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Molecular identification of the secondary endosymbiont *Hamiltonella defensa* in the rose-grain aphid *Metopolophium dirhodum*

MARTA C. TELESNICKI[1], CLAUDIO M. GHERSA[1], MARÍA A. MARTÍNEZ-GHERSA[1], JOEL D. ARNEODO[2*]

[1] IFEVA-Facultad de Agronomía Universidad de Buenos Aires,CONICET, Ciudad Autónoma de Buenos Aires;
[2]IMYZA-INTA Castelar/CONICET, Pcia. de Buenos Aires, Argentina.

[*]Correspondence. E-mail: arneodoj@yahoo.com

ABSTRACT

This is the first report of the association between the rose-grain aphid *Metopolophium dirhodum*, a potentially important cereal pest and the facultative symbiont *Hamiltonella defensa*. The infection with this gamma-proteobacterium was determined by PCR in laboratory-reared and field-collected specimens of an Argentinian population of the aphid. Partial bacterial 16S, IGS and 23S rRNA genes were sequenced and compared to other available *Hamiltonella* sequences by phylogenetic analysis. The present study provides new information on previously unknown *M. dirhodum* microbiota.

Key words: Aphididae, facultative symbiont, gamma-proteobacteria, PCR, sequencing

RESUMEN

Identificación molecular del endosimbionte secundario *Hamiltonella defensa* en el pulgón amarillo de los cereales, *Metopolophium dirhodum*. Esta es la primera comunicación sobre la asociación entre el áfido *Metopolophium dirhodum*, una plaga potencialmente importante de cereales, y el simbionte facultativo *Hamiltonella defensa*. La infección con esta gamma proteobacteria se determinó mediante PCR en ejemplares de una población argentina del áfido, tanto en individuos criados en laboratorio como en ejemplares colectados en campo. Se secuenció parte de los genes 16S y 23S del ARNr, y el espacio intergénico, y se realizó una comparación con otras secuencias disponibles de *Hamiltonella* a través de un análisis filogenético. Este estudio proporciona nueva información sobre la hasta ahora desconocida microbiota de *M. dirhodum*.

Palabras clave: Aphididae, simbionte facultativo, gamma proteobacteria, PCR, secuenciación

Almost all aphid species harbor the obligate, vertically transmitted bacterial endosymbiont *Buchnera aphidicola*, which synthesizes essential amino acids lacking in the insect's diet. In addition to this primary symbiont, aphids may host one or more accessory bacteria, called secondary or facultative endosymbionts. These are also transmitted from mother to offspring, but occasionally move horizontally within and between species (11). Among them, the gamma-proteobacterium *Hamiltonella defensa* has been shown to confer parasitoid resistance in the pea aphid *Acyrthosiphon pisum* by blocking larval development of endoparasitoid wasps *Aphidius ervi* and *Aphidius eadyi* (6). Initially discovered infecting the whitefly *Bemisia tabaci* (4), *H. defensa* was later found in aphids belonging to the genera *Acyrthosiphon*, *Amphorophora*, *Aphis*, *Chaitophorus*, *Cinara*, *Hyperomyzus*, *Macrosiphum*, *Microlophium*, *Periphyllus*, *Sitobion*, *Uroleucon*, *Pemphigus* and *Geopemphigus* (1, 5, 10, 11, 13). *H. defensa* has also been reported in the psyllid *Cacopsylla pyri* and the whitefly *B. argentifolii* (11, 15). Due to its positive effects on insect fitness, the occurrence of this microorganism in other economically or ecologically important insect species still needs to be explored.

The rose-grain aphid *Metopolophium dirhodum* (Walker, 1849) is a common cereal pest that may cause yield reductions by direct feeding and by spreading virus diseases. Although this aphid is of palaearctic origin, it is now globally distributed (3).

Its presence in South America was first recorded during the late 1960s and the 1970s, when it caused considerable damage to wheat crops (2). Despite the relative abundance and economic significance of *M. dirhodum*, basic knowledge concerning its biology and physiology remains incomplete. Data on the insect-associated microorganisms are particularly lacking. A preliminary PCR survey on the microorganisms associated to a natural population of *M. dirhodum* in Buenos Aires, Argentina, revealed both the presence of *Buchnera* and a different species of bacterium (unpublished). On this basis, further studies involving PCR and partial genome sequencing were conducted to elucidate the identity of the accessory bacterium. Thus, this paper presents the first report of the secondary endosymbiont *H. defensa* in *M. dirhodum*.

