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Molecular study of ash psyllids, *Psyllopsis* (Hemiptera: Liviidae), and their *Wolbachia* endosymbiont

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Abstract
Three ash psyllid species, *Psyllopsis repens* Loginova 1963, *Psyllopsis securicola* Loginova 1963 and *Psyllopsis machinosus* Loginova 1963, which have been distributed in the Central Asia and Europe, are important pests on genus *Fraxinus* spp. in the urban area of Iran. They have similar adult and larval morphology as well as same host and biology. On the other hand morphologically identification of adults and nymph, especially for females and damaged adults by removal from sticky traps is difficult. Therefore, a fast and accurate identification method is required. Here we analyzed barcode sequence variation based on two mitochondrial gene regions, cytochrome c oxidase subunit I (*mtCOI*) and cytochrome b (*cytb*) as DNA barcode, and one endosymbiont gene (wsp *Wolbachia* gene) among the species in southern Iran. The results of pairwise genetic distance values showed that *cytb* and *mtCOI* genes had the highest inter-specific variability. Based on the results, two species *P. machinosus* and *P. repens* which are present on one host plant together probably have been infected with similar *Wolbachia* strain.

Key words: species identification, cytochrome c oxidase subunit I (*mtCOI*), cytochrome b (*cytb*)

مطالعه مولکولی پسیل‌های زبان‌گنجشک، ***Psyllopsis*** **(Hemiptera: Liviidae)** و باکتری ***Wolbachia*** همزیست داخلی آن‌ها

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چکیده
سه گونه پسیل زبان‌گنجشک، *Psyllopsis repens* Loginova 1963، *Psyllopsis securicola* Loginova 1963 و *Psyllopsis machinosus* Loginova 1963 که در آسیای مرکزی و اروپا گسترش دارند، از آفات مهم زبان‌گنجشک در مناطق شهری ایران محسوب می‌شوند. حشرات بالغ و پوره‌ها، و همچنین میزبان و زیست‌شناسی آن‌ها بسیار شبیه به هم است. به عبارتی دیگر، شناسایی ریخت‌شناختی آن‌ها، به ویژه ماده‌ها و حشرات بالغ آسیب‌دیده که از روی تله‌های چسبنده جمع‌آوری می‌شوند، دشوار است. بنابراین، یک روش شناسایی سریع و دقیق مورد نیاز است. در این مقاله توالی دو ژن سیتوکروم c اکسیداز زیر واحد یک (*mtCOI*) و سیتوکروم b (*cytb*) به عنوان نواحی بارکد و یک ژن *wsp* مربوط به ژنوم

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باکتری *Wolbachia* همزیست داخلی آن‌ها (ولباکیا) در سه گونه مورد مطالعه بررسی شد. نتایج مقایسه‌های ژنتیکی نشان داد که ژن‌های *cytb* و *mtCOI*، به ترتیب، بیشترین تنوع بین‌گونه‌ای را نشان می‌دهند. بر اساس نتایج، دو گونهٔ *P. machinosus* و *P. repens* که روی یک میزبان با هم زندگی می‌کنند، احتمالاً به‌وسیله یک استرین باکتری ولباکیا آلوده شده‌اند.

واژه‌های کلیدی: شناسایی گونه، سیتوکروم c اکسیداز زیر واحد یک (*mtCOI*)، سیتوکروم b (*cytb*)

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Introduction

The psyllids (Hem.: Psylloidea) with about 3850 described species (Li, 2011) are a sap sucking insects group which are considered as important pests and vectors of plant disease agents (Hodkinson, 1974, Burckhardt & Ouvrard, 2012). They are mainly associated with dicotyledonous plants (Hodkinson, 1974, Ossiannilsson, 1992), and their life cycles are often related to the host plants (Hodkinson, 2009).

