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Exploring the Bacterial Microbiota Associated With Native South American Species of *Aphis* (Hemiptera: Aphididae)

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ABSTRACT Aphids harbor a variety of bacterial endosymbionts, including the obligate symbiont *Buchnera aphidicola* and diverse facultative symbionts. The former supplies its host with essential amino acids. The latter are not indispensable for insect survival, but often improve their host's fitness. To date, the study of such associations was restricted to aphids of Holarctic origin. The bacterial microbiota of seven *Aphis* species from Argentina was investigated. The presence of *B. aphidicola* was assessed by specific PCR. Additional symbionts were identified through PCR with eubacterial universal primers, cloning, and sequencing of nearly complete 16S rRNA gene, intergenic spacer region, and partial 23S rRNA gene and subjected to phylogenetic analysis. Infection with *B. aphidicola* was confirmed in every species analyzed. The facultative symbiont *Serratia symbiotica* was detected in *Aphis malalhuina* Mier Durante, Nieto Nafría & Ortego, 2003, *Aphis senecionicoides* Blanchard, 1944, and *Aphis schinifoliae* Blanchard, 1939, while *Hamiltonella defensa* was identified in *Aphis mendocina* Mier Durante, Ortego & Nieto Nafría, 2006. *Arsenophonus* sp. was found infecting *Aphis melosae* Mier Durante & Ortego, 1999, and a new, undescribed *Aphis* sp. In *Aphis danielae* Remaudière, 1994, no facultative symbionts could be recorded. When analyzing the highly conserved 16S rRNA gene, the phylogenetic tree grouped the *S. symbiotica*, *H. defensa*, and *Arsenophonus* isolates into three well-defined clusters showing little variability among clones corresponding to the same aphid host species. This article reports for the first time the endosymbionts associated with aphids native to South America. Despite their geographic origin, the qualitative composition of their microbiota revealed no evident differences from that described for aphids in the Northern Hemisphere.

RESUMEN Los pulgones albergan una variedad de endosimbiontes bacterianos, incluyendo al simbionte obligado *Buchnera aphidicola* y diversos simbiontes facultativos. El primero aporta a su hospedador aminoácidos esenciales para su dieta, mientras que los últimos no son indispensables para la supervivencia del insecto pero a menudo mejoran su aptitud. Hasta el momento, el estudio de dichas asociaciones estuvo restringido a pulgones de origen Holártico. En este trabajo, se investigó la microbiota bacteriana de siete especies de pulgones del género *Aphis*, autóctonas de Argentina. En primera instancia, se determinó la presencia de *B. aphidicola* por PCR con cebadores específicos. Los simbiontes adicionales fueron identificados mediante PCR con cebadores universales para eubacterias, clonado y secuenciación parcial de los genes ARNr 16S y 23S, así como el espacio intergénico, y análisis filogenéticos. La infección con *B. aphidicola* fue corroborada en todas las especies analizadas. El simbionte facultativo *Serratia symbiotica* fue detectado en *Aphis malalhuina*, *A. senecionicoides* y *A. schinifoliae*, mientras que *Hamiltonella defensa* fue identificada en *A. mendocina*. Bacterias del género *Arsenophonus* fueron encontradas infectando *A. melosae* y una nueva especie de *Aphis* aún no descripta. En *A. danielae*, no se registraron simbiontes facultativos. Al analizar el gen ARNr 16S, altamente conservado, el árbol filogenético reunió a los aislamientos correspondientes a *S. symbiotica*, *H. defensa* y *Arsenophonus* en tres grupos bien definidos, que mostraron poca variabilidad genética entre clones provenientes de una misma especie hospedadora. Este artículo describe por primera vez a los endosimbiontes asociados con pulgones nativos de América del Sur. A pesar de su origen geográfico, la composición cualitativa de su microbiota no reveló diferencias evidentes respecto a la descripta en pulgones del hemisferio norte.

