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Variation in bacterial endosymbionts associated with the date palm hopper, *Ommatissus lybicus* populations

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Abstract

The date palm hopper, *Ommatissus lybicus*, is a key pest of the date palm, which is expected to be comprised of many allopatric populations. The current study was carried out to determine bacterial endosymbiont diversity in the different populations of this pest. Ten date palm hopper populations were collected from the main date palm growing regions in Iran and an additional four samples from Pakistan, Oman, Egypt and Tunisia for detection of primary and secondary endosymbionts using polymerase chain reaction (PCR) assay with their specific primers. The PCR products were directly sequenced and edited using SeqMan software. The consensus sequences were subjected to a BLAST similarity search. The results revealed the presence of '*Candidatus* Sulcia muelleri' (primary endosymbiont) and *Wolbachia*, *Arsenophonus* and *Enterobacter* (secondary endosymbionts) in all populations. This assay failed to detect '*Candidatus* Nasuia deltocephalinicola' and *Serratia* in these populations. '*Ca*. S. muelleri' exhibited a 100% infection frequency in populations and *Wolbachia*, *Arsenophonus* and *Enterobacter* demonstrated 100, 93.04 and 97.39% infection frequencies, respectively. The infection rate of *Arsenophonus* and *Enterobacter* ranged from 75 to 100% and 62.5 to 100%, respectively, in different populations of the insect. The results demonstrated multiple infections by '*Ca*. Sulcia muelleri', *Wolbachia*, *Arsenophonus* and *Enterobacter* in the populations and may suggest significant roles for these endosymbionts on date palm hopper population fitness. This study provides an insight to endosymbiont variation in the date palm hopper populations; however, further investigation is needed to examine how these endosymbionts may affect host fitness.

Keywords: *Arsenophonus*, bacterial endosymbiont, *Enterobacter*, *Ommatissus lybicus*, *Sulcia*, *Wolbachia*

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Introduction

Members of the suborder Auchenorrhyncha in the order Hemiptera have needle-like sucking mouthparts and feeding exclusively on xylem/phloem plant sap. Although xylem or phloem plant sap is rich in carbohydrates, it is devoid of most essential amino acids and many vitamins (Hansen & Moran, 2014). Auchenorrhyncha insects are known to be associated with obligatory symbiotic bacteria, which provide

essential amino acids and other nutrients for their hosts (Bressan *et al.*, 2009; Urban & Cryan, 2012). These endosymbiotic bacteria are vertically transmitted from mother to offspring (Ratzka *et al.*, 2012) and harbored in specialized cells called bacteriocytes. The bacteriocytes typically compose a symbiotic organ called a bacteriome (Matsuura *et al.*, 2012).

Morphological and molecular investigations have revealed that members of Auchenorrhyncha usually harbor the obligatory endosymbiont bacterium '*Candidatus* Sulcia muelleri' (*Bacteroidetes*) and a partner (coprimary symbionts) belonging to the phylum Proteobacteria that are engaged in the synthesis of amino acids (Moran *et al.*, 2005; McCutcheon *et al.*, 2009; Urban & Cryan, 2012; Ishii *et al.*, 2013; Koga *et al.*, 2013; Michalik *et al.*, 2014). Urban & Cryan (2012) surveyed 77 planthopper species representing 18 Fulgoroid families and detected '*Candidatus* Vidania fulgoroidea' (*Betaproteobacteria*) in 40 species and the '*Ca.* Sulcia muelleri' endosymbiont in 30 of the 40 species harboring '*Ca.* Vidania fulgoroidea'.

In addition to obligate bacterial endosymbionts, Auchenorrhyncha also harbor various facultative (secondary) bacterial endosymbionts such as *Arsenophonus*, *Cardinium*, *Wolbachia*, *Rickettsia*, *Diplorickettsia* and *Serratia* (Sacchi *et al.*, 2008; Ishii *et al.*, 2013; Hong-Xing *et al.*, 2015). These bacteria are present in the cells of tissues throughout the host body and can be transmitted maternally and horizontally (Xue *et al.*, 2012). In contrast to obligatory endosymbionts, secondary endosymbionts are not necessary for host survival (White *et al.*, 2013); however, they do have roles ranging from neutral to pathogenic in their hosts (Oliver *et al.*, 2010). They may play a role in host fitness (Dohlen *et al.*, 2013), host resistance to natural enemies (Michalik *et al.*, 2014) and host protection against environmental stresses (Hamilton & Perlman, 2013).

The date palm hopper (DPH), *Ommatissus lybicus* de Bergevin (Hemiptera: Tropiduchidae), is a destructive pest afflicting the date palm in the Middle East and North Africa (Hussain, 1963). Both nymphs and adults of this bivoltine pest cause significant damage to date palms by sucking the phloem sap. Heavy infestations of *O. lybicus* produce extremely large amounts of honeydew followed by the growth of sooty mold, which decreases the photosynthetic activity of the trees (Howard, 2001).

Excessive application of pesticides to date palm orchards to control this pest has contributed to the development of resistance of DPH populations to several conventional chemical insecticides (Ali, 2011). To deter emergence of new resistant populations, new strategies for pest management must be developed. Interference in the endosymbiotic relationship may provide an ecofriendly method for the control of this pest (Douglas, 2007). Thus far, too little attention has been paid to bacterial endosymbionts in the date palm hopper. The current study investigated the diversity of bacterial endosymbionts in DPH populations. The frequency rate of bacterial endosymbionts in the studied populations was also assessed. These findings can provide insight into endosymbiotic infection of *O. lybicus* and open a pathway for further study of *O. lybicus*, its bacterial endosymbiotic interactions and possibly lead to a more ecofriendly strategy to control this pest.

