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Wolbachia in Two Populations of *Melittobia digitata* Dahms (Hymenoptera: Eulophidae)

Claudia S. Copeland[1], Robert W. Matthews[2], Jorge M. González[3], Martin Aluja[4] and John Sivinski[1]

[1]*USDA/ARS/CMAVE, 1700 SW 23rd Dr., Gainesville, FL 32608, USA; cclaudia@bioinf.uni-leipzig.de, john.sivinski@ars.usda.gov*
[2]*Dept. Entomology, The University of Georgia, Athens, GA 30602, USA; rmatthew@uga.edu*
[3]*Dept. Entomology, Texas A & M University, College Station, TX 77843-2475, USA; jmgonzalez@neo.tamu.edu*
[4]*Instituto de Ecología, A.C., Ap. postal 63, 91000 Xalapa, Veracruz, Mexico; martin.aluja@inecol.edu.mx*

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Wolbachia en Dos Poblaciones de *Melittobia digitata* Dahms (Hymenoptera: Eulophidae)

RESUMEN - Se investigaron dos poblaciones de *Melittobia digitata* Dahms, un parasitoide gregario (principalmente sobre un rango amplio de abejas solitarias, avispas y moscas), en busca de infección por *Wolbachia*. La primera población, provenía de Xalapa, México, y fue originalmente colectada y criada sobre pupas de la Mosca Mexicana de la Fruta, *Anastrepha ludens* Loew (Diptera: Tephritidae). La segunda población, originaria de Athens, Georgia, fue colectada y criada sobre prepupas de avispas de barro, *Trypoxylon politum* Say (Hymenoptera: Crabronidae). Estudios de PCR de la región ITS2 confirmaron que ambas poblaciones del parasitoide pertenecen a la misma especie; lo que nos provee de un perfil molecular taxonómico muy útil debído a que las hembras de las diversas especies de *Melittobia* son superficialmente similares. La amplificación del gen de superficie de proteina (*wsp*) de *Wolbachia* confirmó la presencia de este endosimbionte en ambas poblaciones. La ejecución de la secuencia reveló que *Wolbachia* alojada en ambas poblaciones exibe un *wsp* que pertenece a un subgrupo único (denominado aquí como Dig) dentro del supergrupo B de los genes *wsp* conocidos. Este nuevo subgrupo de wsp podría pertenecer o a un lineaje de *Wolbachia* de los previamente conocidos infectando a *Melittobia* o podría ser el resultado de algún evento recombinante. En cualquier caso, los huéspedes conocidos de *Wolbachia* con un wsp en este subgrupo están relacionados taxonómicamente en forma lejana. Se presentan razones posibles del por qué *Melittobia* – un parasitoide fácil de criar y manipular – es prometedor como un organismo modelo conveniente para el estudio de líneas de *Wolbachia* entre diversos huéspedes.

PALABRAS CLAVE: Parasitoide, chalcidoide, endosimbionte, transmisión horizontal

ABSTRACT - We investigated two populations of *Melittobia digitata* Dahms, a gregarious parasitoid (primarily upon a wide range of solitary bees, wasps, and flies), in search of *Wolbachia* infection. The first population, from Xalapa, Mexico, was originally collected from and reared on Mexican fruit fly pupae, *Anastrepha ludens* Loew (Diptera: Tephritidae); the other, from Athens, Georgia, was collected from and reared on prepupae of mud dauber wasps, *Trypoxylon politum* Say (Hymenoptera: Crabronidae). PCR studies of the ITS2 region corroborated that both parasitoid populations were the same species; this potentially provides a useful molecular taxonomic profile since females of *Melittobia* species are superficially similar. Amplification of the *Wolbachia* surface protein gene (*wsp*) confirmed the presence of this endosymbiont in both populations. Sequencing revealed that the *Wolbachia* harbored in both populations exhibited a *wsp* belonging to a unique subgroup (denoted here as Dig) within the B-supergroup of known *wsp* genes. This new subgroup of *wsp* may either belong to a different strain of *Wolbachia* from those previously found to infect *Melittobia* or may be the result of a recombination event. In either case, known hosts of *Wolbachia* with a wsp of this subgroup are only distantly related taxonomically. Reasons are advanced as to why *Melittobia* – an easily reared and managed parasitoid – holds promise as an instructive model organism of *Wolbachia* infection amenable to the investigation of *Wolbachia* strains among its diverse hosts.

KEY WORDS: Parasitoid, chalcidoid, endosymbiont, horizontal transmission

The cosmopolitan genus *Melittobia* comprises 14 species of small (< 2 mm long) gregariously developing chalcidoid ectoparasitoids that principally attack solitary bees and wasps but also are capable of parasitizing a wide range of other insects, including some flies and beetles (Dahms 1984b; González *et al*. 2004a,c). Every species of *Melittobia* so far studied has exhibited highly biased sex ratios, typically with ~95% female offspring (e.g., Schmieder & Whiting 1946, González & Matthews 2002, Cooperband *et al*. 2003, and many others). Eight species are recorded from the New World (Dahms 1984a, González *et al*. 2004b, Sari *et al*. 2006, Mattheus & Gonzáles 2008). Of these, *M. digitata*, widely distributed in North America, is proving particularly amenable for laboratory study (Matthews *et al*. 1996, 1997) in part because of its robust reproduction upon the blow fly *Neobellieria bullata* (Parker) (Diptera: Sarcophagidae), a non-natural but efficacious laboratory host (Silva-Torres & Matthews 2003).

