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ORIGINAL ARTICLE

Bacteriomes of the corn leafhopper, *Dalbulus maidis* (DeLong & Wolcott, 1923) (Insecta, Hemiptera, Cicadellidae: Deltocephalinae) harbor *Sulcia* symbiont: molecular characterization, ultrastructure, and transovarial transmission

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Abstract In this study, we surveyed the bacteriome-associated microbiota of the corn leafhopper *Dalbulus maidis* by means of histological, ultrastructural, and molecular analyses. Amplification and sequencing of 16S rDNA genes revealed that the endosymbiont "*Candidatus* Sulcia muelleri" (Phylum Bacteroidetes) resides in bacteriomes of *D. maidis*. Phylogenetic analysis showed that the sequence was closely allied to others found in representatives of the subfamily Deltocephalinae. We failed to amplify other sequences as "*Candidatus* Nasuia deltocephalinicola," a co-primary symbiont frequently associated to deltocephaline leafhoppers. In addition, a metagenetic analysis carried out in order to investigate the presence of other bacteriome-associated bacteria of *D. maidis* showed that the sequence of *Sulcia* accounted for 98.56 % of all the sequences. Histological and ultrastructural observations showed that microorganisms harbored in bacteriomes (central syncytium and cytoplasm of uninucleate bacteriocytes) look like others *Sulcia* described in hemipteran species and they were transovarially transmitted from mother to offspring which is typical of obligate endosymbionts. The only presence of *Sulcia* in the bacteriomes of *D. maidis* was discussed.

Keywords "*Candidatus* Sulcia muelleri" · Bacteriomes · Corn stunt · Corn leafhopper · Obligate endosymbionts

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Introduction

Most members of the hemipteran suborder Auchenorrhyncha (leafhoppers, cicadas, froghoppers, treehoppers, and planthoppers) feed mainly on plant sap (xylem or phloem), which leads to a notoriously unbalanced nutrition (Moran 1998; Sandstrom and Moran 1999). Mutualistic associations with microorganisms (bacteria or yeasts) provide them with essential amino acids and/or vitamins that complement their diet (Buchner 1965; McCutcheon and Moran 2007; McCutcheon et al. 2009).

Leafhoppers, belonging to the family Cicadellidae, establish obligate symbioses with bacteria that live in the cytoplasm of specialized host cells (bacteriocytes) that form structures known as bacteriomes (Buchner 1965; Baumann 2005; Baumann et al. 2006). Obligate symbionts living within bacteriomes widely distributed in a clade of hosts are referred to as primary symbionts (Baumann 2005) and they are vertically transmitted through infected eggs (Buchner 1965; Moran et al. 2003, 2005; Michalik et al. 2009; Kobialka et al. 2015a). The mutual dependence of endosymbionts and leafhoppers and the way they are transmitted to the offspring lead to their coevolution (Moran et al. 2003; Moran et al. 2005; Takiya et al. 2006; Moran 2007; Bennett and Moran 2013). Mutualistic interactions were established a long time ago through the initial

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infection of an ancestor of the insect group by a free-living microorganism (Baumann 2005; Baumann et al. 2006).

An obligate endosymbiont that is widespread in Auchenorrhyncha and has been documented in hosts from Fulgoroidea, Cicadoidea, Cercopoidea, and Membracoidea (which includes leafhoppers) is "*Candidatus* Sulcia muelleri" (Bacteroidetes) (hereafter *Sulcia*). This symbiont is highly conserved and exhibits a drastic genome reduction (Moran et al. 2005; McCutcheon et al. 2009). Phylogenetic relationships among *Sulcia* strains identified in diverse auchenorrhynchan hosts are congruent with host's phylogenies suggesting that these associations and cospeciation dated back to 260 million years ago from the time these insects emerged (Moran et al. 2005). More recently, Koga et al. (2013) mentioned that the common ancestor of Cicadomorpha and Fulgoromorpha was also infected by a betaproteobacterial symbiont. However during evolution of some hemipterans lineages, the betaproteobacterium was replaced by other bacteria, e.g., gammaproteobacterium (*Baumannia*) in some Cicadellinae and alphaproteobacterium (*Hodgkinia*) in cicadas; therefore, *Sulcia* can coexist, depending on the particular host, with an array of co-primary symbionts belonging to different bacterial divisions (Moran et al. 2003; Takiya et al. 2006; Urban and Cryan 2012; Bennett and Moran 2013; Ishii et al. 2013; Koga et al. 2013; Kobialka et al. 2015a).