Materials and methods

Collection of specimens

A total of 14 populations of DPH were collected from the major date palm growing regions of Iran and four another important date palm growing countries (Pakistan, Oman, Egypt and Tunisia) in 2015. Table 1 lists the sample collection sites and their global positioning system coordinates. All collected insects were kept in an ultra-low temperature freezer at −80°C to avoid DNA degradation until its extraction.

Table 1. Samples of *Ommatissus lybicus* used in this study.

Sample code	Country	Locality	Longitude and latitude
AB	Iran	Abumusa	55°02E, 25°53N
BA	Iran	Bam	58°21E, 29°06N
BU	Iran	Bushehr	51°28E, 28°46N
BE	Iran	Behbahan	50°14′E, 30°35′N
TE	Iran	Tezerj	55°40E, 28°16N
FI	Iran	Fin	55°53E, 27°37N
JI	Iran	Jiroft	58°00E, 28°22N
SH	Iran	Shahdad	57°42E, 30°25N
JA	Iran	Jahrom	53°33E, 30°28N
GH	Iran	Ghasr-e-shirin	45°34E, 34°31N
PK	Pakistan	Pakistan	64°6E, 26°59N
OM	Oman	Oman	58°32E, 23°36N
EG	Egypt	Egypt	31°19E, 29°51N
TU	Tunisia	Tunisia	7°56E, 34°19N

DNA extraction

A single specimen was randomly selected for DNA extraction. The total DNA was extracted using the adjusted CTAB protocol from Reineke *et al.* (1998). Each DPH specimen was ground in liquid nitrogen and subsequently subjected to 500 µl of lysis buffer (100 mM Tris-HCl [pH 8.0], 10 mM EDTA, 2% sodium dodecyl sulfate). The homogenates were incubated in a water bath at 60°C for 1 h. After incubation, 140 µl of 5 M NaCl and 65 µl of 10% CTAB were added to the incubated homogenate and kept at 65°C for 10 min. Next, 700 µl of chloroform:isoamylalcohol (24:1) was added to each sample and they were mixed gently and placed on ice for 30 min. The mixture was centrifuged at 13,000 rpm for 20 min. After centrifugation, the supernatant was collected and 225 µl of 5 M magnesium acetate was added. The solution was mixed gently and kept on ice for 30 min, after which it was again centrifuged at 13,000 rpm for 20 min, the supernatant was discarded and 0.7 volume of cold (4°C) isopropanol was added. Samples were kept at 4°C overnight and then centrifuged for 30 min at 13,000 rpm. After centrifuging, the supernatant was removed, the pellet was washed twice with cold (4°C) 70% ethanol, dried and resuspended in 50 µl double-distilled water. The quantity and quality of the extracted DNA were determined using Thermo NanoDrop 1000 and confirmed by visualization on agarose gel (1%). The extracted DNA was stored at −20°C until use.

PCR assay and DNA sequencing

PCR assay was used to detect the bacterial endosymbionts using universal and specific primer pairs to amplify specified parts of the related bacterial gene (table 2). All PCR reactions were performed in a total volume of 30 µl containing 15 µl buffer mix, 1 µl forward primer (10 pmol μl^{-1}) and 1 µl reverse primer (10 pmol μl^{-1}), 1 µl DNA template and 12 µl double-distilled water.

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Table 2. PCR primers used to identify the bacterial endosymbionts in *Ommatissus lybicus*.

Endosymbiont	Target gene	Primer name	Primer sequence (5′–3′)	Product size (bp)	Reference
Eubacterial general	16S rRNA	16SA1 16SB1	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	1500	Fukatsu & Nikoh (1998)
'*Ca*. Sulcia muelleri'	16S rRNA	10FF 1370R	AGTTTGATCATGGCTCAGGATAA CGTATTCACCGGATCATGGC	1500	Takiya *et al.* (2006)
'*Ca* Nasuia deltocephalinicola'	16S rRNA	MstrNas2F MstrNas1185R	AGTTGACGTTGAATATTCAAAGTA TCAATCTTGCGATATTGCAACT	1200	Ishii *et al.* (2013)
'*Ca* Nasuia deltocephalinicola'	16S rRNA	NasF NasR	GAATTAAAGCGGGGAAAACC AAGTCATCCCCTCCTTCCTC	980	This study
Arsenophonus	16S rDNA	16SA1 Ars16SR	AGAGTTTGATCMTGGCTCAG TTAGCTCCGGAGGCCACAGT	960	Tsuchida *et al.* (2002)
Wolbachia	*wsp*	*wsp*F *wsp*R	TGGTCCAATAAGTGATGAAGAAAC AAAAATTAAACGCTACTCCA	600	Zhou *et al.* (1998)
Enterobacter	16s rRNA	*EnteroF* *EnteroR*	GTGGCTAATACCGCATAAC CCGTGGATGTCAAGAGTA	839	This study
Serratia	16S rRNA	16SA1 PASScmp	AGAGGTTGATCMTGGCTCAG GCAATGTCTTATTAACACAT	500	Douglas *et al.* (2006)

Table 3. PCR conditions to detect bacterial endosymbionts in *Ommatissus lybicus*.