In recent years, a number of insect endosymbionts have been identified and a handful have become the focus of research scrutiny (for an overview, see Bandi *et al*. 2001). Members of this heterogeneous group appear to share a surprising common feature – being transmitted in egg cytoplasm and finding themselves at a reproductive impasse in males, they have evolved ways of distorting host sex ratios for their own benefit. Their methods include inducing asexual reproduction (thelytokous parthenogenesis), feminizing males produced through sexual reproduction, killing males to enhance the survival of female siblings, and developing cytoplasmic incompatibility, with the result that the sperm of infected males sterilizes uninfected female competitors.

The best known of these "reproductive parasites" is the widespread endobacterium *Wolbachia* (Werren 1997a,b; Majerus 2003), estimated to infect up to 76% of all insect species (Jeyaprakash & Hoy 2000, Werren & Windsor 2000, Haine & Cook 2005). By conservative estimates, this would include one to five million arthropod species (Werren *et al*. 1995), encompassing all the major insect orders as well as such related groups as isopods and mites.

Wolbachia were first reported in mosquitoes almost a century ago (Hertig & Wolbach 1924), but their role in inducing unidirectional sexual incompatibility was not made clear until the early 1970s (Yen & Barr 1971). Despite being a close relative of *Ehrlichia* in the Rickettsiales, insect-infecting *Wolbachia* have been found associated mainly with arthropod reproductive tissues, and there is no evidence that they directly cause disease in vertebrates.

A great deal of variation clearly occurs within *Wolbachia*. However, because most rickettsiae cannot be cultured outside of host cells, traditional microbiological phylogenetic studies have been challenging (Werren 1997a). Furthermore, application of the traditional biological species concept to bacteria has always been difficult, particularly if they do not routinely undergo genetic recombination. Thus, until taxonomic issues have been resolved, most researchers have preferred to use the conservative designation of "strains" for different *Wolbachia* isolates, rather than "species" (Werren 1997a).

The diverse strains of *Wolbachia* found in insects were first classified into two supergroups, A and B, on the basis of *ftsZ*, a bacterial gene involved in regulation of cell division (Werren *et al*. 1995). Three years later, characterization of a new gene (*wsp*) encoding the major surface protein of *Wolbachia pipiens* allowed finer phylogenetic classification of different *Wolbachia* strains and populations (Braig *et al*. 1998). On the basis of *wsp*, twelve subgroups of *Wolbachia* were distinguished within the A and B supergroups (Zhou *et al*. 1998). Additional subgroups have subsequently been recognized; van Meer *et al*. (1999) added seven, Ruang-Areerate *et al*. (2003), another eight. However, though its faster rate of mutation has made it useful for fine discrimination between subgroups, recent discoveries of a high recombination propensity may compromise the value of the *wsp* gene as a tool for larger scale phylogenies (Baldo & Werren 2005, Baldo *et al*. 2006).

In this study, we used PCR to search for *Wolbachia* in two populations of *M. digitata* reared on distinct hosts. Because identification of *Melittobia* at the species level by morphology is fairly straightforward with males, but can be difficult on the basis of females only (Dahms 1984a), we also used PCR information to develop a profile that potentially may help others to identify *M. digitata* females. Finally, we present and discuss the advantages of *Melittobia* as a model organism for endosymbiont infection studies.

Material and Methods

***M. digitata* cultures.** Cultures of *M. digitata* were acquired from two sources. The M1 culture, obtained from Martin Aluja (Instituto de Ecología, A.C., Xalapa, Mexico) was originally collected from and continues to be reared at that location on Mexican fruit fly pupae, *Anastrepha ludens* (Loew). The M2 culture, from Robert W. Matthews (University of Georgia, Dept. of Entomology, Athens, GA) was collected from the prepupae of mud dauber wasps, *Trypoxylon politum* Say from Georgia, and continues to be reared on them at that location.

Purification of genomic DNA. Genomic DNA of ~100 *M. digitata* wasps (~1mg tissue) of each population was ground in 600 µl Puregene Cell Lysis Buffer using microfuge pestles made from melted and molded plastic pipette tips and purified using the Puregene® DNA Purification Kit.

Polymerase chain reactions. *Wolbachia* surface protein (*wsp*) fragments were amplified using a high fidelity polymerase chain reaction (PCR) protocol (Jeyaprakash & Hoy 2000) and MJ Mini thermocycler (Bio-Rad). Instead of a single polymerase, a 5:1 mixture of Taq:Tgo polymerase was employed, with a buffer consisting of (10X) 50mM Tris, 16mM ammonium sulfate, 1.75mM $MgCl_2$. Primers for *wsp* (based on those used by Braig *et al*. 1998) had the following sequences: (fwd) 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and (rev) 5'-AAAAATTAAACGCTACTCCA-3'. Primers for ITS2 (based on those of Porter & Collins 1991) had the following sequences: (fwd) 5'-GTGAATTCT GTGAACTGCAGGACACATGAAC-3' and (rev) 5'-ATGCTTAAATTTAGGGGGTA-3'. Five pmol of each primer were added to a 25 µl reaction volume containing

1 µl purified genomic DNA solution, 700 µM dNTPs, 2.5 µl 10x buffer (described above), and 1 µl Taq/Tgo polymerase mix (1U Taq polymerase, 0.2U Tgo polymerase) (Bio-Rad Laboratories, Inc., Hercules, CA; Roche Molecular Biochemicals, Indianapolis, IN). Cycling conditions for both *wsp* and Internal Transcribed Spacer-2 (ITS2) amplifications were as follows. Initial denaturation at 94ºC for 5 min, followed by 10 cycles of 94º C for 10 sec, 56º C for 30 sec, and 68ºC for 2 min, followed by 20 cycles of 94ºC for 10 sec, 56ºC for 20 sec, 68ºC for 2 min + 20 sec/cycle. The PCR setups were carried out in a "PCR clean area", with the bench area, micropipettes, and gloves cleaned with DNAse Away before each reaction setup, and only filter tips were used for pipetting. Negative controls with identical reaction conditions except for the substitution of ddH_2O for genomic DNA were run with each experimental amplification. The negative controls showed no evidence of contamination.