Genus *Psyllopsis* Löw, 1879, which is currently classified in the family Liviidae (Burckhardt & Ouvrard, 2012), is a small West Palaearctic genus associated with various ash species (*Fraxinus* spp.) (Malenovský & Jerinić-Prodanović, 2011). It includes 11 described species in the world (Ouvrard, 2017) which five out of them including *P. narzykulovi* Bajeva, 1964; *P. repens* Loginova, 1963; *P. securicola* Loginova, 1963; *P. machinosus* Loginova, 1963 and *P. fraxini* (Linnaeus, 1758) are distributed in Iran. The species *P. repens*, *P. securicola* and *P. fraxini* have been reported from Kerman Province (Burckhardt & Lauterer, 1993); however, the identification of *P. fraxini* (based on collected samples in 1902) needs to be confirmed (Burckhardt & Lauterer, 1993). Moreover, *P. machinosus* was reported from Kerman Province in 2016 (Lashkari *et al*., 2016). Except for one species, *P. fraxinicola* (Forst.), another Palaearctic species of the genus *Psyllopsis* cause roll leaf galls on ash trees (Hodkinson, 1984). Although, they may damage the foliage of ash trees at high population densities (Hodkinson, 1984), *P. repens* was reported as a serious pest of ash trees from Iran (Rajabi Mazhar *et al*., 2004) and Armenia (Loginova, 1963). Furthermore, other species: *P. fraxini* and *P. fraxinicola* sometimes consider as occasional pests of ash trees in Europe (Burckhardt, 1994). Species in genus *Psyllopsis* have similar adult and larval morphology (Malenovský & Jerinić-Prodanović, 2011). The adults are usually characterized by male paramere and female proctiger (Conci & Tamanini, 1990; Burckhardt & Lauterer, 1993; Malenovský & Jerinić-Prodanović, 2011). In addition, they have the same host (genus *Fraxinus* spp.) and similar biology, as they have two generations per year and overwinter as egg stage (Conci & Tamanini, 1990; Hodkinson, 2009). Therefore, quick and accurate identification of species is very important and necessary. Identification of insect species based on DNA has been used to support or even to replace traditional morphological discrimination (Jenkins *et al*., 2012). DNA barcoding, which builds on the utilization of a standardized DNA region, is used as a label for rapid and accurate species identification (Hebert *et al*., 2003). The 5´ end of mitochondrial *cytochrome c oxidase 1* gene (*mtCOI*) with 658-bp long was selected as DNA barcode (Hebert *et al*., 2003). For superfamily Psylloidea, a 472-bp fragment of this region (the 5'end of *mtCOI*) has been proposed as the barcode (Percy *et al*., 2016).

It is demonstrated that several *Psyllopsis* spp. can be present on one host plant together (Loginova, 1963). In this study, we found that two species *P. machinosus* and *P. repens* were present on one host plant together in all collection sites, but another species, *P. securicola*, was alone on its host plants.

Although the spread of endosymbionts by horizontal transmission has not received well attention, other potential ways of exchange include predators, prey, and parasites and their hosts also have mentioned in the literature (Werren, 1997). Ahmed *et al*. (2016) suggest that specific shared food sources and shared natural enemies are possible routes of horizontal transmission of *Wolbachia* among butterflies and moths (Breinholt *et al*., 2016). *Wolbachia* is a well-studied endosymbiont bacteria in insects that can be transmitted maternally from infected insects to their progeny and cause a number of reproductive manipulations in its hosts (Werren, 1997; Stouthamer *et al*., 1999; Jeyaprakash & Hoy, 2000). The *wsp* gene associated with coding surface proteins of *Wolbachia* has considered to characterize *Wolbachia* strains (Van Meer *et al*., 1999) and used in assessing host-population genetics in Psylloidea (Liu *et al*., 2006; Lashkari *et al*., 2015).

Despite the importance of these species as pests, the morphological characters are lacking or are not sufficient for identification of adults, nymphs, and eggs at the species level as well as for specimens damaged by the trapping mechanism (e.g. sticky traps). Therefore, an accurate and rapid species identification is necessary. In this study; 1. Two mitochondrial genes were used for the three species of ash psyllids, *P. repens*, *P. securicola* and *P. machinosus*, to provide an estimation of mitochondrial genetic distances within different datasets, and 2. One endosymbiont gene (wsp gene of *Wolbachia*) was used to survey the relationship between two species *P. machinosus* and *P. repens* which have observed on the same host plant together.

Material and methods

Numerous psyllids were collected at three sites within Kerman Province during 2016 (Table 1, Fig.1). Collected adults were preserved in 95% ethanol. GPS coordinates were recorded (Table 1) and collection sites were mapped using http://www.simplemappr.net (Fig.1). Five specimens of each species were sent to Naturhistorisches Museum (Basel, Switzerland) and confirmed by Dr. Daniel Burckhardt and then the specimen returned for doing molecular studies.

Table 1. Collection sites, hosts, their geographic coordination of examined specimens.

