CLOSELY RELATED WOLBACHIA (RICKETTSIALES: RICKETTSIACEAE) RECOVERED FROM DIFFERENT GENERA OF MEXICAN THELYTOKOUS FIGITIDAE (HYMENOPTERA)

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The thelytoky in hymenopteran parasitoids is often the result of infection with intracellular endosymbionts, especially the bacterium Wolbachia pipiens (Rickettsiales: Rickettsiaceae) (Werren et al. 2008). The transition from arrhenotoky to thelytoky is due either to prevention of chromosome reduction during meiosis or through the postmeiotic fusion of 2 haploid mitotic products. As a result haploidy is eliminated and adults are all diploid females that can vertically pass the bacteria to offspring in the abundant cytoplasm of their ova (Werren et al. 2008). Wolbachia phylogenies, unlike those of mitochondria, often diverge considerably from those of their eukaryotic hosts (e.g., Bandi et al. 1998). This implies infections are not derived through common descent but are passed from species to species by other means (Werren et al. 1996). Such horizontal transfers can be accomplished by multiparasitism (Huigens et al. 2000; Huigens & Stouthamer 2003), via infected hosts (Heath et al. 1999; Caspi-Fluger et al. 2012) and perhaps intimate contacts in a shared habitat; e.g., Wolbachia from Diptera feeding on mushrooms are more similar than those found in non-mushroom feeding flies (Stahlhut et al. 2010).

Odoniosema anastrephae Borgmeier (Figitidae) is a neotropical/subtropical parasitoid of Anastrepha spp. fruit fly larvae that forages inside fallen fruit, especially guavas (Psidium spp.) (Wharton et al. 1998; López et al. 1999). It has been proposed as a candidate for augmentative releases because of its capacity to search for pest infestations, all of which were conducted under the above environmental conditions. The specimens seen for origin and specialist morphological identification of O. anastrephae cultures see Copeland et al. (2010). Thelytokous (T) and arrhenotokous (A) O. anastrephae colonies from the Instituto de Ecologia, A. C., Xalapa, Mexico, where each strain has been in culture since 2000, were imported into quarantine at the USDA-ARS Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, Florida. Subsequently from 2009 to 2011, the parasitoids were reared on Caribbean fruit fly larvae, Anastrepha suspensa (L.) (Tephritidae) in an environmental chamber at 25 ± 1 °C, 16:8 h L:D and 75% RH. Iso-female T and A lines of O. anastrephae were established and maintained for 2 generations prior to their use in experiments, all of which were conducted under the above environmental conditions. The specimens of A. alujai were obtained in nature from Veracruz State, Mexico and immediately preserved in 100% ethanol.

For DNA extraction, individual specimens were placed singly in 60 mL of 5% Chelex buffer with 4 mL of proteinase K (0.5mg/mL) and crushed with a clean plastic pestle before being incubated at 56 °C for 2 h. Homogenates were then boiled at 99 °C for 3 min to inactivate proteinase K and were used as templates for PCR.
Wolbachia infection status of each female used to establish the iso-female experimental lines was confirmed using PCR of the wsp gene (wsp 81F 5′- TGGTCAATAAGTGATGAAAGAAC-3′ and wsp 691R 5′-AAAAATTTAACGCTACTCCA-3′, ex Zhou et al. 1998; Braig et al. 1998) using the following cycling protocol: 60 s at 92 °C and then 35 cycles of 60 s at 92 °C, 60 s at 55 °C and a 90 s extension at 72 °C, with a final extension of 90 s at 72 °C in a MJ Mini™ thermal cycler (Bio-Rad Laboratories, Hercules, California). Each PCR consisted of 33 mL: 2 mL template DNA, 16.5 µl of AmpliTaq Gold PCR Master Mix (PE Applied Bio-systems, Foster City, California), 1.3 mL of each 10 pmol µL−1 primers and 13.9 mL of sterile water. The specificity of the reactions was confirmed by the lack of product in reactions containing DNA template from females of the O. anastrephae A-iso strain or no DNA template.

In order to assess the presence of other bacteria, primers for 16S eubacterial DNA (for (27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1513R 5′- ACGGTTACCCTTGTTACGACTT-3′, ex Weisburg et al. 1991) were used to produce PCR products from the T iso-female DNA template (following Weeks et al. 2003). PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin) then cloned into a pCR®II-TOPO vector utilizing TOP10 competent cells (Invitrogen, Carlsbad, California). Plasmid DNA from 10 colonies derived from each PCR product were prepared using a Zyppy™ Plasmid Miniprep Kit (Zymo Research, Irvine, California) before being sequenced using T7 primer by Macrogen Corporation (Rockville, Maryland). This experiment was then repeated with 10 further colonies each sequenced with both T3 (Macrogen) and T7 primers to obtain longer reads. Wsp sequences were aligned using ClustalW (Thompson et al. 1994) in MEGA4 (Tamura et al. 2007) to check for multiple infections. The resulting nucleotide sequences were queried using BlastN against the nucleotide database from NCBI (http://www.ncbi.nlm.nih.gov/) to identify homologous sequences.

T iso-female wsp and MLST gene sequences were amplified using standard primers and PCR protocols (available at http://www.pubmlst.org/wolbachia/). The Wolbachia strain was then assigned a sequence type (ST), which was the combination of 5 integers corresponding to the allele numbers at the 5 MLST loci (the allelic profile). No amplification products were observed for wsp or MLST from the A-iso females. Strain and host information is available on the MLST database (http://pubmlst.org/wolbachia/).

