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Source: Florida Entomologist, 96(2) : 649-653

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.096.0238

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

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Thelytoky in hymenopteran parasitoids is often the result of infection with intracellular endosymbionts, especially the bacterium *Wolbachia pipientis* (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).

Odontosema 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 larvae deep in the pulp, an ability not shared by other native non-figitid parasitoids of tephritids in Mexico (Aluja et al. 2009). Thelytokous *O. anastrephae* females are morphologically indistinguishable from arrhenotokous females (Copeland et al. 2010). PCR amplification and sequencing of mitochondrial (COI) and nuclear (ITS2) DNA found thelytokous and arrhenotokous laboratory colonies to be genetically distinct, but no more so than often occurs among populations of completely arrhenotokous species (Copeland et al. 2010). *Aganaspis alujai* Ovruski et al. (Figitidae) is a parasitoid of *Rhagoletis* spp. infesting walnuts (*Juglans* spp.; Fagales: Juglandaceae). Only females of this univoltine species have ever been collected (Ovruski et al. 2007).

We here examined both thelytokous and arrhenotokous *O. anastrephae* and *A. alujai* for *Wolbachia* infection using multilocus sequence typing and through assessment of the DNA sequence alignments, then determined the phylogenetic relationships amongst the bacteria identified. Many previous genetic characterizations of *Wolbachia* have been accomplished by analysis of the *wsp* gene, however, it is now known that *wsp* has relatively high levels of recombination and is under strong selection for divergence (Baldo et al. 2006a). Therefore, it may not be as suitable for comparing genetic similarity and phylogenetic reconstructions as multilocus sequence typing (MLST) (Baldo et al. 2006b). All nucleotide sequences reported herein were submitted to GenBank.

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* (Lowe) (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'- TGGTCCAATAAGTGATGAAGAAAC-3' and *wsp* 691R 5'-AAAAATTAAACGCTACTCCA-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 Biosystems, 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'- ACGGYTACCTTGTTACGACTT-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*, *w*Ana. Based on the *wsp* and MLST sequences from the *w*Ana strain 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.nim.nih.gov/) to identify homologous sequences.

None of the sequences of the *wsp* hypervariable regions (HVR) from *w*Ana were different from those sequences previously reported in the *Wolbachia* MLST database (HVR1 = 11, HVR2 = 9, HVR3 = 15, HVR4 = 25). However, as combined in *w*Ana they presented a novel *wsp* profile (*Wolbachia* MLST database id 522, JX182393). The *wsp* profile was identical to that of the *Wolbachia* strain *w*Alu, infecting another thelytokous figitid parasitoid of tephritids, *A. alujai* (JX182394). However, the MLST profiles for *w*Ana and *w*Alu were not the same. The novel MLST profile of *w*Ana (*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 *w*Ana MLST profile was essentially the same as that in *w*Alu (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 *w*Ana (ST 165) and *w*Alu (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-