KEY WORDS aphid, endosymbiont, *Serratia symbiotica*, *Hamiltonella defensa*, *Arsenophonus*

Aphids and bacteria share a long history of symbiotic associations. The primary symbiont *Buchnera aphidicola*, first discovered by Buchner (1965), is present in almost every aphid species within specialized organs called bacteriomes. This gamma-proteobacterium supplies its host with essential amino acids, and is transmitted to the progeny strictly in a vertical manner (Baumann 2005). At the time of his initial findings, and based on light microscopy, Buchner (1965) also re-

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Table 1. *Aphis* species, host plants, and sampling sites

Aphid species	Host plant	Sampling site
Aphis sp. (undescribed)	*Adesmia pinifolia* Gillies ex Hooker & Arnott	Refugio (Malargüe, Mendoza)
Aphis danielae Remaudière, 1994	*Lycium* sp. L.	La Cruz Negra (Tupungato, Mendoza)
Aphis malalhuina Mier Durante, Nieto Nafría & Ortego, 2003	*Senecio subumbellatus* Philippi	Refugio (Malargüe, Mendoza)
Aphis melosae Mier Durante & Ortego, 1999	*Grindelia chiloensis* Cabrera	RN143, km. 685 (San Rafael, Mendoza)
Aphis mendocina Mier Durante, Ortego & Nieto Nafría, 2006	*Urtica mollis* Steudel	Puesto Los Palacios (Malargüe, Mendoza)
Aphis schinifoliae Blanchard, 1939	*Schinus* sp. L.	Refugio (Malargüe, Mendoza)
Aphis senecionicoides Blanchard, 1944	*Senecio subulatus* Don ex Hooker & Arnott	RN144, km. 741 (San Rafael, Mendoza)

ported the existence of morphologically distinct secondary bacterial symbionts, sometimes living outside the bacteriomes harboring *B. aphidicola.* As new molecular techniques became available, the accessory microbiota of aphids began to be classified into phylogenetic lineages (Unterman et al. 1989). To date, three main secondary symbionts of aphids have been identified: *Regiella insecticola*, *Hamiltonella defensa,* and *Serratia symbiotica,* all included in the gamma-proteobacteria (Moran et al. 2005). Their frequency in the aphid species analyzed so far ranged from 14 to 16%, occasionally co-occurring in mixed infections (Oliver et al. 2010). A fourth gamma-proteobacterium belonging to the genus *Arsenophonus,* initially thought to be extremely rare (Wille and Hartman 2009, Oliver et al. 2010), has been recently reported in as much of 7% of the sampled aphid species (Jousselin et al. 2013). These secondary (or facultative) symbionts are not indispensable for host survival, but often improve the insect's fitness in different ways. Besides transfer from parents to offspring, horizontal transmission of such microorganisms, even across insect taxa, is a well-known feature (Russell et al. 2003, Oliver et al. 2010, Gehrer and Vorburger 2012). In that sense, *H. defensa* has been recorded not only in aphids but also in whiteflies and psyllids (Darby et al. 2001, Russell et al. 2003), while there are reports of *Arsenophonus* sp. infecting whiteflies, psyllids, cixiids, and planthoppers, among others (Thao and Baumann 2004, Hansen et al. 2007, Bressan et al. 2009, Salar et al. 2010). Because the entire research in the matter was carried out on insects of Holarctic origin (generally Palearctic), diversity and phylogenetic studies concerning facultative endosymbionts of aphids from the Southern Hemisphere are crucial to broaden the current knowledge of such interactions. Thus, the aim of this work was to confirm the occurrence of *Buchnera* and to characterize the facultative bacterial microbiota associated with seven *Aphis* species native to southern South America through PCR, cloning, and partial sequencing of the 16S–23S rRNA genes.