			Denaturation		Annealing		Elongation	
Endosymbiont	Primer name	No. cycle	°C	S	°C	S	°C	S
Eubacterial general	16SA1 16SB1	35	94	45	56	45	72	90
'*Ca*. Sulcia muelleri'	SulF SulR	35	95	60	58	60	72	90
'*Ca* Nasuia deltocephalinicola'	MstrNas2F MstrNas1185R	35	95	60	51	60	72	60
'*Ca* Nasuia deltocephalinicola'	NasF NasR	38	94	40	55	30	72	60
Arsenophonus	16SA1 Ars16SR	40	95	30	55	30	72	30
Wolbachia	*wsp*F *wsp*R	34	94	45	55	60	72	90
Enterobacter	EnteroF EnteroR	35	94	35	54.5	35	72	60
Serratia	16SA1 PASScmp	30	94	60	55	60	72	120

The PCR was performed in an Eppendorf thermocycler according to the PCR conditions shown in table 3. A negative control containing no DNA template was also kept with each reaction. The sample of DNA from *Hishimonus phycitis* Distant. (Hemiptera: Cicadellidae) was included in each PCR as a positive control for all investigated bacterial endosymbionts (Hemati *et al.*, unpublished data). PCR products were stained with FluoroDye (Smobio; Taiwan) and subjected to electrophoresis on 1% agarose gel (fig. 1). All PCR products were directly sequenced by Macrogen Sequencing Service (South Korea). All sequences were edited using SeqMan II software (Lasergene, Version 5; DNA Star, Inc, Madison, Wisconsin, USA) and identified based on BLAST similarity searches. All sequences were deposited in the GenBank under accession numbers KX790331 to KX790338 and KY346959.

Molecular phylogenetic analysis

Phylogenetic analysis was performed using a data set of 16S rRNA gene sequences of symbiotic bacteria downloaded from the GenBank. The phylogenetic trees of DPH bacterial endosymbionts were constructed by Bayesian analysis using the MrBayes 3.1.2 program (Ronquist & Huelsenbeck, 2003). Posterior probabilities were calculated for each node used for statistical evaluation in Bayesian analysis.

Results

Endosymbiont diversity

The PCR assay and subsequent sequencing analysis confirmed the presence of a primary endosymbiont, '*Ca.* Sulcia muelleri' (*Bacteroidetes*), in all DPH populations. In addition, the presence of three secondary endosymbionts *Wolbachia* sp. (*Alphaproteobacteria*), *Arsenophonus* sp. (*Gammaproteobacteria*) and *Enterobacter* sp.(*Gammaproteobacteria*) was detected in the studied DPH populations.

By comparing the similarity of these endosymbionts with available sequences of the organism in GeneBank, different patterns of similarity were found for each endosymbiont. '*Ca.* Sulcia muelleri' showed 96% similarity to '*Ca.* Sulcia

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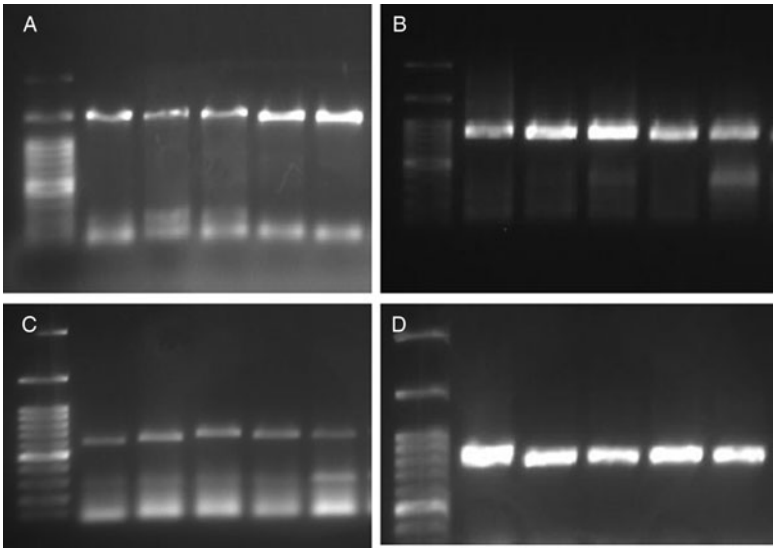

Fig. 1. Diagnostic polymerase chain reaction of the bacterial endosymbionts in *Ommatissus lybicus*. '*Candidatus* Sulcia muelleri'(A: lanes 2–6), *Arsenophonus* (B: lanes 2–6), *Wolbachia* (C: lanes 2–6) and *Enterobacter* (D: lanes 2–6), DNA Marker (Smobio, Taiwan) (Lane 1) comprising band sizes at 3000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp.

Table 4. Prevalence of bacterial endosymbionts in different *Ommatissus lybicus* populations.