Species	Locality	Host	N	E	Alt(m)
P. machinosus	Bardsir, Bahramjerd	*Fraxinus* spp.	29°52′48.32″	56°57′39.88 ″	2088
P. machinosus	Baft, Khabr	*Fraxinus* spp.	28°49′11.48″	56°20′24.83″	2098
P. repens	Bardsir, Bahramjerd	*Fraxinus* spp.	29°52′48.32″	56°57′39.88 ″	2088
P. repens	Baft, Khabr	*Fraxinus* spp.	28°49′11.48″	56°20′24.83″	2098
P. securicola	Bardsir, Bahramjerd	*Fraxinus* spp.	29°52′11.59″	56°57′17.74 ″	2104
P. securicola	Kerman	*Fraxinus* spp.	30°17′48.91″	57°02′33.92″	1757

Fig. 1. Collection sites for each species in Kerman province, Iran

DNA extraction, PCR amplification and sequencing

Two mitochondrial gene regions, cytochrome c oxidase subunit I (*mtCOI*) and cytochrome b (*cytb*) as DNA barcodes (Percy *et al*. 2016), and one endosymbiont gene (*wsp Wolbachia* gene) were amplified. DNA was extracted using DNA Extraction Kit DNP™ according to manufacturer's instructions (SinaClon, Iran) with a single individual in each extraction. Five and two specimens of each species were used for COI and *cytb* genes, respectively. PCR amplification of *mtCOI*, *cytb* and *wsp* genes was performed in a 50 µl reaction mixture containing 17 µl of double distilled water (ddH2O), 25 µl of master mix (Ampliqon, Denmark), 4 µl of DNA solution, 2 µl of forward and reverse primers (10 pmol/µl). A control PCR tube containing all components without genomic DNA was also prepared and run for all genes and species.

MtCOI and *cytb* regions were amplified with specific primers:

mtCOI primers, C1-J-1718 (5'-GGAGGATTTGGAAATTGATTAGTTCC-3') and

C1-N-2191(5'-CCCGGTAAAATTAAAATATAAACTTC-3');

cytb primers, 5'-TGAGGNCAAATATCHTTYTGA-3' and

5'-GCAAATARRAARTATCATTCDG-3' (Simon *et al*., 1994, Percy *et al*., 2016).

The PCR conditions used were: initial denaturation at 94°C for 3 min, then 40 cycles of 92°C for 30 s, 50°C (*mtCOI* gene) or 56°C (for *cytb* gene) for 40 s, and 72°C for 1 min followed by a final extension of 72°C for 10 min (Percy *et al*. 2016).

The *wsp* region was amplified with primer pair:

wsp–81F (5-TGGTCCAATAAGTGATGAAGAAAC-3') and

wsp–691R (5'-AAAAATTAAACGCTACTCCA-3') (Braig *et al*., 1998) under the following PCR conditions: initial denaturation at 94°C for 5min, then 35 cycles of 94°C for 30s, 55°C for 1min, 72°C for 5 min with a final extension of 72°C for 10min (Liu *et al*., 2006).

Amplified fragments (Five specimens of each species) were electrophoresed on 1.2% agarose gels at 80V for 1h in TAE buffer (pH= 8.0) and stained with ethidium bromide. The PCR products were purified and sequenced by Macrogen Inc. (Seoul, Korea). Sequences of *mtCOI* (accession numbers: MG008868- MG008882), *ctyb* (accession numbers: MG008883- MG008888) and *wsp* (accession numbers: MF538793- MF538798) genes were deposited in the GenBank database.

Data analysis

Raw sequences (Five sequences of each species) of *mtCOI*, *cytb* and *wsp* genes of the three species were edited manually to remove primers. Then, all nucleotide sequences aligned using ClustalW in software MEGA v.7.0.21 (Kumar *et al*., 2016). All sequences were compared with those on GenBank using BLASTn (http://www.ncbi.nlm.nih.gov/blast/). Neighbor Joining (NJ) clustering analysis was done in MEGA v.7.0.21 with Bootstrap values (1000 replicates). NJ trees employed the Kimura 2-parameter (K2P) distance model (Kimura 1980) with the pairwise deletion option in software MEGA v.7.0.21. Models of sequence evolution were evaluated for each dataset with JModeltest v.2.1.4 (Darriba *et al*., 2012). Bayesian information criterion (BIC) (Schwarz, 1978) were used to select models. For *cytb* gene dataset, HKY + I model with unequal base frequencies, For *mtCOI* gene dataset, TRN + I with unequal base frequencies; and for wsp gene dataset, TPM3uf with unequal base frequencies were selected. Data were analyzed using Bayesian inference based on a Markov chain Monte Carlo (MCMC) approach in the software package MrBayes v3.2.2 (Ronquist *et al.*, 2012). Four chains starting with a random tree were run for 10 million generations with sampling every 1000 generations and the first 25% of each analysis were discarded as burn-in. The run convergence was monitored by finding the

plateau in the likelihood scores (standard deviation of split frequencies <0.0015) and the potential scale reduction factor (PSRF) approaching one.