The term co-primary symbiont has been referred to as the obligate symbionts that co-occur inside bacteriomes (Moran et al. 2003; Takiya et al. 2006). Recent genomic analysis revealed that these symbionts have the smallest known bacterial genomes, ranging between 112 and 245 kb (Wu et al. 2006; Bennett and Moran 2013; Chang et al. 2015), and interestingly, it was found that the bacteria living in bacteriomes have complementary sets of pathways (Wu et al. 2006; McCutcheon and Moran 2007, 2010; McCutcheon et al. 2009).

Recently, based on the 16S rRNA sequence, bacteriome-associated endosymbionts were identified among species of the subfamily Deltocephalinae (phloem sap-feeders) such as *Nephotettix cincticeps* (Noda et al. 2012); *Matsumuratettix hiroglyphicus* (Wangkeeree et al. 2012), *Macrosteles striifrons*, *Macrosteles sexnotatus* (Ishii et al. 2013), *Macrosteles laevis* (Kobialka et al. 2015a), and *Deltocephalus pulicaris* (Kobialka et al. 2015b). In these species, "*Candidatus* Sulcia muelleri" was reported as a primary symbiont that coexists, within bacteriomes, with a co-primary endosymbiont: "*Candidatus* Nasuia deltocephalinicola" (Betaproteobacteria) (hereafter *Nasuia*). Additionally, minor sequences of other bacteria have been found within bacteriomes of deltocephaline leafhoppers such as a *Rickettsia* species associated with *N. cincticeps* (Noda et al. 2012) and gammmaproteobacteria *Arsenophonus* inside *Sulcia* cells in *Macrosteles laevis* by Kobialka et al. (2015a). Remarkably, it has been found that in some deltocephaline leafhoppers, as occur in some planthopper families, bacterial symbionts have been replaced by yeast symbionts (Noda 1977; Marzorati et al. 2006; Sacchi et al. 2008; Michalik et al. 2009).

The advent of high-throughput next-generation sequencing (NGS) technologies brought new tools to study the genomics at their highest depths at relatively low prices. So far, this new technology was used to sequence genomes of obligate symbionts such as *Sulcia* and *Nasuia* of the Deltocephalinae *Macrosteles quadrilineatus* and *Macrosteles quadripunctulatus* (Bennett and Moran 2013 and Bennett et al. 2016) and *Sulcia* genoma of *Dalbulus maidis* (Chang et al. 2015). Sequencing technologies-based techniques such as metagenetics allow the analysis of the biodiversity of complex genomic samples that might include DNA from both culturable and unculturable organisms. Amplicons of high-throughput partial 16S rDNA are particularly useful to identify unculturable organisms. Therefore the metagenetic analysis is a powerful molecular tool to identify endosymbiotic microorganisms inhabiting bacteriomes, where more than one unculturable bacterial symbiont could co-exist.

The corn leafhopper, *D. maidis* (DeLong & Wolcott) (Cicadellidae: Deltocephalinae), is the major pest of maize *Zea mays* L. in the Americas. It is widely distributed from southern USA to central areas of Argentina (Nault 1990) and becomes a serious pest mainly in subtropical areas (Giménez Pecci et al. 2002; Virla et al. 2004; Carloni et al. 2013). Not much research has been done on the characterization of endosymbionts inhabiting bacteriomes of leafhoppers from the genus *Dalbulus*. As far as we know, only one report described the bacteriomes in *Dalbulus elimatus*, based on morpho-physiological analysis (Galindo Miranda 1994).

Considering that several Auchenorrhyncha have been found associated with an ancient clade of the endosymbiont Bacteroidetes and other bacterial co-primary symbionts, our hypothesis states that the corn leafhopper, *D. maidis*, has bacteriomes containing Bacteroidetes and/or other microorganisms. Therefore, the purpose of this study was to look for the presence of bacteriomes in specimens of *D. maidis*, to survey their associated microbiota and to analyze the phylogenetic relationships with other endosymbionts of sap-feeding insects. In addition, the ultrastructure of endosymbionts and their mode of transmission were examined.