Cloning, sequencing, and sequence analysis. Using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions, PCR products were purified, then cloned into the pCR®II-TOPO vector utilizing TOP10 chemically competent cells (Invitrogen). Plasmid DNA from cells thought to contain inserts was prepared using either the QIAprep Spin Miniprep Kit (Qiagen) or the GenElute HP Plasmid Miniprep Kit (Sigma). After confirmation of the presence of inserts by restriction digest, plasmids were sequenced by NorthWoods DNA sequencing (Solway, MN). Both strands of the inserts were sequenced and sequencing was repeated for each fragment, resulting in four-fold coverage. Sequences were determined from chromatograms using SeqMan software (DNAstar, Madison, WI). GenBank accession numbers for the sequences determined in this study are as follows: *wsp*: DQ487096, EF564624; ITS2: DQ487099, EF580926.

Phylogenetic analysis. Sequences from this study and comparison nucleic acid sequences from GenBank were aligned using Clustal X software (Thompson *et al*. 1997). Alignments were then used to create a bootstrapped tree according to the neighbor-joining algorithm using Clustal X and njplot software (Saitou & Nei 1987). Several positions within the only public sequence for the ITS2 region of *M. digitata* (Acc. U02950) contained "N's" or unknown nucleotides. These positions (for all sequences) were excluded from the analysis. Bootstrap values above 700 (out of 1,000 repetitions) are shown in each phylogenetic tree. In addition, a maximum likelihood analysis was also carried out, which resulted in the same position for the *M. digitata* sequences (not shown).

Results and Discussion

ITS2 sequences from female *M. digitata*. The two strains of parasitoid were determined by male morphology to be *M. digitata*. In addition, the ITS2 ribosomal region amplified from each strain was compared with the only other *M. digitata* ITS2 sequence in the database (Accession U02950, Campbell *et al*. 1993). The M1 and M2 *M. digitata* ITS2 sequences clustered closely with each other, and with the reference sequence (Fig. 1). Because ITS2 is a multi-copy genetic region, slight differences in sequence are to be expected in the same species (Wesson *et al*. 1992, Harris & Crandall 2000, Alvarez & Hoy 2002). The ITS2 sequences for the M1 and M2 *M. digitata* populations varied by 1.04%, a degree of variation similar to the 1.17% intraspecies variation found by Wesson *et al*. (1992) for *Aedes* mosquitoes.

Variation between these populations and the U02950 *M. digitata* sequence was greater: 3.59% for M1 and 2.84% for M2; however, this level of difference was similar to that found in a study of *Ageniaspis* wasps, where sequence variability between individuals ranged from 3.7% to 6.6% (Alvarez & Hoy 2002). The majority of the variation between ITS2 regions in the *Melittobia* sequences was found in a microsatellite region, and microsatellite regions within ITS sequences have been shown to exhibit exceptionally high levels of intragenomic variation (Harris & Crandall 2000).

Taking into account intragenomic and intraspecies

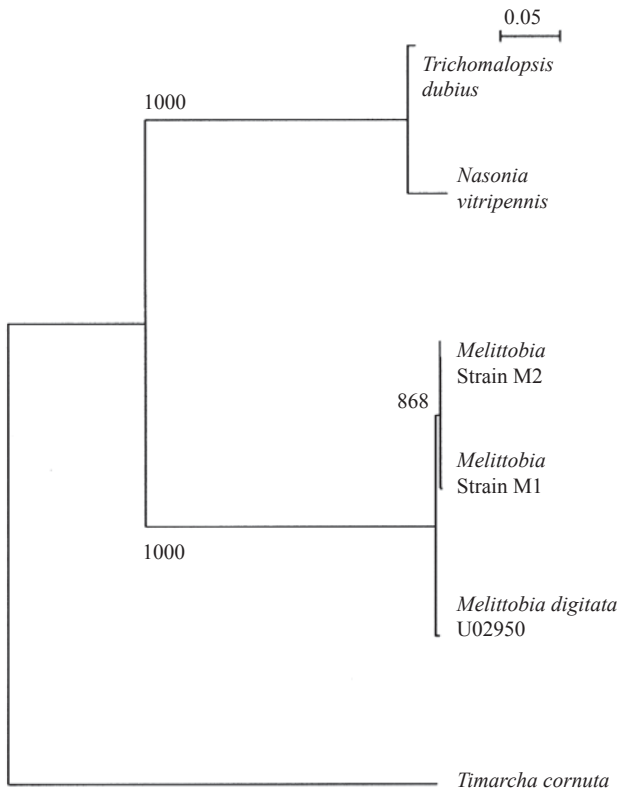

Fig. 1. Phylogenetic tree of ITS2 sequences from the *M. digitata* strains investigated in this study (M1: Xalapa, Mexico; M2: Athens, GA) and four other sequences from GenBank. The four comparison ITS2 sequences were *M. digitata* (Acc. U02950, the only other complete *M. digitata* ITS2 sequence in the GenBank database [Campbell *et al*. 1993]), *Nasonia vitripennis* (Walker) (Acc. U02960) and *Trichomalopsis dubius* (Hymenoptera: Pteromalidae) Ashmead (Acc. U02961) (Campbell *et al*. 1993), and, as outgroup, *Timarcha cornuta* Bechyné (Coleoptera: Chrysomelidae) (Acc. AJ512608) (Gomez-Zurita & Vogler 2003). Bootstrap values above 700 (out of 1,000 replicates) are depicted.

