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

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**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 pupas 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 debido a que las hembras de las diversas especies de *Melittobia* son superficialmente similares. La amplificación del gen de superficie de proteína (*wsp*) de *Wolbachia* confirmó la presencia de este endosimbionte en ambas poblaciones. La ejecución de la secuencia reveló que *Wolbachia* alojada en ambas poblaciones exhibe 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 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* de este subgrupo están relacionados taxonomicamente de manera 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 & González 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 *Neobelliera 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 mosquitos 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 *fisZ*, 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* *pipingens* 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 TaqTgo polymerase was employed, with a buffer consisting of (10X) 50mM Tris, 16mM ammonium sulfate, 1.75mM MgCl₂, 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'-TGGATAATTCTGTGAACTGACGGACACATGAC-3' and (rev) 5'-ATGCTTTAAATTTAGGGGTATA-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 94°C for 5 min, followed by 10 cycles of 94°C for 10 sec, 56°C for 30 sec, and 68°C for 2 min, followed by 20 cycles of 94°C for 10 sec, 56°C for 20 sec, 68°C 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 ddH₂O 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
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. hawaiensis 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 bakeni (Muir) (Acc. AF481180) and Nilaparvata lugens (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 fisZ gene, an unidentified species of Melittobia was found to harbor Wolbachia (Warren 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 byturdid 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).
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) *B. 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 chalcidoïds 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 cycle 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 (Cónsoli & Vinson 2002) and upon eggs and larvae of the host’s other natural enemies (e.g., González et al. 2005). Thus, both multiple ovipositing founding 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 26°C) 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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