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## GENETIC CHARACTERISTICS OF BISEXUAL AND FEMALE-ONLY POPULATIONS OF *ODONTOSEMA ANASTREPHAE* (HYMENOPTERA: FIGITIDAE)

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### ABSTRACT

*Odontosema anastrephae* Borgmeier is a figitid parasitoid of *Anastrepha* fruit fly larvae infesting fallen fruit. It is of potential use in biological control as a complement to parasitoids that attack larvae infesting fruit still on the tree and to parasitoids that can only oviposit into larvae near the surface of the fruit, because *Odontosema* pursues larvae deep within the pulp. A newly discovered Mexican all-female (presumably thelytokous) population, provisionally referred to here as *O. near anastrephae*, appears to be morphologically indistinguishable from arrhenotokous individuals. Thelytokous reproduction can potentially lower costs in mass rearing facilities and increase parasitoid efficacy in the field. PCR amplification and sequencing of mitochondrial (*COI*) and nuclear (*ITS2*) genetic sequences suggested that these populations are genetically distinct, but no more so than often occurs among distinct populations within recognized species. In addition to the description of an all-female population of *Odontosema*, this study presents the first genetic sequence data for members of the genus *Odontosema*, enabling phylogenetic comparison between *Odontosema* and other figitid genera and the development of methods for the identification of *Odontosema* species by PCR. The implications of thelytoky for a cladistic definition of speciation, especially for newly diverging populations such as these, as well as the potential practical implications of our findings for fruit fly biological control, are discussed.

Key Words: parthenogenesis, parasitoid, biological control, *Anastrepha*, *albinerve*

### RESUMEN

*Odontosema anastrephae* Borgmeier es un parasitoide figitido de las larvas de la mosca de la fruta del género *Anastrepha* que infestan frutas caídas. Esto es de un uso potencial en el control biológico como complemento a los parasitoides que atacan larvas que infestan frutas que están todavía en el árbol y a los parasitoides que solamente pueden ovipositar larvas cerca de la superficie de la fruta, esto a que *Odontosema* persigue las larvas que están profundamente dentro la pulpa. Una población mexicana recién descubierta de solo hembras (presumiblemente se reproducen por telitoquia), provisionalmente referida aquí como *O. cerca anastrephae*, parece que es morfológicamente indistinguible de individuos que se reproducen por arrenotoquia. La reproducción por telitoquia puede potencialmente bajar los costos de las facilidades de la cría masiva y aumentar la eficacia del parasitoide en el campo. La amplificación de secuencias genéticas por RCP y la secuenciación de mitocondria (*COI*) y nuclear (*ITS2*) indica que estas poblaciones son genéticamente distintas, pero no más de lo que a menudo sucede entre poblaciones distintas en especies reconocidas. En adición de la descripción de una población de sola hembras de *Odontosema*, este estudio presenta los primeros datos de la secuencia genética para miembros del género *Odontosema*, y permite una comparación filogenética entre *Odontosema* y otros géneros de figitidos, así como el desarrollo de métodos para la identificación de especies de *Odontosema* por medio de RCP. Se discuten las implicaciones de la telitoquia para la definición cladística de especies, especialmente para poblaciones recién divergentes como estas, y también las implicaciones prácticas potenciales de nuestros hallazgos para el control biológico de moscas de la fruta.

Inundative releases of mass-reared parasitoids are increasingly seen as an environmentally benign means of suppressing tephritid fruit fly populations (Sivinski et al. 1996; Montoya et al. 2000; Rendón et al. 2006). New parasitoids (Lopez et al. 2003; Aluja et al. 2007) and release technologies (Sivinski et al. 2000; Baeza-Larios et al. 2002), along with ecological information on the habitat suitability of particular species (Sivinski et al. 1999, 2000; Garcia-Medel et al. 2007), promise significant improvements in efficacy. The relatively high costs of the technique, however, have limited its adoption (Rendón et al. 2006). Thelytoky (parthenogenetic production of daughters) as a mode of reproduction can result in twice as many agriculturally useful parasitoids per generation. If thelytokous strains of parasitoids could be employed, 2 benefits might arise (Stouthamer 1993): (1) lowered production costs and (2) increased population growth rates, in the rearing facility and in the field. These benefits could only arise if equivalent numbers of all-female progeny were produced compared with bisexual populations, and if there were no fitness costs to the thelytoky.