variability between ITS2 copies, the sequence data for these *Melittobia* samples show promise for use of these primers as identification aids. However, future research is needed to determine sequence variability derived from numerous natural populations of *M. digitata*, as well as data from other *Melittobia* species. Hopefully, such studies will show that PCR based on ITS2 can be a worthwhile tool to add to the options provided by other molecular methods such as RAPD, which has been shown to be effective in distinguishing *M. australica* Girault from *M. hawaiiensis* Perkins (Sari *et al*. 2006). Unlike the RAPD technique, PCR comparison to previously characterized sequences such as the ones introduced here have the potential to identify a single sample, rather than simply determining whether two samples represent the same or different species.

***Wolbachia*.** When the two populations of *M. digitata* were examined for the presence of *Wolbachia* endosymbionts by PCR, both produced robust bands of ~600bp in length. Cloning and sequencing of these bands revealed a gene encoding a surface antigen protein (pfam 01617 of the NCBI conserved domains database) with homology to the surface protein of *Wolbachia* isolated from diverse arthropods The amplified fragments were confirmed to encode the *Wolbachia* surface protein through conceptual translation of their DNA sequences and comparison of the translation to closely related *Wolbachia* surface protein sequences. Identical amino acid sequences were obtained for both populations.

Based on phylogenetic analysis (Fig. 2), *Wolbachia* from both populations appear to belong to the B supergroup. In terms of the subgroups classified by Zhou *et al*. (1998) and others, they appear to be closely related to the Spe, Btom1, and Diacir2 *Wolbachia*, but form a separate branch with high bootstrap values, indicating that, along with *Wolbachia* from *By. ochraceus*, they may represent an independent subgroup within the B supergroup. A BLAST analysis and literature search focusing on the branch of B supergroup of *Wolbachia* giving rise to the Ori, For, and Spe subgroups uncovered additional *wsp* sequences that represented *Wolbachia* from this branch of the B supergroup, some previously classified by subgroup, some unclassified. Due to the relatively small size of some of the comparison sequences, the sequence data for this alignment were ~100bp shorter than for the M1 and M2 sequences. In spite of this, bootstrap values were robust and *wsp* sequences segregated into clear subgroups, all but one of which had been previously defined (Zhou *et al*. 1998, van Meer *et al*. 1999, Kittayapong *et al*. 2003, Malloch & Fenton 2005). Note that although Kittayapong *et al*. (1999) assigned unique strain names to the *Wolbachia* found in *Nilaparvata bakeri* (Muir) (Acc. AF481180) and *Nilaparvata lugen* (Stål.) (Homoptera: Delphacidae) (Acc. AF451181), the *Wolbachia* found in these two species are clearly not from distinct strains, and can be grouped within the For subgroup defined by van Meer *et al*. (1999). The three strains are over 98% similar, fulfilling the criterion of Zhou *et al*. (1998) for inclusion in a single subgroup.

The results shown in Fig. 2 suggest that the *Wolbachia* strains found in *M. digitata* M1 and M2, along with *Wolbachia* found in the fig wasp *Blastophaga psenes* (L.) (Hymenoptera: Agaonidae) (Acc. AY567560) Haine & Cook 2005) and the beetle *Byturus ochraceus* (Scriba) (Coleoptera: Byturidae) (Acc. AJ585379) (Malloch & Fenton 2005), are not members of the Spe, Btom1, and Diacir2 subgroups. Instead, the *Wolbachia* from the three hymenopterans (and possibly also that from *By. ochraceus*) appear to form a separate, previously undescribed subgroup, which we have named "Dig" to reflect the root of its classification in *M. digitata*. The Dig subgroup, along with the closely related Spe, Btom1, and Diacir2 subgroups, appears to form a distinct lineage within the B supergroup *Wolbachia*, separate from the Ori, For, and other B subgroups, and with greater host diversity than the others.

When we did a simple nucleotide sequence percentage analysis, the M1 and M2 nucleotide sequences differed from the Spe sequences by over 3.5% and differed from the Btom1 sequences by over 5%. M1 and M2 were also 98.2% similar to the *wsp* from *By. ochraceus* and 99.8% similar to the *wsp* from *B. psenes*. Based on the criteria of Zhou *et al*. (1998) that subgroup members should be at least 97.5% similar to each other and at least 2.5% different from members of other subgroups, the two new *Wolbachia* populations found in *M. digitata*, together with the *Wolbachia* from *B. psenes* and *By. ochraceus*, would appear to represent a new subgroup of B-supergroup *Wolbachia*.