Materials and methods

Insects were collected during samplings performed in 2009–2010 on maize grown at "El Manantial" (26°50′03, 41S–65°16′30, 62 W 435), an area within Chaco Subhúmedo (Tucumán province, Argentina). Insects were preserved in 96° ethanol and stored at −20° until they were dissected for molecular studies. Some adults of *D. maidis* collected from the field were used to establish a colony under controlled conditions (L16/D8 photoperiod; 24 ± 1 °C; 40–50 % RH) in a rearing

room at División Entomología, Facultad de Ciencias Naturales y Museo, UNLP. Females at reproductive stage from the colony were used for light and electron microscopy analyses.

Genomic DNA preparation

One hundred bacteriome-like structures from 50 *D. maidis* females were obtained through the following procedure. Surface microorganisms were removed by sterilizing individuals with 70 % EtOH and 6 % sodium hypochlorite for 3 min, followed by three washes with sterilized water. Females were then dissected with fine needles under a stereomicroscope and bacteriome-like structures were placed into 96 % ethanol until processed later. Total genomic DNA was extracted with DNeasy Blood and Tissue Kit (QIAGEN, GmbH, Germany). Bacteriomes were crushed with a sterile iron pestle and homogenized in the buffer provided by the kit and the extraction followed the manufacturer's instructions. The quality and quantity of genomic DNA were assessed by electrophoresis in 0.7 % agarose gel stained with ethidium bromide. Gels were photographed and analyzed with an image analyzer. Extracted DNA was stored at −70 °C until analysis.

16S rDNA diagnostic PCR and sequencing

The polymerase chain reaction (PCR) for detection of symbionts of *D. maidis* was performed using specifics primers. The presence of *Sulcia* symbiont was assessed using the primers 10_CFB FF (5′-AGA GTT TGA TCA TGG CTC AGG ATG-3′) and 1515_R (5′-GTA CGG CTA CCT TGT TAC GAC TTA G-3′) based on the protocol developed by Moran et al. (2005). We also run reactions aimed at amplifying *Nasuia* symbiont (Betaproteobacteria) using the primers NcBeta_16S/f1 (5′-AAG GAT AAA AGC GGG GAA AAC C-3′) and NcBeta_16S/r1 (5′-ACA CCA CTA AAA AAA ATT TTT AAC AG-3′) (Noda et al. 2012). Total DNA, extracted from bacteriomes of specimens of *N. cincticeps* harboring *Nasuia* (kindly provided by Dr. Hiroaki Noda), was included in the reactions as a positive control. Reactions were performed in 25 μl volume containing 50 ng of template DNA, 12 pmol of forward and reverse primers, 2.5 μl 10× reaction buffer (500 mM KCl; 100 mM Tris–HCl, pH 9.0 a 25 °C; 1 % Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 units of Taq polymerase (Inbio Highway®, Buenos Aires, Argentina). The thermocycler (PTC-0150 MiniCycler; MJ. Research. Watertown, MA, USA) was programmed as follows. (a) For the former reaction, an initial denaturation step at 94 °C for 2 min; followed by 35 cycles of a denaturing step at 94 °C for 1 min, an annealing step at 58 °C for 1 min, and an extension step at 72 °C for 2 min, followed by a final extension step at 72 °C for 6 min. (b) For the latter reaction, a denaturing step at 94 °C for 2 min followed by 35 cycles of a denaturing step at 94 °C for 30 s, an annealing step at 52 °C for 30 s, and an extension step at 72 °C for 2 min, followed by a final extension step at 72 °C for 6 min. PCR products were resolved by 1 % agarose gel electrophoresis stained with ethidium bromide and visualized by UV illumination.

The amplicons were precipitated by adding 1 vol of isopropanol and 0.1 vol of Na Ac. The DNA was sequenced by the dideoxy termination method (Sanger et al. 1977) using the BigDye Terminator Cycle Sequencing Ready Reaction kit and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems, Macrogen, Seoul, Korea).