		Population code												
Bacterial endosymbionts	AB	BA	BU	BE	TE	FI	JI	SH	JA	GH	PK	OM	EG	TU
'*Ca*. Sulcia muelleri'	+	+	+	+	+	+	+	+	+	+	+	+	+	+
'*Ca* Nasuia deltocephalinicola'	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arsenophonus	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Wolbachia	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Enterobacter	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Serratia	–	–	–	–	–	–	–	–	–	–	–	–	–	–

muelleri' endosymbiont from different species of the Fulgoridae family. The *Wolbachia* symbiont exhibited 100% similarity to the *Wolbachia* symbiont isolated from *Diaphorina citri*. The most similar *Arsenophonus* with 97% similarity has been reported as a secondary endosymbiont in *Ornithomya avicularia*. Two *Enterobacter* sequences from the Oman and Abumusa populations showed 100% similarity to the *Enterobacter* endosymbiont from *Rhynchophorus ferrugineus* and *Oniticellus cinctus*, respectively.

All samples were searched for the presence of '*Candidatus* Nasuia deltocephalinicola' and *Serratia*, but no evidence of these endosymbionts was detected in the studied populations (table 4). Using universal primers, the bacteria *Rickettsiella* sp., *Staphylococcus* sp., *Pseudomonas* sp. and *Erwinia* sp. were detected in the DPH populations.

Infection rate in DPH populations

'*Ca*. Sulcia muelleri' was detected in all DPH populations at a 100% infection rate. The average infection rate for the *Arsenophonus* endosymbiont was 93.04% (107 infected individuals out of 115 specimens), but its prevalence varied among populations. The highest infection rate was observed in the BA, BU, FI, SH, JA, PK, OM and TU populations. The JI population showed the lowest infection rate (six out of eight infected individuals; 75%). The secondary endosymbiont *Wolbachia* exhibited a 100% infection rate in all populations. The *Enterobacter* infection rate ranged from 62.5 to 100% in the different DPH populations (table 5).

Molecular phylogenetic analysis

Phylogenetic analysis of the 16S rRNA gene sequences revealed a monophyletic group for '*Ca*. Sulcia muelleri' sequenced from the DPH population in the '*Ca*. Sulcia muelleri'of the family Fulgoridae with 0.87 posterior probability values (fig. 2). The *Arsenophonus* symbiont of DPH was closely related to the *Arsenophonus* sequences from the flies of the Hippoboscidae family with 1.00 posterior probability (fig. 3). Phylogenetic analysis based on the *wsp* gene indicated that the DPH *Wolbachia* symbiont was associated with the *Wolbachia* sequence from *Nilaparvata lugens* with 0.87 posterior probability (fig. 4). The *Enterobacter* symbiont of DPH was closely affiliated (1.00 posterior probability) with the *Enterobacter* isolated from *Rhynchophorus ferrugineus* (Col: Curculionidae) (fig. 5). The *Rickettsiella* 16S rRNA sequence of DPH was closely related (1.00 posterior probability) to

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Table 5. Infection frequencies of primary and secondary endosymbionts in different populations of *Ommatissus lybicus*.

Population code	No. of infected insects/total no. of insects examined (% detection rate)			
	'Ca. Sulcia muelleri'	*Arsenophonus*	*Wolbachia*	*Enterobacter*
AB	10/10 (100)	9/10 (90)	10/10 (100)	10/10 (100)
BA	10/10 (100)	10/10 (100)	10/10 (100)	10/10 (100)
BU	10/10 (100)	10/10 (100)	10/10 (100)	10/10 (100)
BE	8/8 (100)	8/8 (87.5)	8/8 (100)	8/8 (100)
TE	7/7 (100)	6/7 (85.71)	7/7 (100)	7/7 (100)
FI	10/10(100)	10/10 (100)	10/10 (100)	10/10 (100)
JI	8/8 (100)	6/8 (75)	8/8(100)	8/8 (100)
SH	8/8 (100)	8/8 (100)	8/8 (100)	5/8 (62.5)
JA	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)
GH	10/10 (100)	8/10 (80)	10/10 (100)	10/10 (100)
PK	8/8 (100)	8/8 (100)	8/8 (100)	8/8 (100)
OM	10/10 (100)	10/10 (100)	10/10 (100)	10/10 (100)
EG	10/10(100)	8 (10) (80)	10/10 (100)	10/10 (10)
TU	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)
Total	115/115 (100)	107/115 (93.04)	115/115 (100)	112/115 (97.39)