Three other studies of *Wolbachia* in *Melittobia* have been published. Based on sequencing of the *ftsZ* gene, an unidentified species of *Melittobia* was found to harbor *Wolbachia* (Werren *et al*. 1995) belonging to the A supergroup. Fialho & Stevens (1997) also found *Wolbachia* from *M. digitata* to fall only within supergroup A. Recently, *Wolbachia* has also been reported from *M. australica* (Abe *et al*. 2003) but no supergroup was assigned. Thus, it appears that different populations of *Melittobia* might be capable of harboring phylogenetically distinct populations of *Wolbachia*, suggesting that these infections might have occurred relatively recently in evolutionary time. However, recent studies (e.g., Baldo *et al*. 2005, 2006) have uncovered significant recombination in many *Wolbachia* strains and shown it to be extensive in the *wsp* region. Furthermore, in byturid beetles, evidence of genetic transfer between A and B supergroups has also been found (Malloch & Fenton 2005). Further studies will be needed to discern whether the Dig subgroup represents a different strain from the previously identified A supergroup *Wolbachia*, or the presence of a unique *wsp* gene sequence within these previously discovered A supergroup *Wolbachia*, acquired through recombination; our study was not designed to distinguish between these possibilities.

Whereas the phylogeny of the mutualistic C and D supergroup *Wolbachia* found in nematodes generally follows host phylogeny, suggesting stable infection maintained by vertical transmission, the phylogeny of the A and B supergroup *Wolbachia* found in arthropods generally is not congruent with that of their hosts (Bandi *et al*. 2001). This suggests that the A/B supergroup may have a different mode of transmission than the C/D supergroup. Incongruence between host and symbiont phylogeny is one generally accepted indication of horizontal transmission, which can occur between the most intimate symbionts, even nuclear genetic elements (e.g., Copeland *et al*. 2005).