16S rDNA metagenetic analysis

An Illumina-based 16S rDNA amplicon diversity study was performed (Mr. DNA Shallowater, TX, USA). Bacterial 16S rDNA of total DNA from bacteriomes, isolated as described before, was amplified using primers 27F (5′-AGRGTTTG ATCMTGGCTCAG-3′) and ill519R (GTNTTACNGCG GCKGCTG) with barcode on the forward primer. A single-step 30-cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) was carried out with the following cycling program: denaturation at 94 °C for 30 s, annealing at 53 °C for 40 s, and elongation at 72 °C for 1 min for 28 cycles. The PCR reaction was resolved in 2 % agarose gel electrophoresis and amplicons were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, Danvers, MA, USA). Libraries were prepared with a TruSeq DNA library preparation kit. Sequencing was performed using an Illumina MiSeq 2000 sequencing system at MR DNA (www.mrdnalab.com, Shallowater, TX, USA). Data derived from sequencing were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Briefly, sequences were depleted of barcodes and primers and sequences <150 bp as well as sequences with ambiguous base calls were removed. Sequences were denoised and chimeras were removed. Operational taxonomic units (OTUs) were defined by clustering at 3 % divergence (97 % similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII, and NCBI (www.ncbi.nlm.nih.gov; DeSantis et al. 2006; http://rdp.cme.msu.edu) and compiled into each taxonomic level.

Phylogenetic analysis

Phylogenetic analyses were performed using the obtained sequence of 16S rDNA with the primers describe by Moran et al. (2005) and similar sequences available at the GenBank database (Moran et al. 2005; Takiya et al. 2006; Noda et al. 2012; Wangkeeree et al. 2012; Ishii et al. 2013; Kobialka et al. 2015a).

Sequences were aligned using the ClustalW (Larkin et al. 2007) and the multiple alignment tool included in the Geneious 9.1.2 package (Biomatters Ltd, Auckland, New Zealand). The alignment was automatically curated using

Gblocks 0.91b (Talavera and Castresana 2007) using default settings except for the minimum length of a block which was set to 2. Best-fit model of evolution was selected with jModelTest 2.1.7 (Darriba et al. 2012) and data matrices were analyzed under maximum likelihood criteria in PhyML 3.0 (Guindon and Gascuel 2003). The support of the groups within the tree was evaluated through bootstrap with 1000 replications (Felsenstein 1985).

Light and electron microscopy

Ten females at reproductive stage (4–6 days old) from the colony were fixed in glutaraldehyde 2 %, postfixed in osmium tetroxide 1 %, dehydrated in ethanol series (50–100 %), and embedded in Epoxy resin for 36 h at 35, 50, and 60 °C. Serial semithin sections (2–3 μm) were obtained by cutting them with a diamond knife in an Ultracut Reichert J-Supernova ultramicrotome. Then, samples were stained with 0.05 % O-Toluidine blue and examined with a light microscope Nikon YS2-H equipped with a digital camera Nikon D40. Ultrathin sections (60 nm) were mounted on copper grids, contrasted with uranyl acetate and lead citrate, and observed with a Jeol JEM 1200 EX II electron microscope. The micrographs were taken with a digital camera Erlangshen ES 1000W.

Results

Bacteriome-like structures

All the specimens of *D. maidis* observed with the stereomicroscope presented bacteriome-like structures that were bilaterally paired and located in the lateral margins of the first and second abdominal segments (Fig. 1a). These structures were bean-shaped, small (0.4–0.6 mm long), yellow colored (Fig. 1b), and supplied with abundant tracheae.

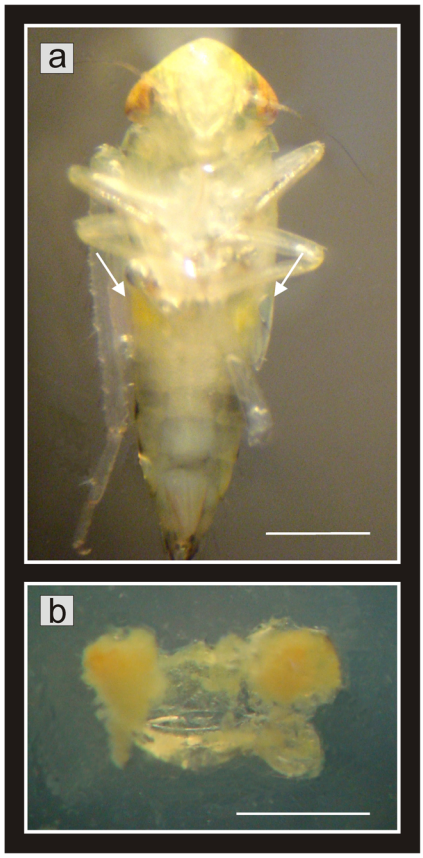

Fig. 1 Bacteriome-like structures in *D. maidis*. **a** Habitus of an adult female showing the localization on each side of the abdomen (*arrows*). *Scale bar* = 1 mm; **b** yellow-colored dissected bacteriome-like structures. *Scale bar* = 0.6 mm