Thelytoky occurs in a number of agriculturally important parasitoid families, including natural enemies of tephritid larvae (see Ovruski et al. 2000; Argov et al. 2000). It is particularly common among the Cynipoidea, including the Figitidae, a family that includes a number of parasitoids of Diptera and several well-known natural enemies of tephritids (Ovruski et al. 2000). However, until now, all-female broods have not been described in any specialist parasitoids of pestiferous fruit flies. Here, we genetically characterize an all-female strain of the Mexican tephritid parasitoid *Odontosema anastrephae* Borgmeier, provisionally referred to here as *O. near anastrephae*.

*Odontosema anastrephae* is one of most common frugivorous-fruit fly parasitoids in the New World (Ovruski et al. 2000). A member of the Eucolilinae, a monophyletic subfamily of the Figitidae (Fontal-Cazalla et al. 2002; Buffington et al. 2007), *O. anastrephae* is part of a diverse guild of solitary, koinobiont endoparasitoids that attack third instars of *Ceratitidis capitata* (Wiedemann) and several species of *Anastrepha*, particularly as they occur in guavas (*Psidium* spp; Myrtaceae) (Wharton et al. 1998; López et al. 1999; Sivinski et al. 1997, 2000). *Odontosema anastrephae* is almost always associated with fallen fruit that are rarely intact, and either enters through breaks in the skin or chews a hole to pursue hosts within the pulp (Ovruski 2004). It is therefore of potential use in biological control as a complement to parasitoids that are only able to attack larvae near the fruit's surface (Sivinski et al. 2000).

We have investigated the degree of genetic similarity between 2 morphologically indistinguishable populations of *O. anastrephae*, one bisexual, the other (*O. near anastrephae*), entirely female.

## MATERIALS AND METHODS

### *Odontosema near anastrephae* Cultures

All cultures stemmed from wild-collected larvae, as follows. *Odontosema anastrephae* (bisexual) was collected in 1998 from infested guavas (larvae of *Anastrepha striata* and the Mexican form of *Anastrepha fraterculus*) in Llano Grande and Tejería, Veracruz, Mexico (Aluja et al. 2009). Cultures of the all-female *O. near anastrephae* stemmed from infested guavas (mainly larvae of *A. striata*) collected in the year 2000 in La Mancha, Santiago Tuxtla and in San Andrés Tuxtla (all Veracruz, Mexico) and in the vicinity of Tapachula, Chiapas, Mexico (Aluja et al. 2009). Specimens collected in 2000, all from Veracruz, Mexico, were mixed, so the exact geographic origin (La Mancha, Santiago Tuxtla, or San Andrés Tuxtla) of the all-female strain is not known. Field-collected samples were reared in the laboratory and maintained in culture on third instars of the Mexican fruit fly, *Anastrepha ludens* Loew, at the Instituto de Ecología, A.C. in Xalapa, Mexico (1998 to date for the bisexual strain and 2000 to date for the all-female strain; details in Aluja et al. 2009) and the Caribbean fruit fly, *Anastrepha suspensa* Loew, at the USDA Center for Medical, Agricultural, and Veterinary Entomology in Gainesville, FL (2005 to 2007).

In order to confirm that these 2 populations were morphologically indistinguishable, the samples were examined by figitid specialists Sergio Ovruski (Instituto Superior de Entomología, San Miguel de Tucumán, Tucumán, Argentina) and Matt Buffington (Systematic Entomology Laboratory, Smithsonian Institution, Washington, D.C). This examination did not reveal any differences between *O. anastrephae* and *O. near anastrephae*. It should be noted that Guimaraes et al. (2005) suggested that *O. anastrephae* be synonymized with *O. albinerve* Kieffer. Unfortunately, Guimaraes et al. (2005) did not comment in their synonymy on the diagnostic characters used to separate *albinerve* from *anastrephae*, namely the width to length ratio of the propodeum (Borgmeier 1935). Further examination of both the primary types of both species and numerous specimens from other collections, including Texas A&M University (TAMU), Entomology Research Museum, University of California at Riverside (UCRC), California Academy of Sciences, San Francisco (CASC) and the Smithsonian National Museum of Natural History

(NMNH), should be conducted prior to recognizing this synonymy (Matt Buffington, personal communication). Mating studies and/or molecular analyses may also shed light on this dilemma. Until these studies have been completed, we refer here to this species as *O. anastrephae*. Voucher specimens may be obtained from the colonies maintained by MA.

#### Purification of Genomic DNA

Genomic DNA was purified with the Puregene® DNA purification kit with minor modifications. Each wasp (about 1 mg frozen tissue) was homogenized in 600  $\mu$ L Puregene Cell Lysis Buffer using microfuge pestles made from melted and molded plastic pipette tips. Other volumes that differ from the kit's protocol were: Protein Precipitation Solution: 200  $\mu$ L; 100% isopropanol for DNA precipitation: 600  $\mu$ L; 70% ethanol for washing: 600  $\mu$ L.