Materials and Methods

Aphid Sampling and Identification. Specimens belonging to the genus *Aphis* were collected in late November 2012 from naturally occurring populations growing on spontaneous native plants in the province of Mendoza, western Argentina. Collecting sites corresponded to areas with little anthropic impact and away from agricultural activities. Preliminary identification of insects was made in the field based on ecological features and morphological characteristics, with the aid of magnifying lenses. Further microscopic examinations (including slide mounting, if necessary) were performed at the laboratory, according to the corresponding descriptions and keys (Remaudière 1994, Mier Durante and Ortego 1999, Mier Durante et al. 2003, Mier Durante et al. 2006). In total, seven *Aphis* species were considered in this study (one population per sampling site per species). *Aphis* species and collection details are summarized in Table 1.

DNA Extraction, PCR, Cloning, and Sequencing of Bacterial 16S–23S rDNA. Metagenomic DNA was extracted from single adult aphids (whole body) following a cetyltrimethylammonium bromide (CTAB)-based protocol, modified after the method first described by Doyle and Doyle (1987). The DNA yield and purity was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). A first PCR assay with *Buchnera*-specific primer pair Buch16S1F and Buch16S1R (Tsuchida et al. 2002) was performed, both to confirm its presence in the native South American *Aphis* species and to evaluate the suitability of the bacterial DNA for subsequent analysis. Then, amplifications were carried out with the universal primers 10F and 480R, which amplify nearly complete 16S rRNA gene, intergenic spacer (IGS) region, and partial 23S rRNA gene of most bacterial taxa except *Buchnera* (Sandström et al. 2001). Four specimens of each aphid species were individually subjected to PCR for amplification of *Buchnera* and other bacterial sequences on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Amplicons were resolved in 1% agarose gels, stained with ethidium bromide. In the case of 10F- and 480R-primed products, the bands were excised from the gels and the DNA was purified with QIAEX II Gel Extraction kit (Qiagen GmbH, Hilden, Germany). Amplified fragments were cloned into chemically competent *E. coli* DH5α cells using PCR 2.1 TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Transformed bacteria were grown overnight at 37°C on LB agar plates containing ampicillin. A colony PCR was conducted to verify the integration of the insert, and for each plate (four per *Aphis* species), 10 clones were screened by

restriction fragment-length polymorphism with HindIII to estimate intra- and interspecific diversity. Plasmid purification was done with a QIAprep Spin Miniprep kit (Qiagen GmbH). Two to four clones of each *Aphis* species obtained from different individuals (except for *A. senecionicoides,* where only one clone was analyzed) were sequenced in an ABI PRISM 3500 XL genetic analyzer (Applied Biosystems, Foster City, CA) using plasmid primers M13F and M13R (Invitrogen), and new sets of internal primers that will be described later.

Phylogenetic Analysis. An initial BLAST search (Altschul et al. 1990) was performed to determine whether the obtained 16S–23S rRNA sequences corresponded to previously unreported bacterial species or to already known endosymbionts. Then, a phylogenetic analysis was performed to establish evolutionary relationships. Owing to the limited availability of related sequences in GenBank, the analysis was restricted to the 16S rRNA. The majority of the 16S rRNA sequences of *S. symbiotica, H. defensa,* and *Arsenophonus* sp. infecting *Aphididae* from different geographical origins annotated so far were included in this study. Only sequences longer than 1,200 bp were considered. Some sequences of *S. symbiotica* hosted by aphids in the subfamily *Lachninae* were discarded because of the big amount of data concerning this particular group. A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) with MEGA version 5.2 (Tamura et al. 2011). Bootstrap test was performed with 1000 replicates. The alpha-proteobacteria *Wolbachia* sp. was used as an outgroup.