16S rDNA and phylogenetic analysis

PCR reaction using the primers 10_CFB FF and 1515_R amplified a 1368-bp-long DNA fragment that corresponded to the 16S rDNA of the putative endosymbiont of *D. maidis*. The sequence was annotated at the NCBI database (JX514697). A Blast analysis showed that the sequence was 99 % similar to those of the Bacteroidetes *Sulcia*. Additionally, PCR reactions with primers designed to amplify 16S sequences homologous to *Nasuia* only gave a positive result when the DNA extracted from bacteriomes of *N. cincticeps* was used as a control.

Phylogenetic analysis of the 16S rDNA sequences of *Sulcia* symbiont of various representatives of Cicadomorpha and one representative of Fulgoromorpha generated a tree showing that the sequence of *Sulcia* from *D. maidis* was closely related to *Sulcia* endosymbionts from leafhoppers species in the same subfamily, Deltocephalinae (Fig. 2).

16S rDNA metagenetic analysis

Since other auchenorrhynchans harbor more than one endosymbiont within bacteriomes, we made a metagenetic analysis of bacteriome's DNA using 16S rDNA primers as described in materials and methods and because of this we called it metaribosomic analysis. Cleaning raw data rendered a total number of 77.518 sequences, which were organized in 102 OTUs. The sequence of *Sulcia* was highly represented and accounted for 98.56 % of all sequences. The remaining 1.44 % of sequences included 101 OTUs different from *Sulcia* and, among these low-abundant sequences, we did not find the presence of any other reported Deltocephalinae endosymbiont nor any other closely related organism. These sequences were considered contaminants and might correspond to sequences of microorganisms that make up the microbiome of the insect and microorganisms acquired during the processing of samples (Fig. 3).

