad *et al*., (2011) reported low intraspecific variability in the biological parameters of *Trichogramma* wasps, Samara *et al*. (2008) reported a high degree of variation between *T. aurosum* populations, similar to the variation recorded among different species. This high intraspecific variation in Samara *et al*. (2008) could be related to the collection of samples from a wide geographic region (seven European countries).

The age-specific fecundity and survival rate patterns of *T. brassicae* did not differ among populations and they were not affected by *Wolbachia* infection. Obtained patterns in our study resemble those found in other studied *Trichogramma* species (Boivin, 2001; Samara *et al*., 2008). Increased female fecundity during the first three days of the reproductive life followed by its decreased until female death (figure 1) is typical of pro-ovigenic species (Pak and Oatman, 1982; Pak, 1988; Li *et al.*, 1993; Kidd and Jervis, 1996). Pro-ovigenic parasitoids complete oogenesis either before or very soon after adult emergence by using the larval food source and adults feed only for their maintenance (Jervis *et al*., 1993; Jervis and Copland, 1996; Heimpel and Rosenheim, 1998).

All field collected populations had a female-biased progeny when reared in the laboratory. Two *Wolbachia*-infected populations reproduced by thelytokous parthenogenesis. Thelytoky in Iranian *T. brassicae* associated with *Wolbachia* infection has been previously reported (Farrokhi *et al*., 2010; Poorjavad *et al*., 2012). Based on our results, life table parameters of this species were affected positively by *Wolbachia* infection, especially in population five. Enhancement of survival rate and intrinsic rate of increase by *Wolbachia* infection have been reported in *Trichogramma kaykai* Pinto et Stouthamer (Tagami *et al*., 2001; Miura and Tagami, 2004). A neutral effect of *Wolbachia* infection on the functional response of *T. brassicae* to host density was reported by (Farrokhi *et al*., 2010). Wu *et al.* (2016) showed that *Wolbachia* infected *T. ostriniae* population had lower fecundity than a naturally uninfected populations. In order to study the effects of *Wolbachia* infection on host fitness, antibiotic- treated wasps have typically been used, while in our experiment, naturally uninfected populations were compared with *Wolbachia*-infected wasps. Thus, observed differences in the biological parameters may be partly due to differences between populations, not only because of *Wolbachia* infection. On the other hand, in the experiments with antibiotic treatments, toxic effects of the antibiotic on the female wasps can never be completely avoided (Huigens and Stouthamer, 2003).

Analysis of genetic variations among populations revealed that populations in this study differed from European populations and two other Iranian populations of which COI sequences are available. The genetic proximity of two previously sequenced Iranian samples with European populations (figure 2) may be due to the introduction of *Trichogramma* wasps from Germany against SSB in 1974 (Attaran and Dadpour, 2011). Considering that *T. brassicae* is an indigenous species for parts of Europe (Pintureau, 1990) and has been produced and released commercially against European corn borer in Germany since 1980 (Hassan and Zhang, 2001), probably the *Trichogramma* population introduced to Iran belonged to *T. brassicae*. Also, this species has been successfully used for controlling European corn borer in several western European countries since 1975 (Bigler, 1986). The more phylogenetic distant position of our *T. brassicae* populations compare to other Iranian samples from European populations (figure 2) could be related to the origin of our samples that were collected from regions with no history of *Trichogramma* release programs. No correlation was found between the constructed phylogenetic tree based on the COI sequences and calculated fertility life table parameters.

In conclusion, the observed variation between fertility life table parameters of our *T. brassicae* populations could be partly related to the sampling location and *Wolbachia* infection status. This relationship can be considered as a critical point in selecting an appropriate *Trichogramma* population for achieving a successful biological control program. Because population growth parameters of these wasps were evaluated under laboratory conditions on *E. kuehniella* eggs, further research is needed to determine other influencing factors such as host (when *E. kuehniella* is substituted with *C. suppressalis*), plant structure, dispersal and foraging ability of the wasps especially under field conditions.

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