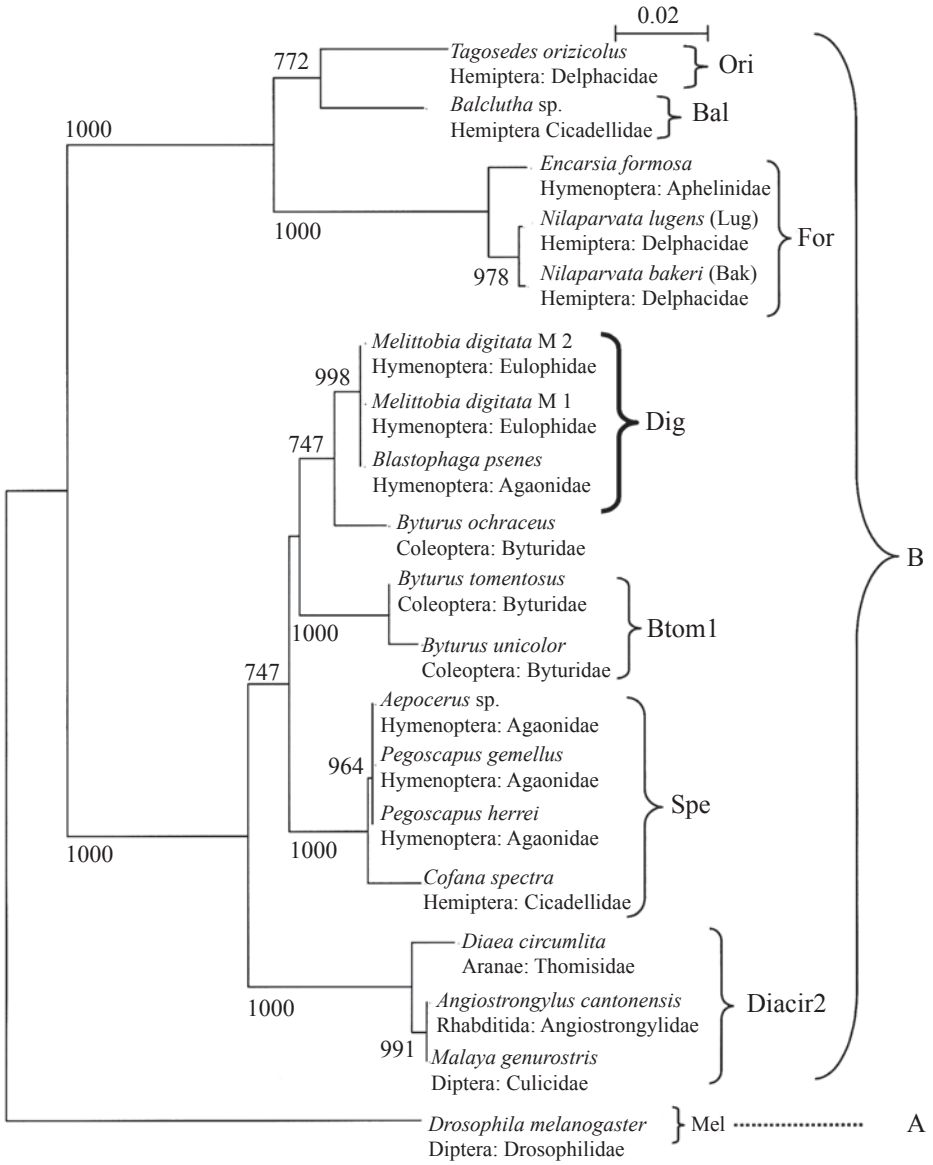

Fig. 2. Phylogenetic tree of *wsp* sequences from the *M. digitata* strains investigated in this study (M1: Xalapa, Mexico; M2: Athens, GA) and 16 closely related B supergroup strains, rooted with a Mel subgroup (A supergroup) (Acc. AF020065) (Zhou *et al*. 1998) *wsp* sequence as outgroup. Bootstrap values above 700 (out of 1,000 replicates) are depicted. Size bar reflects phylogenetic divergence in genetic distance units. Accession numbers are as follows: *Balclutha* sp.: AF481172; *Byturus tomentosus* (Degeer): AJ585376; *Byturus unicolor* Say: AJ585378; *Angiostrongylus cantonensis* (Chen): AY508981; *Diaea circumlita* (Likoch): AY486092; *Malaya genurostris* Leicester: AY462865; *B. psenes*: AY567560; *By. ochraceus*: AJ585379; *M. digitata-WM1*: DQ487096; *M. digitata-WM2*: EF564624; *Encarsia formosa* Gahan: AF071918; *N. bakeri*: AF481180; *N. lugens*: AF481181; *Drosophila melanogaster* Meigen: AF020065; *Tagosedes orizicolus* (Muir): AF020085; *Aepocerus* sp.: AF521149; *Cofana spectra* (Distant): AF481173; *Pegoscapus gemellus* (Wiebes): AF521152; *Pegoscapus herrei* Wiebes: AF521150.

The observation that the *M. digitata* sequences in this study are members of a phylogenetic branch including highly diverse host species also raises the possibility that this lineage of *Wolbachia* may be particularly suited to transfer horizontally from one species to another. The host species within the Dig subgroup, *M. digitata*, *B. psenes*, and (possibly) *By. ochraceus*, are much less closely related than their *Wolbachia* endosymbionts (Fig. 2).

Even more striking, though, is the diversity of hosts of *Wolbachia* from the three subgroups clustering together with Dig: Btom1, Spe, and Diacir2. The hosts of Diacir2 are especially phylogenetically distant (arachnid, dipteran insect, and parasitic nematode). Though differences in phenotypic reproductive effects on host species have not been found to correlate with *Wolbachia* strain phylogeny (van Meer *et al*. 1999), it is plausible that some *wsp* subgroups of *Wolbachia* transfer more easily than others, since the wsp gene is an outer surface protein that interacts with the host environment. It

would be useful to know whether the hosts were themselves infected with *Wolbachia* prior to being parasitized; however, because *Melittobia* is an ectoparasitoid that lays its eggs on the naked host's cuticle and its young entirely consume the host, it would be difficult to determine the host's *Wolbachia* status without rendering it unsuitable for the parasitoid.

Parasitoids as vectors of endosymbiont transfer. If in fact *M. digitata* harbors *Wolbachia* endosymbionts that are highly similar to those found in the phylogenetically distant fig wasp *B. psenes* and significantly similar to those from the beetle *By. ochraceus* (Fig. 2), this raises the possibility that *M. digitata* or an ancestor might have acted as a vector of endosymbiont transfer between arthropod hosts. Significantly, *Wolbachia* infection appears to be much higher among parasitoid Hymenoptera than in the general insect population, and parasitoids (and chalcidoids specifically) have been proposed as plausible candidates for the role of such "vectors" for a variety of insect hosts (Werren *et al*. 1995, Noda *et al*. 2001, Rokas *et al*. 2002, Kikuchi & Fukatsu 2003). Malloch & Fenton (2005) suspected parasitoids as possible *Wolbachia* vectors leading to the infection of *By. ochraceus*, and in fact, a recent study (Hanni & Luik 2006) indicates byturids can be highly parasitized in the wild. That study did not identify the parasites, but other coleopterans can be parasitized by *Melittobia* (Dahms 1984b, Thompson & Parker 1927).

Like *Wolbachia* itself, *Melittobia* is a generalist upon a wide variety of species in several insect orders. In addition, its life style brings it into touch with a broad range of other arthropods, for the host nests of these ubiquitous parasitoids provide a teeming community comprised of parasites, predators, commensals, and incidental visitors and occupants (Matthews 1997). *Melittobia*'s mode of contact with these hosts – which is similar not only to parasitism but also to a predatory relationship – is probably quite representative of the types of horizontal encounters that may have generally served to spread *Wolbachia* across the insect world.

On many occasions, we have documented multiple ovipositions, both conspecific and heterospecific, by *Melittobia* species both upon their primary hosts and upon other host nest cohabitants as well as upon hosts not normally attacked in nature (see Deyrup *et al*. 2003). Molumby (reviewed in Matthews *et al*. 2005) regularly found superparasitism of field collected *T. politum* hosts by up to five (ave. = 1.83) females of *M. femorata* Dahms, and we have recorded females of two or three different species simultaneously attacking the same host in the field (Matthews & Deyrup 2007). *Melittobia* larvae also are freely cannibalistic, both upon their own kind (Cônsoli & Vinson 2002) and upon eggs and larvae of the host's other natural enemies (e.g., González *et al*. 2005). Thus, both multiple ovipositing foundress females and cannibalism would offer significant avenues of opportunity for infection and genetic exchange.

As a potential *Wolbachia* vector model, *Melittobia* wasps also offer many practical advantages. *Melittobia* will readily mate under laboratory conditions and oviposit upon a variety of hosts (for an example, see Table 1 in González *et al*. 2004b). Their life cycle (about 25 days at 26ºC) is short, and gregarious development yields up to several hundred individuals per host. They are commercially available (Carolina Biological Supply, Burlington NC) at modest cost, as are their laboratory hosts, *N.. bullata* (several sources). Additionally, because *Melittobia* are ectoparasitoids, obtaining immature forms does not require dissecting a host; instead, they can simply be removed with a fine brush.

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References

Abe, J., Y. Kamimura, N. Kondo & M. Shimada. 2003. Extremely female-biased sex ratio and lethal male-male combat in a parasitoid wasp, *Melittobia australica* (Eulophidae). Behav. Ecol. 14: 34-39.