#### PCR Amplifications

*COI* and *ITS2* gene fragments were amplified with a high fidelity PCR protocol (Jeyaprakash & Hoy 2000) with an MJ Mini thermocycler (Bio-Rad). Instead of a single DNA polymerase, a 5:1 mixture of Taq:Tgo polymerase was employed, with a buffer consisting of (10X) 50 mM Tris, 16 mM ammonium sulfate, 1.75 mM MgCl<sub>2</sub>. Primers for *COI* were designed based on the general insect *COI* primers of Kambhampati & Smith (1995) and modified upon alignment with *COI* sequences from other Figitidae; they had the following sequences: (fwd) 5'- TGACCAAATT-TATAAT-3' and (rev) 5'- CATCTAAAAATTT-TAATTCNGTDDG-3' (degenerate). Primers for *ITS2* were those of Porter & Collins (1991), with the following sequences: (fwd) 5'- GTGAATTCT-GTGAAGTGCAGGACACATGAAC -3' and (rev) 5'- ATGCTTAAATTTAGGGGGTA -3'. Five pmol of each primer were added to a 25- $\mu$ L reaction volume containing 1  $\mu$ L of purified genomic DNA solution, 700 mM dNTPs, 2.5  $\mu$ L 10X buffer (described above), and 1  $\mu$ L Taq/Tgo DNA polymerase mix (1U Taq polymerase, 0.2U Tgo polymerase) (Bio-Rad Laboratories, Inc., Hercules, CA; Roche Molecular Biochemicals, Indianapolis, IN). Cycling conditions for all amplifications were: initial denaturation at 94°C for 5 min, followed by 10 cycles of 94°C for 10 s, 56°C for 30 s, and 68°C for 2 min, followed by 20 cycles of 94°C for 10 s, 56°C for 20 s, 68°C for 2 min + 20 s/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 filtered pipette tips were used. Negative controls with identical reaction conditions except for the substitution of ddH<sub>2</sub>O for genomic DNA were run alongside each experimental amplification.

#### Cloning and Sequencing

We used the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin) according to the manufacturer's instructions, to purify PCR products and cloned them into the pCR®II-TOPO vector utilizing TOP10 chemically competent cells (Invitrogen, Carlsbad, California). Plasmid DNA from cells thought to contain inserts was prepared by either the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) or the GenElute HP Plasmid Miniprep Kit (Sigma, St. Louis, MO). After confirmation of the presence of inserts by restriction digest, plasmids were sequenced by Cleveland Genomics (Cleveland, OH, USA) or NorthWoods DNA sequencing (Solway, MN). Both strands of the inserts were sequenced.

#### Sequence Analysis

Sequences were determined from chromatograms based on Chromas (Technelysium Pty. Ltd.) and SeqMan software (DNASTar, Madison, WI). Coverage was between two-fold and five-fold for all sequences. GenBank accession numbers for the sequences determined in this study are as follows: *COI*: DQ399309- DQ399318; *ITS2*: EU090919- EU090936; *wsp*: EU088133; *actin*: EU215416-EU215422, EU178819-EU178822. For *COI*, 5 individual wasps were used, and each sequence represents a separate individual wasp. For *ITS2*, 5 individuals were also used, but different copies of *ITS2* were sometimes found in the same individual. These were treated as separate *ITS2* sequences in the analysis.

#### Phylogenetic Analysis

Sequences from this study and comparison nucleic acid sequences from GenBank were aligned with Clustal X software (Thompson et al. 1997). Alignments were then used to create bootstrapped trees according to the neighbor-joining algorithm with Clustal X and njplot software (Saitou & Nei 1987). The *COI* sequence alignment did not contain any gaps, but did include ambiguous residues. These positions were removed from the analysis. For the *ITS2* sequences, major differences between sequences were the result of microsatellites with identical repeat sequences, but varying lengths. The resulting gaps were therefore informative. For this reason, gaps were included in these trees. Whether gaps were included or excluded, branching order was the same, and branch lengths were only slightly changed. Bootstrap values above 700 (out of 1,000 repetitions) are shown in each phylogenetic tree. In addition, Maximum Likelihood analyses were carried out with the PHYLIP package (Felsenstein 1989), which resulted in the same positions for the *O. anastrephae* and *O. near anastrephae* sequences (not shown).