Results

Extraction of nucleic acids from single aphids yielded sufficient quantity and quality of DNA to carry out subsequent molecular analysis. As expected, specific PCR assays demonstrated the occurrence of the obligate symbiont *Buchnera* in every specimen processed, regardless of the aphid species, revealing a fragment of ≈0.43 kb (not shown). Furthermore, amplification with primer pair 10F and 480R, intended to target bacteria other than *Buchnera,* resulted in products of the predicted size (2.25–2.6 kb) in six out of seven *Aphis* species analyzed. The exception was *A. danielae,* which tested repeatedly negative (not shown). HindIII digestion of the 10F- and 480R-primed amplicons evidenced different profiles between some of the bacterial isolates corresponding to distinct *Aphis* species, but the restriction patterns of isolates from the same host were always identical (not shown).

Cloning and sequencing of the amplified partial 16S–23S rRNA genes allowed the classification of these *Aphis*-associated bacteria: the secondary symbiont *S. symbiotica* was detected in *A. malalhuina, A. senecionicoides,* and *A. schinifoliae,* while *H. defensa* was identified as the facultative symbiont of *A. mendocina.* In addition, a bacterium in the *Arsenophonus* clade was found infecting *A. melosae* and the undescribed *Aphis* sp. collected from *Adesmia.* Owing to the length of the amplified fragments, the following primers were designed for sequencing the internal portions of the inserts: SerrIntF (5′-GCATTTGAGACTGGCAAGCT-3′) and SerrIntR (5′-GTCACCATGACAGCTTGACC-3′) for *S. symbiotica;* and ArsIntF (5′-AAGAAGCACCGGCTAACTCC-3′) and ArsIntR (5′-GCCACTCGTCTGCCTATTTG-3′) for *Arsenophonus.* In the case of *H. defensa,* the primers HintForw (5′-CATTTGAAACTGGGTCGCTA-3′) and HintRev (5′-TGTCCTAGGCCTCTAGACGAA-3′) were used (Telesnicki et al. 2012). Sequences ranging from 2254 to 2623 kb (depending on the length of the IGS region) were obtained. Sequence data were deposited at GenBank under the following accession numbers: KF824521, KF824522, KF824523, and KF824524 (*Arsenophonus* from *A. melosae,* clones 5.1, 5.2, 5.3, and 5.4, respectively); KF824525 (*S. symbiotica* from *A. senecionicoides,* clone 13.2); KF824526, KF824527, KF824528, and KF824529 (*Arsenophonus* from *Aphis* sp., clones 19.1, 19.2, 19.3, and 19.4, respectively); KF824530 and KF824531 (*S. symbiotica* from *A. malalhuina,* clones 20.1 and 20.2, respectively); KF835611, KF835612, and KF835613 (*S. symbiotica* from *A. schinifoliae,* clones 21.3, 21.5 and 21.8, respectively); KF835614 and KF835615 (*H. defensa* from *A. mendocina,* clones 26.1 and 26.2, respectively). These and other related 16S rRNA sequences available at GenBank enabled the construction of a phylogenetic tree that clearly grouped the different *S. symbiotica, H. defensa,* and *Arsenophonus* isolates into three main clusters with high bootstrap support. Clones of the bacterial 16S rRNA from the native South American *Aphis* spp. tended to group together according to their insect host species. Furthermore, clones of *S. symbiotica* infecting *A. senecionicoides* and *A. schinifoliae,* and *Arsenophonus* sp. infecting *A. melosae* and *Aphis* sp. collected from *Adesmia* were closely related, compared with other reported bacterial isolates from different aphid hosts around the world (Fig. 1).

To further investigate the prevalence of the endosymbionts, and to detect the possible occurrence of mixed infections, metagenomic DNA was extracted from six additional specimens of each *Aphis* species. Then, 10 individuals per *Aphis* species were analyzed by PCR with the specific primer combinations 16SA1 and PASScmp for *S. symbiotica* (Fukatsu et al. 2000), PABSF and 16SB1 for *H. defensa* (Darby et al. 2001, Douglas et al. 2006), and Ars2F and NC-Arsen16S-R for *Arsenophonus* (Russell et al. 2012, Dergousoff and Chilton 2010). Primer pair U99F and 16SB1 for specific detection of *R. insecticola* (Douglas et al. 2006) was used as well. The results corroborated the previous findings, and revealed that all 10 tested specimens of each *Aphis* species (excepting *A. danielae*) were infected with the corresponding secondary endosymbiont mentioned above. PCR assays demonstrated the absence of mixed infections. Furthermore, reactions failed to amplify *R. insecticola* sequences in any of the analyzed samples (not shown).