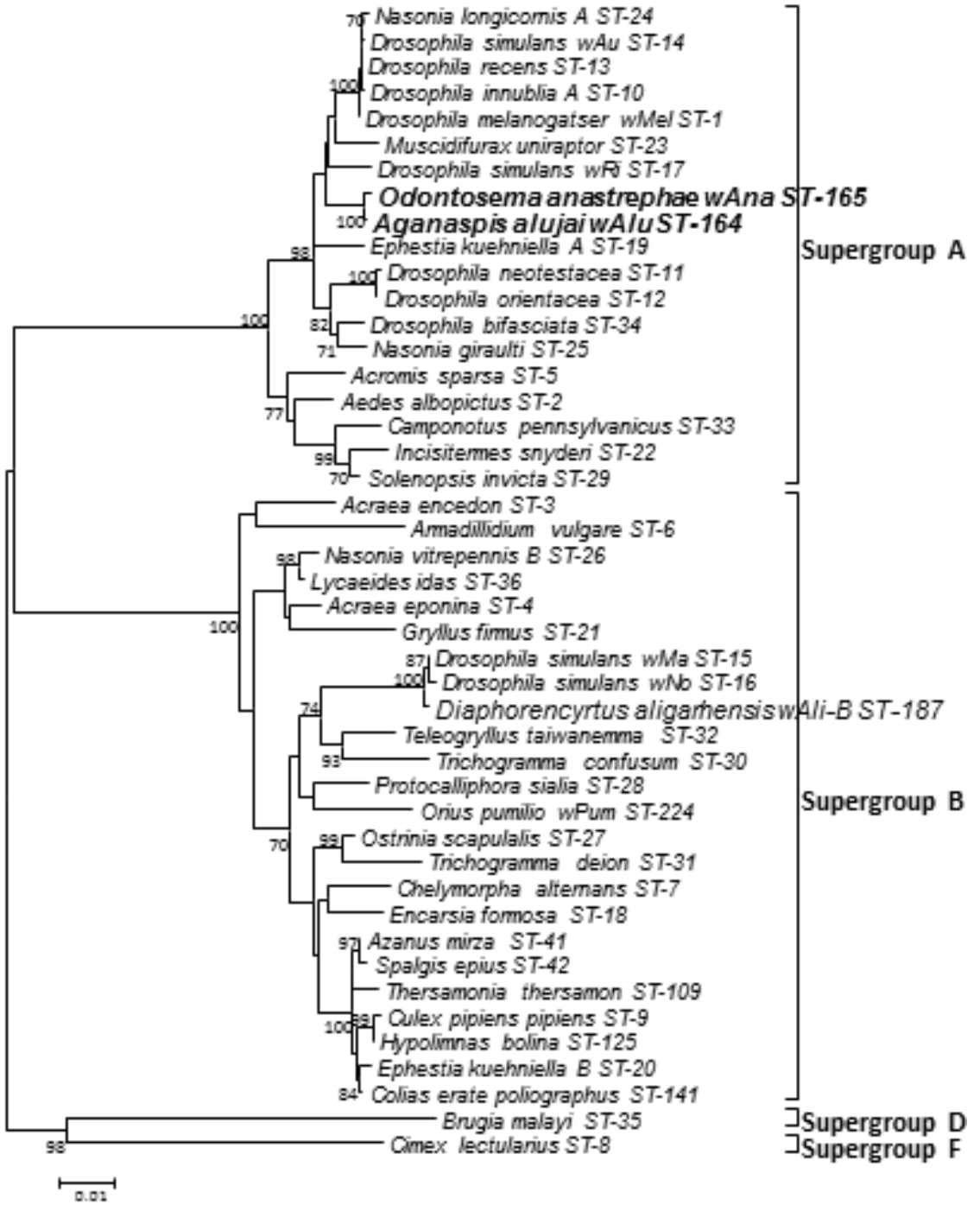

Fig. 1. Neighbor joining phylogenetic tree constructed using concatenated multi-locus sequence types (ST's) of *Wolbachia* strains with Kimura's 2-parameter correction, bootstrap testing (1000 replications) and ST-35 (ex *Brugia malayi*) and ST-8 (ex *Cimex lectularius*) as outgroups. *Wolbachia* Supergroup 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 & Zchori-Fein 2001, Fig 1).

Thelytoky 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). Thelytokous 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 attacking the same tephritids, foraging in 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.

We thank Charlie Stuhl, Heidi Burnside, Marc Schetelig, Nirmala Xavier, Al Handler, Jeff Shapiro, Paul Shirk and Kristie Morris for technical support and in Mexico Larrisa Guillen, Andrea Birke and Juan Rull for supplying parasitoids. This research was funded by a USDA-ARS Postdoctoral Fellowship.

Summary

Closely related novel *Wolbachia* strains were recovered from the thelytokous 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 figitidos telitoquios, *Odontosema anastrephae* Borgmeier y *Aganaspis alujai* Ovruski et al. No se detectó *Wolbachia* en una cepa bi-sexual de *O. anastrephae*. Mientras que la presencia o ausencia de *Wolbachia* no demuestra que *Wolbachia* es responsable por la falta de machos producidos por hembras infectadas, la tipificación de secuencia multilocus no identificó otros endosimbiontes que podrían haber causado las distorsiones en la proporción de sexos. Las filogenias de los insectos y su *Wolbachia* a menudo no son paralelas y bacterias estrechamente relacionadas que infectan hospederos aparentemente más distantes sugiere que la transferencia horizontal sucedió después de la divergencia de las avispas. Teniendo en cuenta la ecología actual de los figitidos, hay pocas oportunidades obvias para su transferencia por medio del hospedero o el hábitat compartido.

Palabras Clave: *Aganaspis, Odontosema, Anastrepha, Rhagoletis,* control biológico

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