Alvarez, J.M. & M.A. Hoy. 2002. Evaluation of the ribosomal ITS2 DNA sequences in separating closely related populations of the parasitoid *Ageniaspis* (Hymenoptera: Encyrtidae). Ann. Entomol. Soc. Am. 95: 250-256.

Baldo, L., N. Lo & J.H. Werren. 2005. Mosaic nature of *wsp* (*Wolbachia* surface protein). J. Bacteriol. 187: 5406-5418.

Baldo, L., S. Bordenstein, J.J. Wernegreen & J.H. Werren. 2006. Widespread recombination throughout *Wolbachia* genomes. Mol. Biol. Evol. 23: 437-449.

Bandi, C., A.M. Dunn, G.D. Hurst & T. Rigaud. 2001. Inherited microorganisms, sex-specific virulence and reproductive parasitism. Trends Parasitol. 17: 88-94.

Braig, H.R., W. Zhou, S.L. Dobson & S.L. O'Neill. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J. Bacteriol. 180: 2373-2378.

Campbell, B.C., J.D. Steffen-Campbell & J.H. Werren. 1993. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. Insect Mol. Biol. 2: 225-237.

Cônsoli, F.L. & S.B. Vinson. 2002. Larval development and feeding behavior of the wing dimorphics of *Melittobia digitata* Dahms

(Hymenoptera: Eulophidae). J. Hym. Res. 11: 188-196.

Cook, J.M. & R.D.J. Butcher. 1999. The transmission and effects of *Wolbachia* bacteria in parasitoids. Res. Popul. Ecol. 41: 15-28.

Cooperband, M.F., R.W. Matthews & S.B. Vinson. 2003. Factors affecting the reproductive biology of *Melittobia digitata* and failure to meet the sex ratio predictions of Hamilton's local mate competition theory. Entomol. Exp. Appl. 109: 1-12.

Copeland, C.S., V.H. Mann, M.E. Morales, B.H. Kalinna & P.J. Brindley. 2005. The *Sinbad* retrotransposon from the genome of the human blood fluke, *Schistosoma mansoni*, and the distribution of related *Pao*-like elements. B.M.C. Evol. Biol. 5: 20.

Dahms, E.C. 1984a. Revision of the genus *Melittobia* (Chalcidoidea: Eulophidae) with the description of seven new species. Mem. Queensland Mus. 21: 271-336.

Dahms, E.C. 1984b. A review of the biology of species in the genus *Melittobia* (Hymenoptera: Eulophidae) with interpretations and additions using observations on *Melittobia australica*. Mem. Queensland Mus. 21: 337-360.

Deyrup, L.D., M. Deyrup & R.W. Matthews. 2003. Paralyzation and developmental delay of a factitious host by *Melittobia digitata* (Hymenoptera: Eulophidae). J. Entomol. Sci. 38: 533-535.

Fialho, R.F. & L. Stevens. 1997. Molecular evidence for single *Wolbachia* infections among geographic strains of the flour beetle *Tribolium confusum*. Proc. R. Soc. Lond. B 264: 1065-1068.

González, J.M., J. Abe, & R.W. Matthews. 2004c. Offspring production and development in the parasitoid wasp *Melittobia clavicornis* (Cameron) (Hymenoptera: Eulophidae) from Japan. Entomol. Science 7:15-19.

González J.M., J.A. Genaro & R.W. Matthews. 2004a. Species of *Melittobia* (Hymenoptera: Eulophidae) established in Bahamas, Costa Rica, Cuba, Hispaniola, Puerto Rico, and Trinidad. Fla. Entomol. 87: 619-620.

González, J.M., J.B.Terán & R.W. Matthews. 2004b. Biology of *Melittobia acasta* (Hymenoptera: Eulophidae). Carib. J. Sci. 40: 52-61.

González, J.M. & R.W. Matthews. 2002. Development and sex ratio of *Melittobia australica* and *M. digitata* (Hymenoptera: Eulophidae) on *M. rotundata* (Hymenoptera: Megachilidae) and *Trypoxylon politum* (Hymenoptera: Sphecidae). Great Lakes Entomol. 35: 85-91.

González, J.M. & R.W. Matthews. 2005. An annotated bibliography of *Melittobia* (Hymenoptera: Eulophidae) Caribbean J. Sci., Special Pub. 8: 1-48.

González, J.M., R.W. Matthews & J.R. Matthews. 2005. Comparative development and competitive ability of *Dibrachys pelos* Grissell (Hymenoptera: Pteromalidae) on various potential hosts. Fla. Entomol. 88: 49-54.

Guinan, J.A., C.S. Beck, L.S. Bloomer & R.W. Matthews. 2004. Competition within and between species of parasitoid wasps. In M.A. O'Donnell (ed.), Tested studies for laboratory teaching, vol. 26. Proceedings, 26th Workshop/Conference, Association for Biological Laboratory Education (ABLE), June 8-12, 2004, p. 213-232.

Haine, E.R. & J.M. Cook. 2005. Convergent incidences of *Wolbachia* infection in fig wasp communities from two continents. Proc. R. Soc. London B 272: 421-429.

Hanni, L. & A. Luik. 2006. Parasitism of raspberry beetle (*Byturus tomentosus* F.) larvae in different cropping techniques of red raspberry. Agron. Res. 4: 187-190.

Harris, D.J. & K.A. Crandall. 2000. Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): Implications for phylogenetic and microsatellite studies. Mol. Biol. Evol. 17: 284-291.