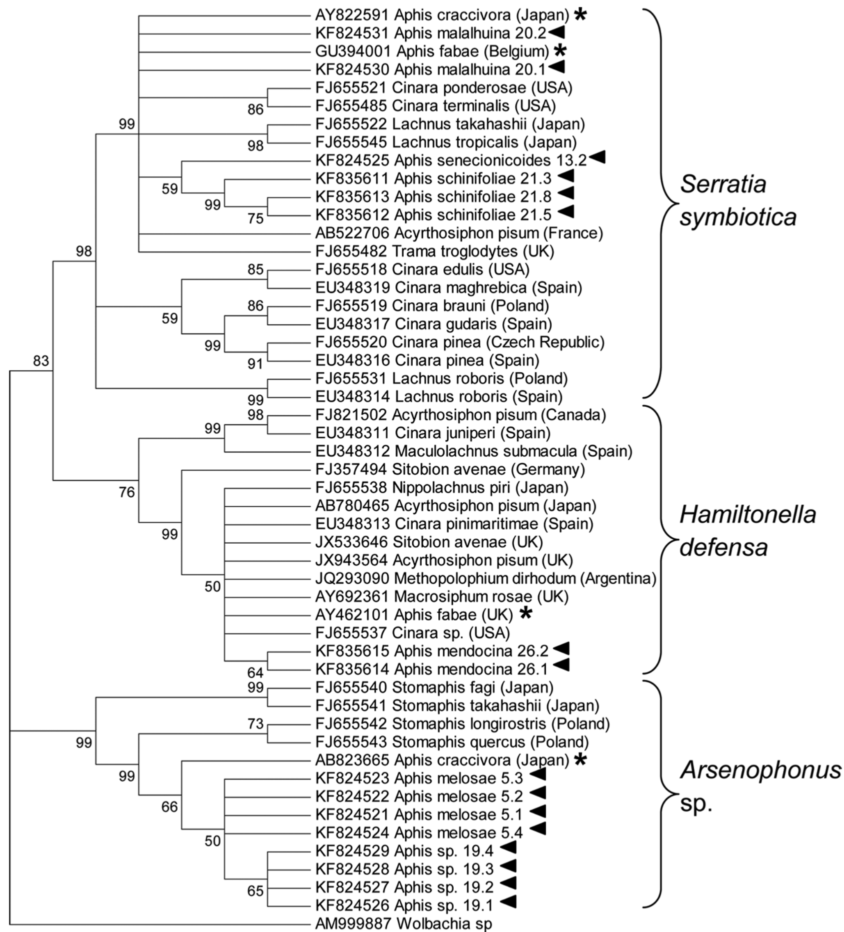

Fig. 1. Phylogenetic analysis of 16S rRNA gene of secondary symbionts found in native South American species of *Aphis* collected in Mendoza, Argentina (arrowheads), and previously reported sequences of *S. symbiotica*, *H. defensa*, and *Arsenophonus* sp. infecting *Aphididae*. Asterisks highlight species in the genus *Aphis*, other than those explored in the current study. Taxonomic names refer to host species, except for *Wolbachia* (outgroup), and collection countries appear in parentheses. For the sequences obtained in this work, clone numbers are shown. GenBank accession numbers are given at the branch tips. Branches with <50% bootstrap support are collapsed. Bootstrap values are indicated at the nodes (percentages after 1000 replicates).