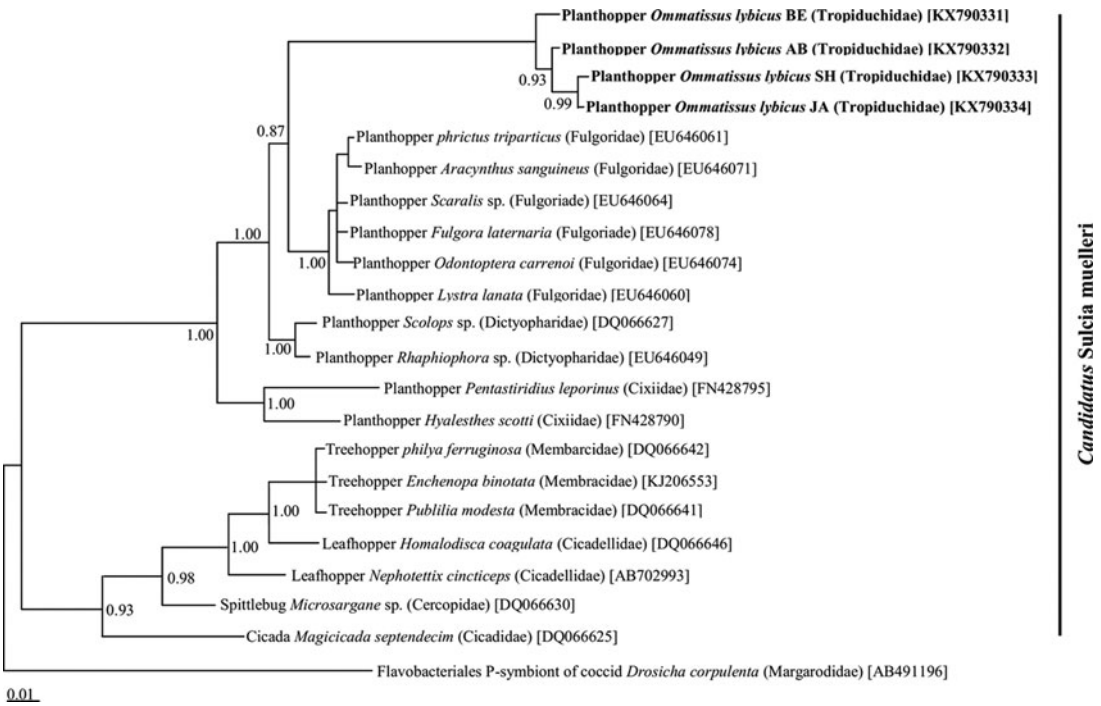

Fig. 2. Phylogenetic relationship of '*Candidatus* Sulcia muelleri' identified from *Ommatissus lybicus* and other hemipteran insects on the basis of 16S rRNA gene sequences. The tree was constructed using Bayesian analysis and the numbers near nodes are posterior probabilities. The sequences obtained from *O. lybicus* in this study are in bold type, wherein insect species, insect family in parentheses, and GenBank accession numbers in brackets are indicated. Flavobacteriales P-symbiont of *Drosicha corpulenta* was used as the out group.

Rickettsiella isolated from *Acyrthosiphon pisum* (Hem: Aphididae) (fig. 6).

Discussion

A substantial number of sap-feeding insects in the suborder Auchenorrhyncha are associated with symbiotic microorganisms (Ishii *et al*., 2013). Other than Cicadomorpha, little information is available about the bacterial endosymbionts of Fulgoromorpha (Urban & Cryan, 2012). One study revealed the presence of endosymbionts in 217 species of Fulgoroidea and showed that the a-symbiont was present in the most species (Müller, 1940, 1962). They hypothesized that the a-symbiont is ancient and was acquired by the common ancestor of the Fulgoroidea. Moran *et al.* (2005) identified '*Ca.* Sulcia muelleri', an ancient symbiont lineage belonging to the *Bacteroidetes*, in 30 Auchenorrhyncha species. This matched Müller's description and illustrations of the a-symbiont.

'*Ca*. Sulcia muelleri' as an endosymbiont was also reported in Cixiidae, Delphacidae, Dictyopharidae and Fulgoridae

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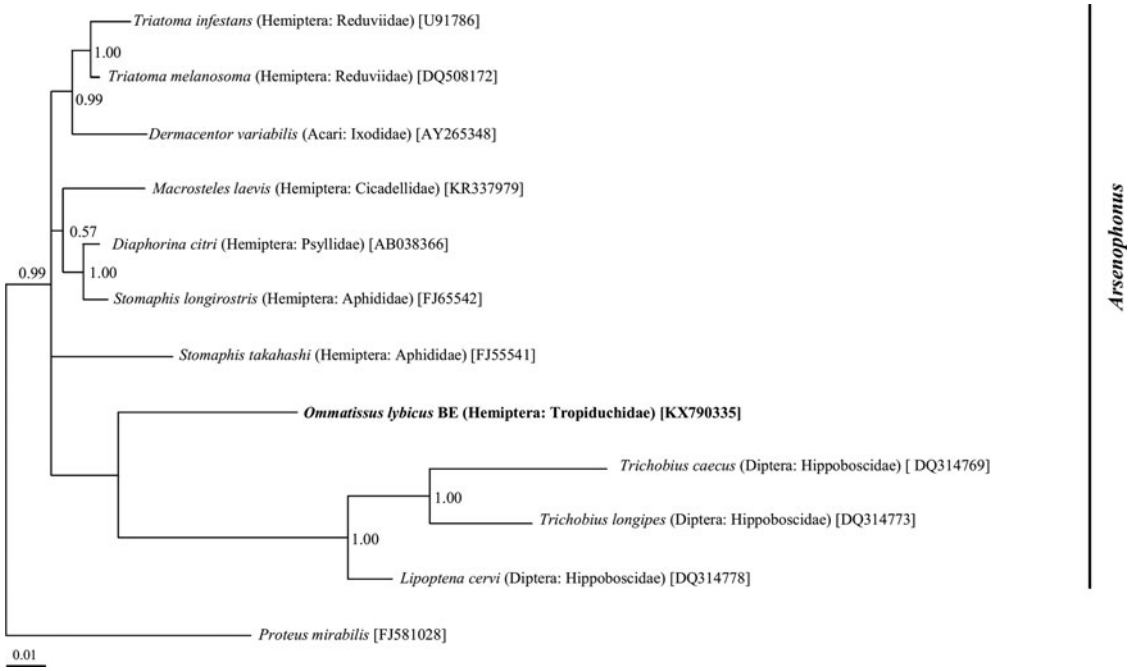

Fig. 3. Phylogenetic relationship of *Arsenophonus* symbiont identified from *Ommatissus lybicus* on the basis of 16S rRNA gene sequences. The tree was constructed using Bayesian analysis and the numbers near nodes are posterior probabilities. The sequences obtained from *O. lybicus* in this study are in bold type, wherein insect species, insect family in parentheses, and GenBank accession numbers in brackets are indicated. Sequence from *Proteus mirabilis* was used as out group.