The sequences of the *mtCOI* gene obtained in this study were also compared with sequences of the *Psyllopsis fraxini* Linné, 1758 (Hem.: Liviidae) (accession number: KU517187.1) deposited in the gene bank, NCBI. Moreover, sequences of *Trioza erytreae* (Del Guercio) (Hem.: Triozidae) (accession number: KU517195.1), *Trioza zimmermani* Tuthill, 1942 (Hem.: Triozidae) (accession number: KY294653.1) were chosen as the outgroups for the analysis of *mtCOI* and *cytb* genes, respectively.

Results

The total genes in all individuals belonging to the three psyllid species were successfully amplified (Fig. 2) and sequenced. Two mitochondrial gene regions used in this study are both short and typical, 385bp for *cytb* and 472bp for *mtCOI*, but this makes them easy to amplify and sequence even for fairly degraded material. Results of BLAST revealed close sequence matches (>90% nucleotide identity) for all studied genes. The mean pairwise genetic distance values (K2P) calculated by in software MEGA v.7.0.21 are listed in Table 2. Mean intra-specific distance was 0.0000 in all genes among all species due to no genetic variation among individuals, while mean inter-specific divergence was greater (Table 2) which is in agreement with the requirement for ideal DNA barcodes. For *cytb* gene sequences, the maximum inter-specific distance (0.253) was between *P. repens* and *P. securicola*, while the minimum distance (0.019) was found between *P. machinosus* and *P. repens* (Table 2). The results of pairwise genetic distance values showed that *cytb* and *mtCOI* genes had the highest inter-specific variability, respectively (Table 2).

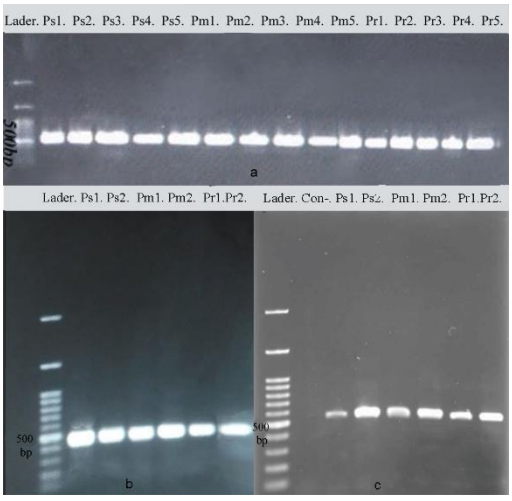

Fig. 2. Gel electrophoresis of *mtCOI* (a), *cytb* (b) and *wsp Wolbachia* (c) in four ash psyllids.

Table 2. Inter-specific Kimura 2-parameter distance values, with standard errors, among three psyllid species.

Gene	AD±SE	Minimum	Maximum
mtCOI	0.098±0.012	0.121	0.146
cytb	0.139±0.018	0.253	0.019
wsp	0.100±0.011	0.187	0.187

For *mtCOI* gene sequences, the maximum inter-specific distance (0.149) was between *P. fraxini* and *P. securicola*, while the minimum distance (0.121) was between *P. machinosus* and *P. repens* (Table 2). Results of clustering methods were similar, thus only the tree obtained from Bayesian inference method is shown (Fig. 3-5). Similar phylogenetic relationships were found in the analysis of *mtCOI* and *cytb* genes (Figs. 3 and 4), as the closest relationship was observed between *P. machinosus* and *P. repens*, while the largest association was found between *P. repens* and *P. securicola*.