Individuals of *M. dirhodum* were collected in January and February 2011 from a spontaneous population growing on *Lolium multiflorum* and other Poaceae at the Instituto de Investigaciones Fisiológicas y Ecológicas Vinculadas a la Agricultura (IFEVA)-University of Buenos Aires experimental field (34° 35'S, 58° 29'W). The insects were further raised on wheat plants at 22 °C ± 1 °C and LD 12:12 h photoperiod. Additional *M. dirhodum* specimens were sampled in October 2011 at the same IFEVA field and immediately processed. Total DNA was extracted from whole single aphids following a CTAB-based protocol modified after Doyle & Doyle (9). DNA yield and quality were determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington DE, USA).

Amplification of nearly complete 16S rRNA gene, intergenic spacer region and partial 23S rRNA was performed with universal eubacterial primer pair 10F/480R (13). These primers amplify most bacterial taxa except *Buchnera*, in which the 16S gene is not linked to the 23S gene by an IGS (13). *Hamiltonella* specific forward primer PABSF and general reverse primer 16SB1 (4, 8) were used to amplify part of the 16S rRNA gene. Reactions were carried out on an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) thermal cycler under the following conditions: 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 68 °C for 3 min when using primer pair 10F/480R; and 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min when using primer pair PABSF/16SB1. Amplicons were run in agarose (1 %) gel and stained with ethidium bromide. Nine laboratory-reared and three randomly field-collected *M. dirhodum* specimens were tested by PCR with both primer sets. Negative controls consisted of total DNA extracted from the aphid *Schizaphis graminum*, which is known not to contain secondary symbionts (13).

Total DNA extracted from a single laboratory-reared aphid which tested positive for *Hamiltonella* was used for amplification with primers 10F and 480R. The amplified product was purified from agarose gel and cloned into *E. coli* DH5α cells using PCR 2.1 TOPO TA cloning kit (Invitrogen, Carlsbad CA, USA). Transformed bacteria were grown on ampicillin-containing LB medium and plasmid DNA was purified with the QIAprep® Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany). Two clones were sequenced at the Instituto de Biotecnología (INTA Castelar, Argentina) in an ABI PRISM 3500 XL genetic analyzer (Applied Biosystems, Foster City CA, USA). Sequencing was performed with plasmid primers M13F and M13R (Invitrogen, Carlsbad CA, USA), and a new primer set designed for the central portion of the insert: HintForw (5'-CATTTGAAACTGGGTCGCTA-3') and HintRev (5'-TGTCCTAGGCCTCTAGACGAA-3'). The sequence obtained was compared to other *H. defensa* sequences from different insect hosts and geographical origins available in genome databases.

The twelve *M. dirhodum* individuals assessed were positive in PCR assays targeting either eubacteria or *H. defensa* (Figure 1), showing the expected amplicon sizes of *ca.* 2.25 kb and 1.6 kb, respectively.

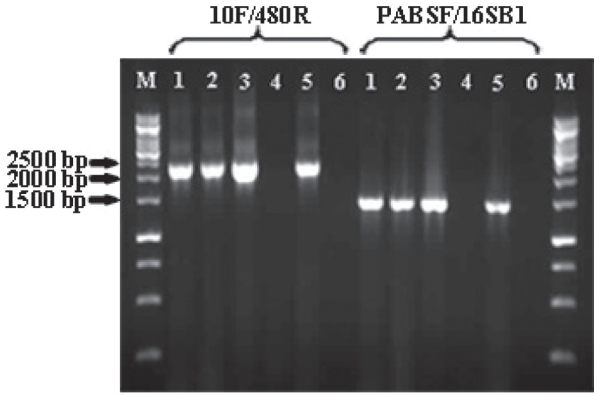

Figure 1. PCR detection of eubacteria (primer pair 10F/480R) and *Hamiltonella defensa* (primer pair PABSF/16SB1) in *Metopolophium dirhodum* specimens. M= molecular weight marker (GeneRuler™ 1 kb DNA ladder, Fermentas), 1 and 2= laboratory-reared aphids, 3= field-collected aphid, 4= *Schizaphis graminum* negative control, 5= laboratory-reared aphid from which partial bacterial genome sequence was obtained, 6= water.

None of the *S. graminum* negative controls or water revealed any amplification signal. Thus, the specific PCR with PABSF/16SB1 demonstrated the occurrence of the secondary endosymbiont *H.*

defensa in both laboratory-reared and field-collected insects. Although an extensive sampling should be performed to determine whether this secondary endosymbiont is fixed in the *M. dirhodum* population studied, its presence in every specimen analyzed provides evidence of high infection frequency. In this context, previous works had reported intermediate frequencies of *H. defensa* in *A. pisum* populations, while *H. defensa* was shown to be fixed in *Uroleucon ambrosiae* collected across the United States (13).