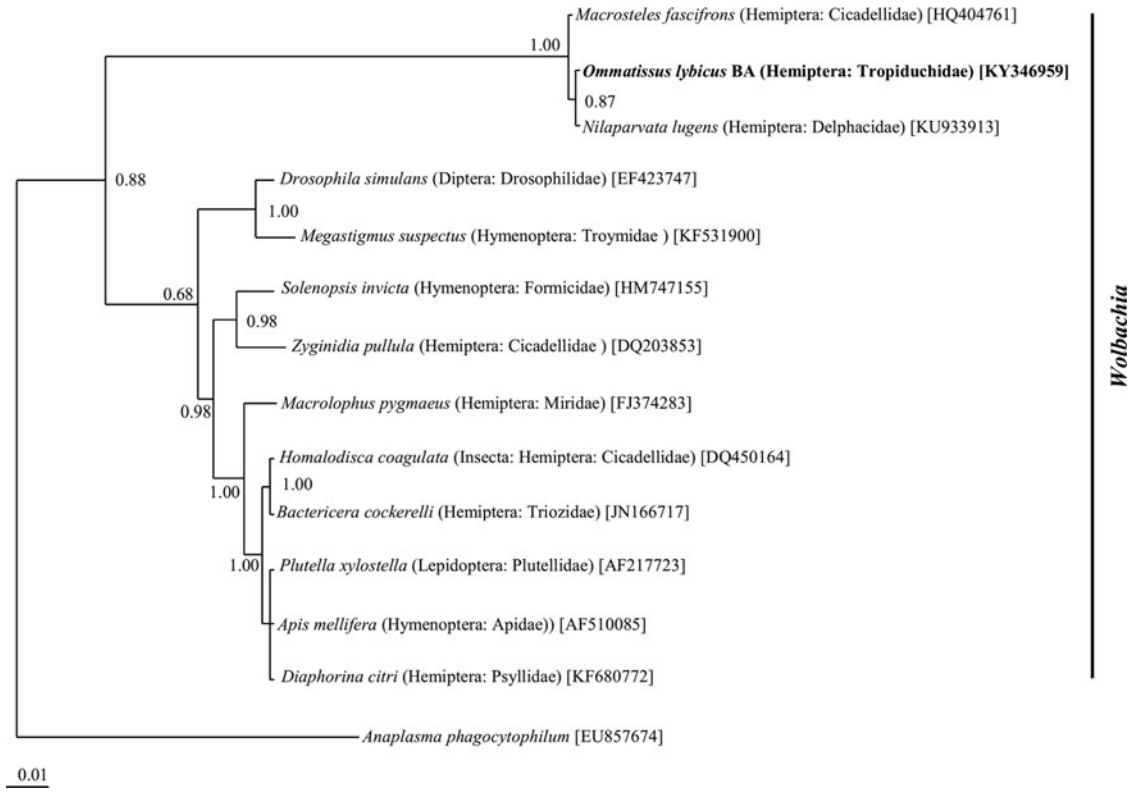

Fig. 4. Phylogenetic relationship *Wolbachia* symbiont identified from *Ommatissus lybicus* on the basis of *wsp* gene sequences. The tree was constructed using Bayesian analysis and the numbers near nodes are posterior probabilities. The sequences obtained from *O. lybicus* in this study are in bold type, wherein insect species, insect family in parentheses, and GenBank accession numbers in brackets are indicated. Sequence from *Anaplasma phagocytophilum* was used as outgroup.

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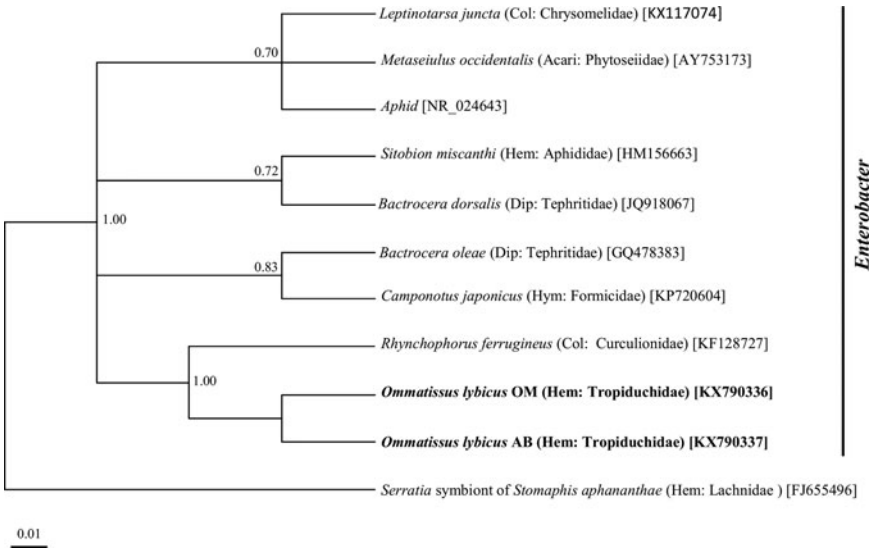

Fig. 5. Phylogenetic relationship of *Enterobacter* symbiont of *Ommatissus lybicus* on the basis of 16S rRNA gene sequences. The tree was constructed using Bayesian analysis and the numbers near nodes are posterior probabilities. The sequences obtained from *O. lybicus* in this study are in bold type, wherein insect species, insect family in parentheses, and GenBank accession numbers in brackets are indicated. *Serratia* symbiont of *Stomaphis aphananthae* was used as the out group.