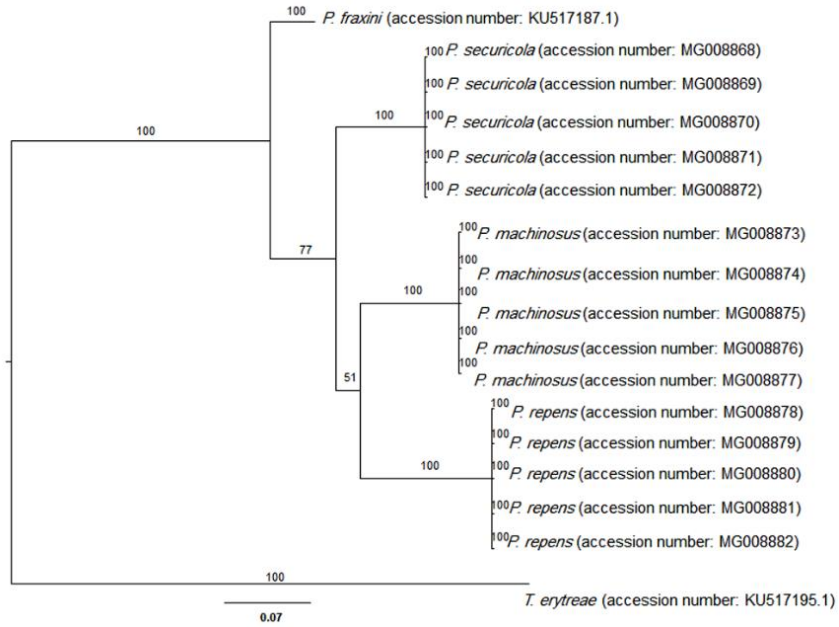

Fig. 3. NJ analysis of *mtCOI* gene sequences compared with data available in GenBank for ash psyllids. Bootstrap values (1000 replicates) are shown above the branches. The scale bar shows K2P distances.

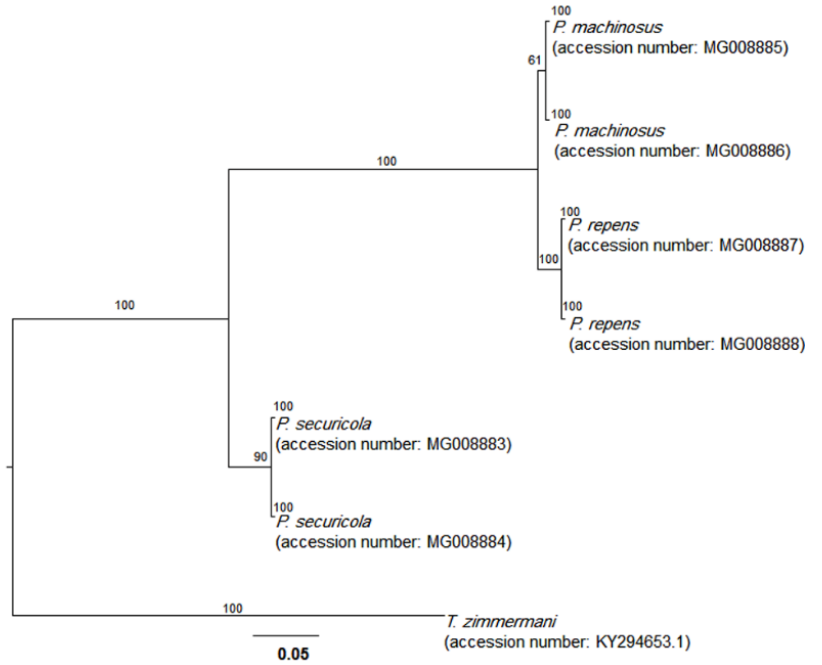

Fig. 4. NJ analysis of *cytb* gene sequences for ash psyllids. Bootstrap values (1000 replicates) are shown above the branches. The scale bar shows K2P distances.