Discussion

The molecular and functional characterization of the facultative microbiota associated with aphids and other hemipterans has received increasing attention during the last decade. A significant effect on insect development and survival has been demonstrated for some of the secondary endosymbionts of aphids identified so far. For instance, *H. defensa* has been shown to protect the pea aphid *Acyrthosiphon pisum* (Harris) against parasitoid wasps, while *S. symbiotica* has been reported to improve the fitness of *A. pisum* after heat stress (Oliver et al. 2010). Although their function in other aphid species needs to be further examined, facultative endosymbionts can be regarded as playing a key ecological role. Thus, it is relevant to study their occurrence in a variety of aphid species colonizing different agroecosystems. This article constitutes the first molecular screening of bacterial symbionts of aphids native to South America. Despite being relatively isolated (both in terms of geographic location and coexistence with introduced species), the survey performed on seven autochthonous species in the *Aphis* genus collected in natural landscapes in western Argentina failed to detect new bacteria other than those already described for the aphids of Holarctic origin. Indeed, this study revealed the presence of *H. defensa* and *S. symbiotica* in one and three of the *Aphis* species analyzed, respectively. These results showed that the later endosymbiont is widespread among native *Aphis* populations, at least in the sampled area. Regarding the bacterium in the *Arsenophonus* clade, which was found in two of the species evaluated, a recent study published during the course of this research reported *Arsenophonus* infections to be common in *Aphis* species (Jousselin et al. 2013). The high incidence recorded at that time (31%) is in agreement

with the results presented here in *Aphis* spp. collected in a distant region of the globe. Although members of the *Arsenophonus* group have been detected in several plant and insect hosts, their phenotypic effects in aphids are still unknown (Bressan et al. 2009, Jousselin et al. 2013, Brady and White 2013). No mixed infections were recorded in the current study, but this possibility cannot be excluded, as different populations from the same aphid species can differ in the composition of their microbiota (Oliver et al. 2010, Brady and White 2013). The same goes for the absence of secondary symbionts in *A. danielae.* A larger survey should be performed to elucidate these issues. New samplings should include different aphid populations from a variety of habitats. In that sense, *A. danielae*, *A. malalhuina*, *A. melosae*, and *A. mendocina* are rather restricted to the provinces of Mendoza and Neuquén (central western Argentina), while *A. schinifoliae* has a wider distribution comprising the Argentine pampas and up to Bolivia. In turn, *A. senecionicoides* is found to the south throughout the Patagonian region (J. O., unpublished data).

The phylogenetic studies conducted on the highly conserved bacterial 16S rRNA gene clustered together the bacterial clones corresponding to each aphid host analyzed. When including the more variable IGS region, a considerable intraspecific genetic diversity was found in the endosymbionts hosted by the different aphid species (not shown). Besides, 16S rRNA sequences of *Arsenophonus* sp. infecting native *A. melosae* and *Aphis* sp. were more similar among them than to any other known sequences of this bacterium genus available at GenBank. This was also the case of *S. symbiotica* harbored by *A. senecionicoides* and *A. schinifoliae*, although *S. symbiotica* from *A. malalhuina* was less closely related. The few published bacterial sequences from *Aphis* spp. in the Northern Hemisphere were relatively not very distant from those reported in this article, but much more information is required to come to a valid conclusion on this subject. Additional sequence data would be necessary to better assess the significance of the taxonomic position of the host, its geographic situation on the composition of the insect's facultative microbiota, or both. Furthermore, as it has been proposed for *Arsenophonus*, new sets of genes other than the ribosomal RNA genes should be incorporated into the phylogenetic analysis of the bacterial lineages (Jousselin et al. 2013). This, together with an insect phylogeny of the native South American *Aphis* spp, would help to reconstruct the coevolutionary history of these aphid–symbiont associations and thus to improve the current understanding of the processes that shaped such interactions.

This article represents a valuable contribution to the literature on the nature of the aphid–endosymbiont associations, as this is, to the author's knowledge, the first description of the bacterial symbionts of non-Holarctic aphid species. Future work will focus on the characterization of the microbiota harbored by different aphid genera exclusive to the Neotropical region.

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