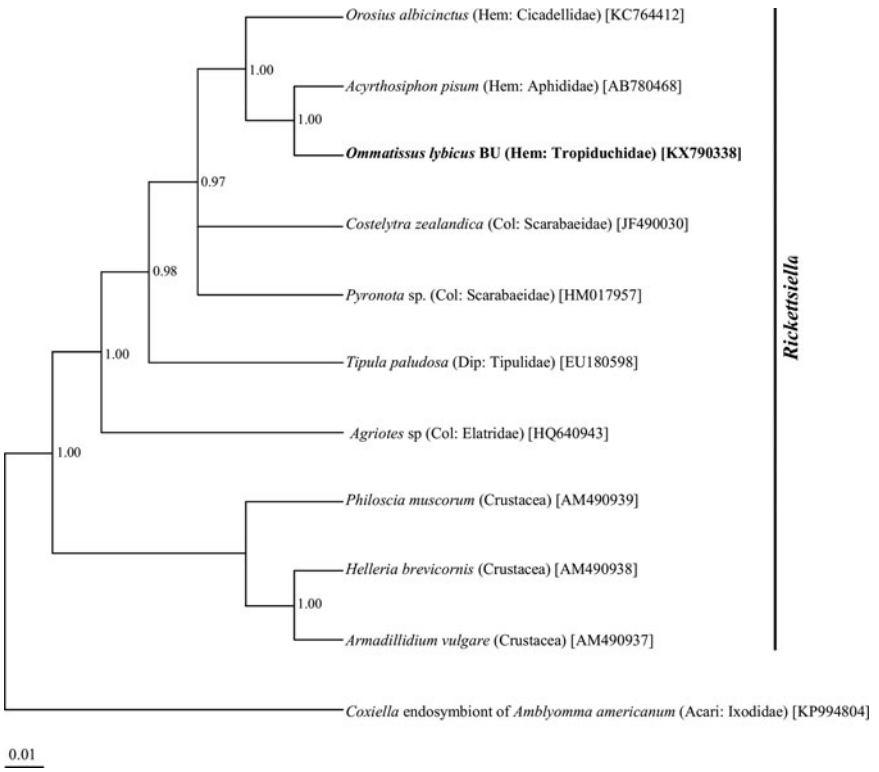

Fig. 6. Phylogenetic relationship of *Rickettsiella* symbiont of *Ommatissus lybicus* on the basis of 16S rRNA gene sequences. The tree was constructed using Bayesian analysis and the numbers near nodes are posterior probabilities. The sequences obtained from *O. lybicus* in this study are in bold type, wherein insect species, insect family in parentheses, and GenBank accession numbers in brackets are indicated. *Coxiella* endosymbiont of *Amblyomma americanum* was used as the out group.

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families belonging to Fulgoroidea (Urban & Cryan, 2012). Several studies have demonstrated and identified the role of '*Ca.* Sulcia muelleri' as an obligatory endosymbiont that retains genes for the synthesis of essential amino acids absent in plant sap, the food of the insects (McCutcheon & Moran, 2010). The results of the current study revealed 100% '*Ca.* Sulcia muelleri' infection in all DPH populations, which is in line with previous findings for the Fulgoroidea superfamily. The sap-feeding behavior of the DPH lets us hypothesize that '*Ca.* Sulcia muelleri' is similarly involved in synthesizing essential nutrients lacking in phloem sap for this species.

Recent investigations suggest that the members of Auchenorrhyncha, in addition to '*Ca.* Sulcia muelleri', harbor other bacterial endosymbionts that complement each other in providing essential amino acids for their hosts. These include *Baumannia cicadellinicola* (*Gammaproteobacteria*) in leafhoppers (Membracoidea: Cicadellidae), *Hodgkinia cicadicola* (*Alphaproteobacteria*) in cicadas (Cicadoidea: Cicadidae) and *Zinderia insecticola* (*Betaproteobacteria*) in spittlebugs (Cercopoidea) (Koga *et al*., 2013).

'*Ca.* Nasuia deltocephalinicola' (*Betaproteobacteria*) is an obligate bacterial endosymbiont, which coexists with '*Ca.* Sulcia muelleri' in the subfamily Deltocephalinae. This bacterium produces cofactors and several essential amino acids that '*Ca.* Sulcia muelleri' is not able to synthesize (Noda *et al*., 2012; Wangkeeree *et al*., 2012; Ishii *et al*., 2013). The current study failed to detect this endosymbiont bacterium in DPH populations. This can either be evidence of the lack of this endosymbiont in this species or that further study is required to identify its presence.