Hertig, M. & S.B. Wolbach. 1924. Studies on *Rickettsia*-like microorganisms in insect. J. Med. Res. 44: 329-374.

Jeyaprakash, A. & M.A. Hoy. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9: 393-405.

Kikuchi, Y. & T. Fukatsu. 2003. Diversity of *Wolbachia* endosymbionts in heteropteran bugs. Appl. Environ. Microbiol. 69: 6082-6090.

Kittayapong, P., W. Jamnongluk, A. Thipaksorn, J.R. Milne & C. Sindhusake. 2003. *Wolbachia* infection complexity among insects in the tropical rice-field community. Mol. Ecol. 12: 1049-1060.

Majerus, M.E.N. 2003. Sex wars: Genes, bacteria, and biased sex ratios. Princeton, Princeton University Press, 280p.

Malloch, G. & B. Fenton. 2005. Super-infections of *Wolbachia* in byturid beetles and evidence for genetic transfer between A and B super-groups of *Wolbachia*. Mol. Ecol. 14: 627-637.

Matthews, R.W. 1997. Teaching ecological interactions with mud dauber nests. Am. Biol. Teach. 59: 152-158.

Matthews, R. W. & J. M. González. 2008. The *acasta* conundrum: Polymorphism and synonymy in the parasitoid genus *Melittobia* (Hymenoptera: Eulophidae). Zootaxa 1854: 45-54.

Matthews, R. W. & L.D. Deyrup. 2007. Female fighting and host competition among four sympatric species of *Melittobia* (Hymenoptera: Eulophidae). Great Lakes Entomol. 40: 52-62.

Matthews, R.W., L.D. Deyrup & J.M. González. 2005. Increased male sex ratio among brachypterous progeny in *Melittobia femorata*, a sib-mating parasitoid wasp (Hymenoptera: Eulophidae). Insect Sci. 12: 443-448.

Matthews, R.W., L.R. Flage & J.R. Matthews. 1997. Insects as teaching tools in primary and secondary eduction. Annu. Rev. Entomol. 42: 269-289.

Matthews, R.W., T.R. Koballa, L.R. Flage & E.J. Pyle. 1996. Wowbugs: New life for life science. Athens, Riverview Press, 318p.

Noda, H., T. Miyoshi, Q Zhang, K. Watanabe, K. Deng & S. Hoshizaki. 2001. *Wolbachia* infection shared among

planthoppers (Homoptera: Delphacidae) and their endoparasite (Strepsiptera: Elenchidae): A probable case of interspecies transmission. Mol. Ecol. 10: 2101-2106.

Porter, C.H. & F.H. Collins. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera:Culicidae). Am. J. Trop. Med. Hyg. 45: 271-279.

Rigaud, T. & P. Juchault. 1995. Success and failure of horizontal transfers of feminizing *Wolbachia* endosymbionts in woodlice. J. Evol. Biol. 8: 249-255.

Rokas, A., R.J. Atkinson, J.L. Nieves-Aldrey, S.A. West & G.N. Stone. 2002. The incidence and diversity of *Wolbachia* in gallwasps (Hymenoptera;Cynipidae) on oak. Mol. Ecol. 11: 1815-1829

Ruang-Areerate, T., P. Kittayapong, V. Baimai & S.L. O'Neill. 2003. Molecular phylogeny of *Wolbachia* endosymbionts in Southeast Asian mosquitoes (Diptera: Culicidae) based on *wsp* gene sequences. J. Med. Entomol. 40: 1-5.

Saitou, N. & M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-25.

Sari, E.H.R., M.G. Tavares & L.A.O. Campos. 2006. RAPD variation within and among species of *Melittobia* Westwood (Hymenoptera: Eulophidae). Neotrop. Entomol. 35: 201-205.

Schmieder, R.G. & P.W. Whiting. 1946. Reproductive economy in the chalcidoid wasp *Melittobia*. Genetics 32: 29-37.

Silva-Torres, S.A. & R.W. Matthews. 2003. Development of *Melittobia australica* Girault and *M. digitata* Dahms (Parker) (Hymenoptera: Eulophidae) parasitizing *Neobellieria bullata* (Parker) (Diptera: Sarcophagidae) puparia. Neotrop. Entomol. 32: 645-651.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin & D.G. Higgins. 1997. The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876-4882.

Thompson, W.R. & H.L. Parker. 1927. The problems of host relations with special reference to entomophagous parasites. Parasitology 19: 1-34.

van Meer, M.M.M., J. Witteveldt & R. Stouthamer. 1999. Phylogeny of the arthropod endosymbiont *Wolbachia* based on the *wsp* gene. Insect Mol. Biol. 8: 399-408.

Werren, J.H. 1997a. Biology of *Wolbachia*. Annu. Rev. Entomol. 42: 587-609.

Werren, J.H. 1997b. *Wolbachia* run amok. Proc. Nat. Acad. Sci. USA 90: 11154-11155.

Werren, J.H. & D.M. Windsor. 2000. *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? Proc. Biol. Sci. 267: 1277-1285.

Werren, J.H., W. Zhang & L.R. Guo.1995. Evolution and phylogeny of Wolbachia: Reproductive parasites of arthropods. Proc. Biol. Sci. 261: 55-63.

Wesson, D.M., C.H. Porter & F.H. Collins. 1992. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). Mol. Phylogenet. Evol. 1: 253-269.

Yen, Y.H. & A.R. Barr. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens*. Nature 232: 657-658.

Zhou, W., F. Rousset & S. O'Neil. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. Biol. Sci. 265: 509-515.

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