RESULTS

Cytochrome C Oxidase I Sequences

PCR was used to amplify the *Cytochrome C Oxidase I (COI)* mitochondrial gene from samples of the 2 *Odontosema* populations. *COI* sequences from 5 individual wasps of each strain revealed similar, but clearly distinct, sequences for this gene (Fig. 1). The consensus nucleotide sequences for the 2 population samples differed at 10 nucleotide positions out of 830 bp of sequence, corresponding to a difference of 1.2%. *COI* amino acid sequences did not differ for the 2 populations (not shown). The tree depicted in Fig. 1 was drawn by the neighbor-joining method.

ITS2 Sequences

To further investigate the genetic differences between these 2 populations, approximately 600 bp of the ITS2 ribosomal region were amplified from each population. Surprisingly, these ITS2 sequence data provided neither a clear separation between the all-female and the bisexual wasps, nor did all of the sequences cluster together. Instead, there seemed to be 2 major branches of

ITS2, with the first found only in the all-female wasps and the second in both all-female and bisexual wasps (Fig. 2). ITS2 is a multi-copy genetic sequence and therefore slight differences in sequence are to be expected in the same species (Wesson et al. 1992; Harris & Crandall 2000; Alvarez & Hoy 2002). However, though there was variation between individual ITS2 sequences, mostly due to differing lengths of repeats within microsatellite insertions, this variation was much smaller in magnitude than the clear separation of these 2 branches (Fig. 2). Clone P2 appeared to be unique in that it did not fit neatly into either group. Further examination of P2 revealed a sequence resembling a chimera of the 2 other types rather than a unique sequence, with the first 410 bp of the alignment resembling the bisexual+all-female sequence and the last 203 bp resembling the all-female-only sequence.

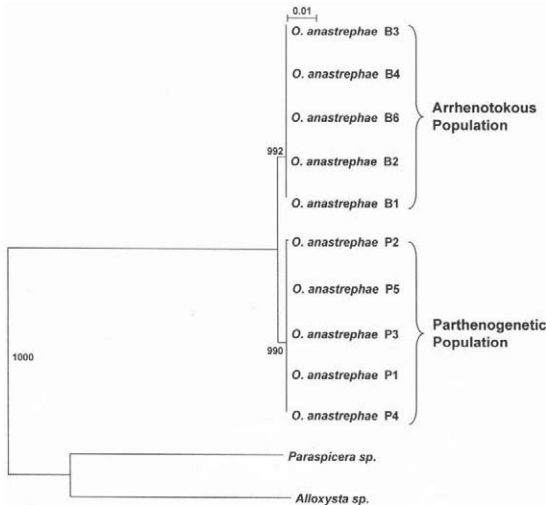


Fig. 1. Phylogenetic neighbor-joining tree of *Cytochrome C Oxidase I* gene sequences from the *Odontosema* strains investigated in this study, rooted with *COI* sequences from 2 different genera of figitid wasps, *Paraspicera* sp. (Hymenoptera: Figitidae) and *Alloxysta* sp. (Hymenoptera: Figitidae). B1-B6: individual wasps from the arrhenotokous population of *Odontosema*; P1-P5: individual wasps from the all-female population of *Odontosema*. Bootstrap values shown are for 1,000 replicates. Size bar reflects phylogenetic divergence in genetic distance units. Accession numbers are as follows: *Alloxysta* sp.: DQ012618; *Paraspicera* sp.: AY675842; *Odontosema* (near) *anastrephae*: DQ399309-DQ399318.