Urban & Cryan (2012) detected another obligatory bacterial endosymbiont, *Vidania fulgoroideae*, coexisting with '*Ca.* Sulcia muelleri' in some families belonging to Fulgoroidea. The present study did not investigate the presence of *Vidania fulogoroidea* in DPH populations. Because DPH belongs to this superfamily, future studies are recommended to implement such a survey.

Wolbachia, *Arsenophonus* and *Enterobacter* were detected in all 14 DPH populations. The occurrence of these endosymbionts in all DPH populations suggests significant roles for these endosymbionts on DPH population fitness and their coevolution. Although in some populations (JA and TU), the sample size was too low to conclude 100% infection rates for the bacterial endosymbionts.

The presence of the gammaproteobacterium *Arsenophonus* has been evidenced in a diverse array of insects from Hemiptera, Hymenoptera and Diptera (Taylor *et al*., 2011; Russell *et al*., 2012; Jousselin *et al*., 2013; Duron *et al*., 2014). Studies have shown that this bacterium imposes significant effects on the ecology and life history of their arthropod hosts (Bressan *et al*., 2012). *Arsenophonus* may act as a primary endosymbiont and provide essential nutrients for its host (Trowbridge *et al*., 2006; Perotti *et al*., 2007; Nováková *et al*., 2015) or as a secondary endosymbiont (Chiel *et al*., 2007). One study conferred a host protection role for *Arsenophonus* against parasitoid wasps (Hansen *et al*., 2007) and to promote the adaptation of the insect to specific host plants (Chiel *et al*., 2007). Other *Arsenophonus* bacteria can be insect-vectored plant pathogens (Bressan, 2014) or parasitic agents (Gherna *et al*., 1991).

In the current study, *Arsenophonus* infection was detected in all DPH populations at different frequencies. The lack of *Arsenophonus* in some specimens can be explained by a difference in the genetic pattern of the DPH populations, the low titer of this bacterium in the specimens or the sensitivity of the detection method. The presence of *Arsenophonus* endosymbiont has been reported in different populations of brown planthoppers, *Nilapavata lugens* (Hemiptera: Delphacidae), in recent studies (Qu *et al*., 2013; Xu *et al*., 2014). *Arsenophonus* was also detected in *Macrosteles laevis* (Hemiptera: Cicadellidae) by Kobialka *et al*. (2016).

Wolbachia has been reported from many species within Auchenorhyncha, such as *Laodelphax striatellus* (small brown planthopper), *Sogatella furcifrea* (white back planthopper) (Liu, 2011), *Nilaparvata lugens* (brown planthopper) (Qu *et al*., 2013) and *Macrosteles* leafhoppers (Ishii *et al*., 2013).

A number of effects for *Wolbachia* on host reproduction have been reported, including cytoplasmic incompatibility, parthenogenesis, male killing and feminization (Werren *et al*., 2008). They may increase the fitness of the insect host, provide protection against parasitization (Xue *et al*., 2012) and inhibit defense gene expression in plants (Barr *et al*., 2010).

Several studies of the genus *Enterobacter* have reported that this bacterium as endosymbiont from Acari (Jeyaprakash & Hoy, 2010) and other insect species such as the oriental fruit fly (*Bactrocera dorsalis*) (Liu *et al*., 2016), *Bemisia tabaci* (Singh *et al*., 2012) and rice brown planthopper (*Nilaparvata lugens*) (Wang *et al*., 2015).

Almost all established endosymbiotic relations of this bacterium have a beneficial function on its insect host. These include synthesis of essential nutrients (Ben-Yosef *et al*., 2008), degradation of toxic purine compounds of the host plants (Lauzon *et al*., 2000) and production of antiparasitic compounds like prodigiosin (Moss, 2002). Wang *et al*. (2016) demonstrated the suppression of plant defenses by *Enterobacter* bacteria in the oral secretions of the false potato beetle (*Leptinotarsa juncta*). All samples in the current study showed evidence of at least one secondary endosymbiotic bacteria, which is an indication of the crucial role of these organisms in the survival and fitness of DPH populations. The rapid resistance of the DPH populations to several insecticides could be closely related to these endosymbionts.

PCR assay failed to detect the *Serratia* endosymbiont in the studied DPH populations. This could result from the lack of this endosymbiont in these populations or the low titer of the bacterium in the tested samples. Lack of success in endosymbiont detection because of the low titer of the endosymbiont has been frequently documented (de Leon *et al*., 2006). Further investigation with more sensitive methods like real time PCR are needed to determine the presence/absence of *Serratia*.

Other groups of bacteria with less-established functions in their endosymbiotic relationships with insects were detected in some populations in this study. Members of the *Rickettsiella* genus have been described as facultative symbionts of diverse insects (Tsuchida *et al*., 2014). The *Erwinia* sp. is a common plant pathogenic bacteria and *Staphylococcus* sp. has been reported as an endosymbiont of some insect species (Peloquin & Greenberg, 2003; Indiragandhi, *et al*., 2010).

Identification of major endosymbionts in insect hosts is an important step toward symbiotic control (Ricci *et al*., 2012). The current study determined the main bacterial endosymbionts in natural populations of DPH that are assumed to effect the life history traits of their hosts. This is the first study to verify and establish the endosymbiont flora of the DPH as a key pest of the date palm with a worldwide distribution. Additional study is required to understand the role of these endosymbionts on the life history traits of the DPH and

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provide a pathway to establishing ecofriendly pest management through symbiotic control of this pest.

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Disclosure

The authors declare no conflict of interest.

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