The phylogenetic relationship between the Wolbachia extracted from T iso-female O. anastrephae- Wolbachia and other Wolbachia strains was examined based on concatenated MLST gene nucleotide sequences. Alignments of the MLST sequences were performed using ClustalW in MEGA4. The phylogenetic associations were constructed in MEGA5, using the Neighbor-Joining method (Saitou & Nei 1987) with Kimura's 2-parameter correction (Kimura 1980) with bootstrapping (1000 replicates) and MLST sequences from the nematode, Brugia malayi (Brug) (Spirurida: Onchoceridae), and Cimex lectularius L. (Hemiptera: Cimicidae) as outgroups.

As per Copeland et al. (2010), we found that based on the wsp sequences the thelytokous and arrhenotokous iso-female O. anastrephae experimental lines were Wolbachia infected and uninfected, respectively. Subsequent generations were tested for infection using the same method and with the same and consistent results. Seven 536 bp nucleotide sequences (GenBank accession numbers JX182373 - JX182379), from clones of the T iso-female wsp PCR product, were identical, suggesting the original specimen used to establish the T iso-female line was infected with a single strain of Wolbachia, wAna. Based on the wsp and MLST sequences from the wAna strain it showed it to be included in Supergroup A.

As determined by comparisons of 16S sequences, no other bacteria strains were identified in O. anastrephae. Thirteen 16S sequences derived from T iso-female template DNA (JX182380 - JX182392) closely matched rRNA nucleotide sequences previously derived from Wolbachia (98 – 100% coverage) when queried with BlastN against the nucleotide database from NCBI (http://www.ncbi.nlm.nih.gov/) to identify homologous sequences.

None of the sequences of the wsp hypervariable regions (HVR) from wAna were different from those sequences previously reported in the Wolbachia MLST database (HVR1 = 11, HVR2 = 9, HVR3 = 15, HVR4 = 25). However, as combined in wAna they presented a novel wsp profile (Wolbachia MLST database id 522, JX182393). The wsp profile was identical to that of the Wolbachia strain wAlu, infecting another thelytokous figitid parasitoid of tephritids, A. alujai (JX182394). However, the MLST profiles for wAna and wAlu were not the same. The novel MLST profile of wAna (Wolbachia MLST database ST 165 = gatB 54, JX182397; coxA 52, JX182395; hcpA 62, JX182399; ftsZ 3, JX182401; fbpA 164, JX182403) contained a unique fbpA isolate (fbpA 164). The wAna MLST profile was essentially the same as that in wAlu (ST 164 = gatB 54, JX182398; coxA 52, JX182396; hcpA 62, JX182400; ftsZ 82, JX182402; fbpA 62, JX182404), but also contained a different ftsZ sequence (ftsZ 82).

A phylogenetic analysis of the concatenated MLST sequences from wAna (ST 165) and wAlu (ST 164) showed them to be closely aligned and within Supergroup A (Fig. 1). ST 23 (Baldo et al. 2006b) from the only other closely related Wolbachia strain in this analysis is known to induce thel-
Fig. 1. Neighbor joining phylogenetic tree constructed using concatenated multi-locus sequence types (ST's) of Wolbachia strains with Kimura’s 2-parameter correction, bootstrap testing (1000 replications) and ST-35 (ex Brugia malayi) and ST-8 (ex Cimex lectularius) as outgroups. Wolbachia Supergroup designations are provided. Numbers next to branches indicate bootstrap values greater than 70% and the tree is drawn to scale (given), with branch length units the same as the evolutionary distances used to infer the phylogeny. ST-165 (ex Odontosema anastrephae) is in large bold text with other novel ST's (ST-164 ex Aganaspis alujai and ST-187 ex Diaphorencyrtus aligarhensis) in large text only.
ytoky in its host, *Muscidifurax uniraptor* Kogan & Legner (Gottlieb & Zhori-Fein 2001, Fig 1).

Theytoky in *O. anastrephae* and *A. alujai* may have been, but was not proven to be, due to the Wolbachia infections detected by molecular means (Copeland et al. 2010). Theytokous females of both *O. anastrephae* and *A. alujai* were infected while bisexual *O. anastrephae* were not. There was no genetic evidence of other endosymbionts that might cause sex-ratio distortion.

The nearly identical Wolbachia infecting *O. anastrephae* and *A. alujai* raises the question of how such similar endosymbionts came to occupy such different parasitoids. To our knowledge, there are no published records of these 2 different wasps at-

tending to the same host fruit or sharing hosts and microhabitats with another parasitoid that could have carried the bacteria and so acted as a bridge and provide a vehicle for the horizontal transfer of Wolbachia between *O. anastrephae* and *A. alujai*. If the similarity of their Wolbachia is due to common descent then there must have been an unusual degree of genomic conservation (stabilizing selection in both bacteria populations) that left the bacteria nearly identical while their hosts diverged to the point of becoming members of different genera.

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**Summary**

Closely related novel Wolbachia strains were recovered from the theytokous figitids, *Odontosema anastrephae* Borgmeier and Aganaspis alujai Ovruski et al. No Wolbachia were detected in a bi-sexual strain of *O. anastrephae*. While the presence or absence of Wolbachia does not demonstrate that Wolbachia is responsible for the lack of males produced by infected females, multilocus sequence typing failed to identify other endosymbionts that might have caused sex-ratio distortions. The phylogenies of insects and their Wolbachia are often not parallel and closely related bacteria infecting apparently more distantly related hosts suggests a horizontal transfer occurred after the divergence of the wasps. Given the figitids present ecology there are few obvious opportunities for transfer through host or habitat-sharing.

**Key Words:** Aganaspis, Odontosema, Anastrepha, Rhagoletis, biological control

**RESUMEN**

Se recuperaron nuevas cepas de Wolbachia estrechamente relacionadas dentro de los figiti-