Complete sequence identity was observed between the two clones analyzed. The partial 16S rDNA, intergenic spacer region and partial 23S rDNA sequence of a total of 2254 bp corresponding to clone HdMd2 is available at the GenBank under Accession Number JQ293090. Based upon available *Hamiltonella* 16S rDNA sequences, a phylogenetic analysis was conducted with the neighbor-joining method (12) using MEGA version 4 (14). For this purpose, only sequences longer than 1300 bp were considered. The bootstrap consensus tree (Figure 2) inferred from 1000 replicates strongly supported (100 %) the inclusion of clone HdMd2 from *M. dirhodum* in the *Hamiltonella* group. Furthermore, the new *Hamiltonella* sequence was more closely related to sequences obtained from the other aphid species and the psyllid *C. pyri*, as validated by a significant bootstrap value (87 %). The secondary endosymbionts isolated from whiteflies *B. tabaci* and *B. argentifolii* formed a different cluster within *Hamiltonella*. The high degree of similarity among sequences is not unexpected, because this is a conserved region, but also because the horizontal transfer of facultative endosymbionts is a well documented feature (11). In this sense, partial *Hamiltonella* sequences show high nucleotide identity even between isolates from taxonomically distinct hosts.

The molecular identification of *H. defensa* in the genus *Metopolophium* extends the reported host range of this endosymbiont. To the author's knowledge, this is also the first study on microbiota associated to *M. dirhodum*. Since hymenopteran *Aphidius ervi* has already been cited parasitizing *M. dirhodum* in the field (7), the defensive role of *H. defensa* in this aphid species and the effect of these tritrophic interactions on the population dynamics of the pest deserve further research.

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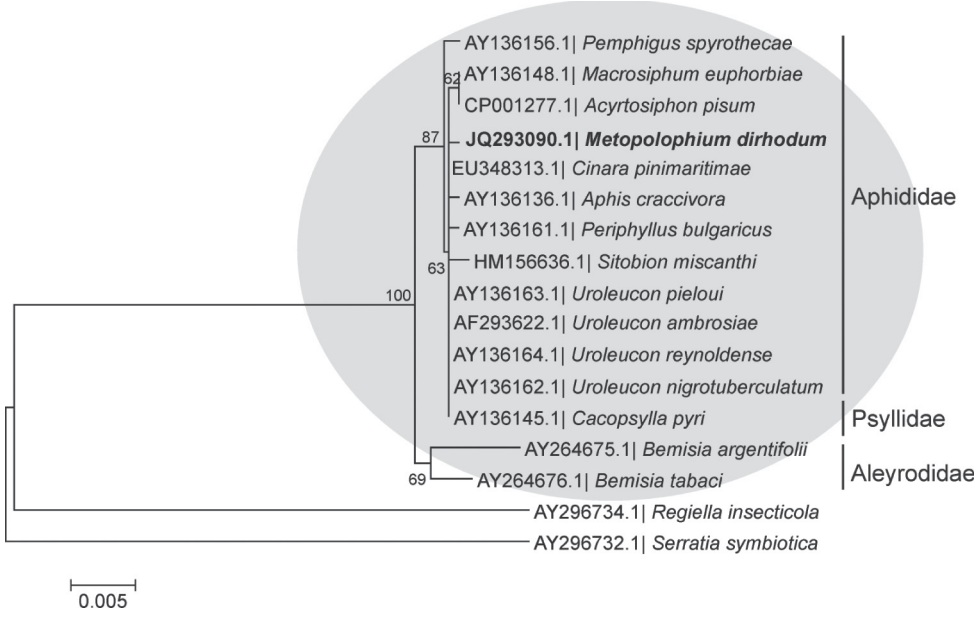

Figure 2. Neighbor-joining phylogenetic analysis showing the position of *Hamiltonella* isolated from *M. dirhodum* (in bold). The tree is based on available *Hamiltonella* 16S rRNA gene sequences (light grey shaded area), with the two additional aphid secondary endosymbionts *Regiella insecticola* and *Serratia symbiotica* as related outgroups. Branches with <50% bootstrap support are collapsed. Taxonomic names for *Hamiltonella* isolates refer to host species. Associated labels correspond to GenBank accession numbers. Bootstrap values (1000 replicates) reported as percentages are indicated at the nodes. The scale bar represents the number of base substitutions per site.

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