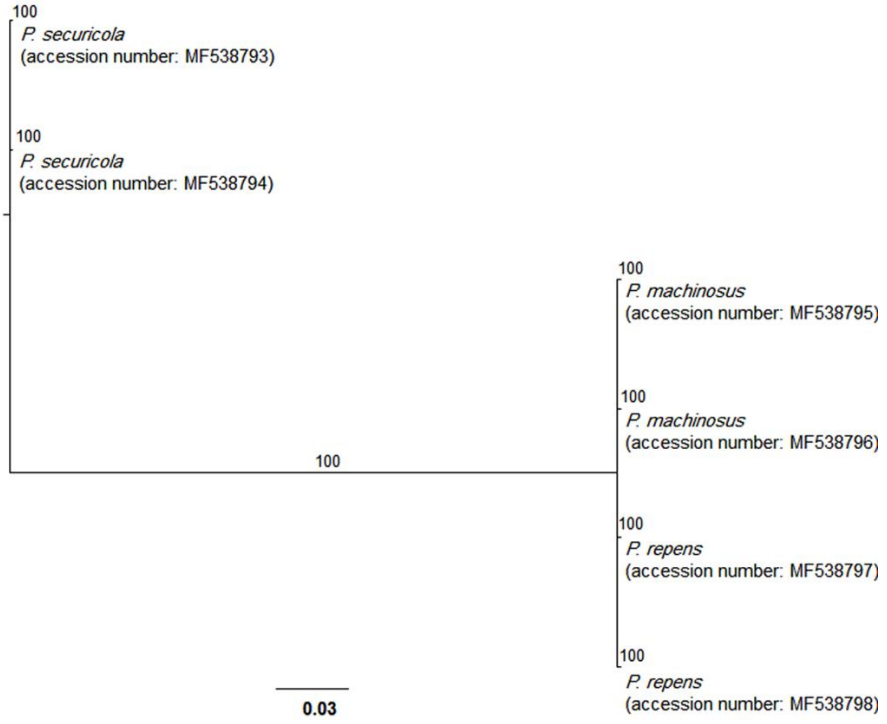

Fig. 5. NJ analysis of *wsp Wolbachia* gene associated with ash psyllids. Bootstrap values (1000 replicates) are shown above the branches. The scale bar shows K2P distances.

Based on the result of phylogenetic analysis of the *Wolbachia* strains , it seems that *P. machinosus* and *P. repens* had been infected with the same *Wolbachia* strain, while *P. securicola* infected with a separate strain, but it needs more evidences (Fig. 5). The maximum inter-specific distance (0.187) was between *Wolbachia* strains infected *P. securicola* and two species *P. machinosus* and *P. repens*.

Discussion

The psylloidea fauna of Iran is poorly known and also no molecular data was available before this study. Recently, efforts to build a DNA barcode for psyllids have begun (Percy *et al*., 2016), but currently just five species of the known species of Liviidae have a barcode record which one of them is *P. fraxini*. Moreover, none of these reference sequences derived from Iran. Therefore, it shows the need to prepare a complete database with reference sequences from carefully identified specimens. In this study, mitochondrial DNA barcodes for the *P. repens*, *P. securicola* and *P. machinosus* are provided. Although the climate conditions were different at collection sites, intra-specific genetic distance among the individuals was not found in each species. No presence of intra-specific genetic distance in Psylloidea have also been shown in some previous studies (Lashkari *et al*., 2015). Lashkari *et al*. (2015) demonstrated that all geographic populations of *Diaphorina citri* (Psylloidea: Liviidae) from Iran have same *mtCOI* sequence without intra-specific distance; they used two adult insects per population in their study. Although the three species were present in all urban and rural areas of Kerman province, there is no information about the dominant species of *Psyllopsis.* Based on the evidence it seems that *P. securicola* is dominant species in central part of Kerman and other species, *P. machinosus* and *P. repens*, showed a much broader distribution range in West area of Kerman Provinces. *P. fraxini* is another species which has been reported from Kerman province, but it was not detected at the studied sites. Burckhardt and Lautere (1993) mentioned that the record of this species from Iran needs verification; therefore it seems that this species in not present in Kerman province and Iran.

Little information is available about the systematic of *Psyllopsis*. Loginova described them in 1963 without systematic relationship (Loginova, 1963). Burckhardt and Lautere (1993) separate *P. repens* from *P. machinosus* and *P. repens*, according to the black spots on head or thorax, and they also separate *P. machinosus* and *P. repens* based on the shape of male parameters in an identification key (Burckhardt & Lauterer, 1993). The molecular results presented here revealed that *P. machinosus* and *P. repens* have the closest relationship and they are separated from *P. securicola* which was not congruence with morphological data.

Moreover, a similar *Wolbachia* strain in these species might rule out the effect of reproductive isolation caused by *Wolbachia*; therefore it might be due to other microorganisms (Thao *et al*., 2000). Psylloidea, *Wolbachia* strains data only have hitherto been published for families Liviidae (Jeyaprakash & Hoy, 2000; Meyer & Hoy, 2008; Wang, 2010; Guidolin & Consoli, 2013; Lashkari *et al*., 2015) and Triozidae (Liu *et al*., 2006). It would be interesting to explore the *Wolbachia* strains from other psyllid families, to survey of *Wolbachia* diversity, mode of transmission and rules within this superfamily; for example increasing the resistance against natural pathogens by *Wolbachia* strain has been shown in *Drosophila melanogaster* (Teixeira *et al*., 2008). Correct and rapid identification of these pests is the best first step in pest management programs.

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