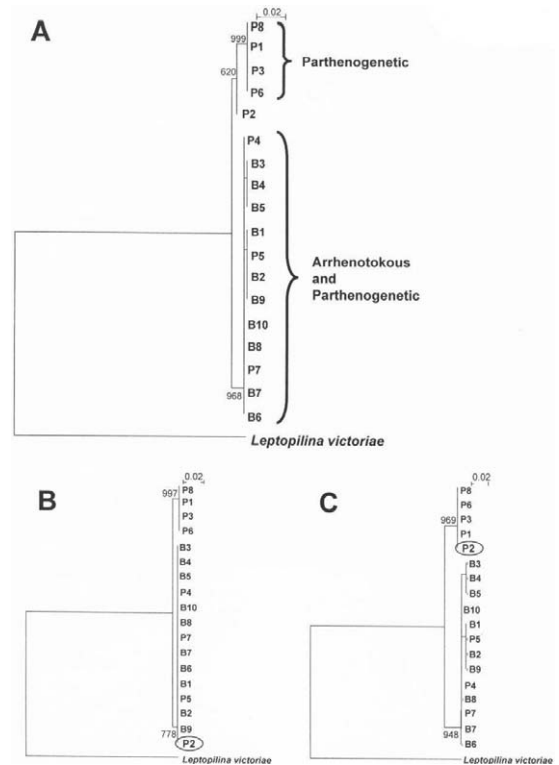


Fig. 2. Phylogenetic neighbor-joining tree of ITS2 gene sequences from the *Odontosema* strains investigated in this study, rooted with the ITS2 sequence of the figitid *Leptopilina victoriarie* (Hymenoptera: Figitidae). P1-P8: ITS2 clones from the all-female population of *Odontosema*; B1-B10: ITS2 clones from the arrhenotokous population of *Odontosema*. Bootstrap values shown are for 1,000 replicates. Size bar reflects phylogenetic divergence in genetic distance units. Accession numbers are as follows: *Leptopilina victoriarie*: AY124554; *Odontosema* (near) *anastrephae*: EU090919- EU090936.

Excluding P2, the all-female-only sequences were identical, and the sequences from both populations differed in only 2 locations, with all discrepancies except one corresponding to gaps generated by differences in repeat lengths. In contrast, the consensus sequences of the 2 major ITS2 branches exhibited discrepancies in 18 nucleotide positions out of 618, corresponding to a variation of 2.75%.

In addition to *COI* and ITS2, actin gene fragments (2 isoforms) were also amplified and sequenced, but no differences were found between *O. anastrephae* and *O. near anastrephae* on the basis of these sequences (not shown). This was not an unexpected result, since actin is highly conserved and would not be expected to distinguish between closely related cryptic species or subspecies (Baldauf et al. 2000).

#### DISCUSSION

Thelytokous populations can co-exist with bisexual congeners and survive over evolutionary time when ecological conditions are favorable, particularly in marginal, biologically depauperate environments (Cuellar 1977; Aeschlimann 1990; Stouthamer & Luck 1993; Jensen et al. 2002). The 2 populations of *Odontosema* investigated here, although apparently morphologically identical (S. M. Ovruski and M. L. Buffington, personal communication), appear to exhibit some differences in host-searching behavior (Ramirez-Romero et al. unpublished data). In addition, in preliminary mating observations, males of the bisexual strain do not mate with females of the all-female strain, though more rigorous behavioral experiments under varying conditions are needed before concluding that these 2 strains cannot mate. These possible differences in behavior suggest the possibility of genetic divergence and so indirectly provide a measure of whether non-sexual reproduction in this instance has been consistently adaptive and so has survived as a long-term phenomenon. Addressing this issue, we compared mitochondrial (*COI*) and nuclear (ITS2) genetic sequence samples from each population.

The *COI* sequences from the 2 populations separated into 2 populations that are closely related but, nevertheless, genetically distinct. The discrepancies in nucleotides between the 2 consensus sequences totaled 1.2%. For comparison, other studies based on *COI* have found the majority of within-species variation to be less than 1%, but up to 2%, *vs.* between-species variation within the Hymenoptera averaging 11.5% (Hebert et al. 2003). Cryptic species of (bisexual) *Aphelinus* parasitoids, however, have been shown to differ by 0.5% or less, even though they are reproductively incompatible (Heraty et al. 2007).

The other genetic region we investigated, ITS2, also showed 2 distinct branches, but with a

surprising twist. One ITS2 type was present only in the all-female population but the other was present in both populations. The 2 ITS2 types exhibited clear differences. The consensus sequences exhibited a variation of 2.75%, somewhat greater than the mean 1.17% intraspecific variation found by Wesson et al. (1992) for *Aedes* mosquitoes, and also greater than that found in a study of *Ageniaspis* wasps, where sequence variability between conspecific individuals and between ITS2 copies within individuals was found to range from 0.14% to 1.18% (Alvarez & Hoy 2002). In contrast to the 2.75% difference between groups, the all-female-only sequences were identical, and the sequences found in both populations differed in only 2 locations (excluding P2). All of the discrepancies within these locations, except one, corresponded to gaps generated by differences in repeat lengths in microsatellite regions. Microsatellite regions within ITS sequences have been shown to exhibit exceptionally high levels of intragenomic variation (Harris & Crandall 2000).

How genetically divergent then are *O. anastrephae* and *O. near anastrephae*? Though it is spurious to draw conclusions about speciation on the basis of DNA sequence differences alone (Cognato 2006; Feguson 2002), our results suggest that these 2 insect populations have diverged to a lesser extent than is typically found between well recognized species, but to a similar extent to that sometimes found between closely related cryptic species (Kankare et al. 2005). In any case, the genetic discrepancies between these 2 *Odontosema* populations indicate true differences, and may reflect short, but nevertheless unique, evolutionary histories.

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