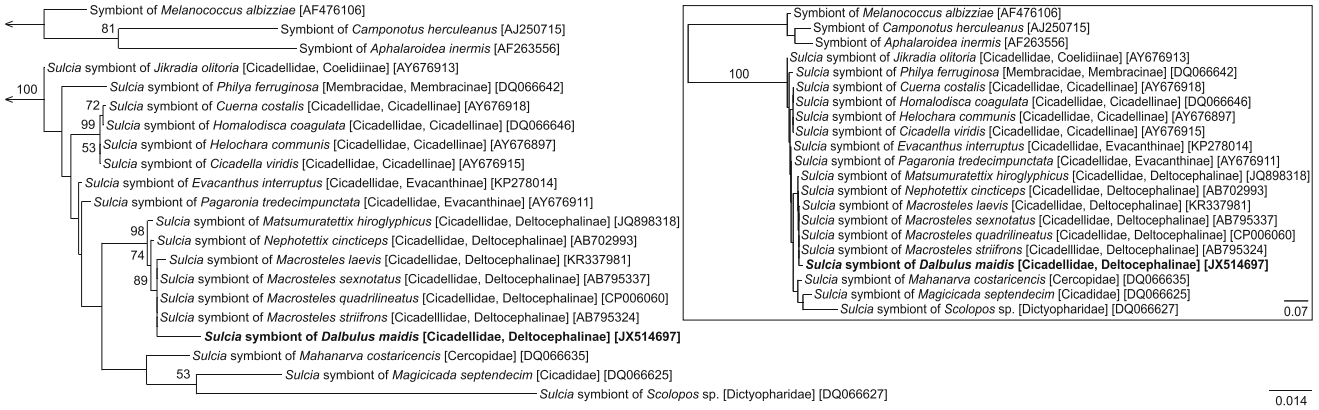

Fig. 2 Maximum likelihood (ML) tree based on 16S rDNA gene sequences of the Bacteroidetes symbiont "*Candidatus* Sulcia muelleri" obtained from bacteriomes of *D. maidis* (*in bold*) and others selected sequences of auchenorrhynchan species. The name of the host species and family and subfamily (*in brackets*) are shown as each taxon label. GenBank accession numbers of sequences of 16S rRNA for symbionts are given *in brackets* too. Outgroups included a secondary symbiont of *Melanococcus albizziae* (Maskell, 1892) (Hemiptera: Pseudococcidae) (AF476106) and *Aphalaroidea inermis* Crawford, 1914 (Hemiptera: Psyllidae) (AF263556) and "*Candidatus* Blochmannia herculeanus" endosymbiont of *Camponotus herculeanus* (Linnaeus, 1758) (Formicidae) (AJ250715). Bootstrap values (%) were obtained from a search with 1000 replicates. *Numbers above nodes* indicate bootstrap values with more than 50 % support

Light and electron microscopy analyses

Bacteriomes of *D. maidis* were composed of a monolayered epithelium with large translucent cells with large nucleus and nucleolus, a syncytium, and an aggregate of uninucleate cells (Fig. 4a–c). The organisms localized in the syncytium stained intensely with methylene blue. Ultrastructural studies revealed large (3–4 μm wide and 8–12 μm long), irregular in shape, electron-dense, pleomorphic organisms that are found in close vicinity to mitochondria (Fig. 5a–c). Organisms quite different in shape and in staining behavior (relative to the cytoplasm and the nucleus) filled the cytoplasm of uninucleate cells (Fig. 5c); these organisms were surrounded by membranes and presented an electron-dense body in their cytoplasm (Fig. 5d).

In semithin sections of mature females, we observed that bacteriomes were located close to the ovaries. Ovaries of *D. maidis* are composed by six ovarioles (for further details concerning morphology of the reproductive system of *D. maidis*, see Tsai and Perrier 1996). In the terminal part of each ovariole, there was an oocyte at the stage of advanced vitellogenesis (Fig. 6a). In addition, different stages of transovarial transmission of symbiotic bacteria were observed. Bacteria gathered at the terminal syncytial zone of the bacteriome, leave the cytoplasm of the syncytia (Fig. 6a, b), and invade the posterior pole of oocytes through the follicular cells (Fig. 6c).

Discussion

This is the first study describing bacteriome-associated endosymbionts of *D. maidis*, a leafhopper vector of three important phytopathogens: mollicutes *Spiroplasma kunkelli*, *Maize bushy stunt phytoplasma*, and *Maize rayado fino virus* that either alone or in combination are the causative agents of "corn stunt," a disease complex that has become a limiting factor for maize production in tropical and subtropical America (Nault and Ammar 1989; Oliveira et al. 1998; Summers et al. 2004; Virla et al. 2004; Carloni et al. 2013).

The morphology and localization of the structures described within all specimens of *D. maidis* as well as their similar appearance to others described for deltocephaline leafhoppers such as *Helochara communis* (Cicadellidae) (Chang and Musgrave 1972), *D. elimatus* (Galindo Miranda 1994), *N. cincticeps* (Noda et al. 2012), and *Macrosteles laevis* (Kobialka et al. 2015a) lead us to conclude that the structures observed in *D. maidis* are typical of bacteriomes that host endosymbiotic organisms.

Amplification and sequencing of the 16S rDNA using bacteriomes DNA as a template showed that bacteriomes host *Sulcia* and the sequence was 99 % similar to other Bacteroidetes. Furthermore, the *Sulcia* sequence of *D. maidis* belongs to the same clade that includes *Sulcia* from other Deltocephalinae leafhoppers such as *Matsumuratettix hiroglyphicus*, *N. cincticeps, Macrosteles* sp., and *Macrosteles laevis* reported by Wangkeeree et al. (2012), Noda et al. (2012), Ishii et al. (2013), and Kobialka et al. (2015a), respectively, reflecting the host-symbiont phylogenetic concordance. Molecular studies of symbionts associated with the three genera of the deltocephalines mentioned above showed that bacteriomes harbor bacteroidetes *Sulcia* and the betaproteobacterial *Nasuia* as well. Besides, other minor sequences of bacteria have been found (Noda et al. 2012; Ishii et al. 2013; Kobialka et al. 2015a, b). We unsuccessfully tried to amplify through PCR the 16S rDNA of *Nasuia*. Accordingly,

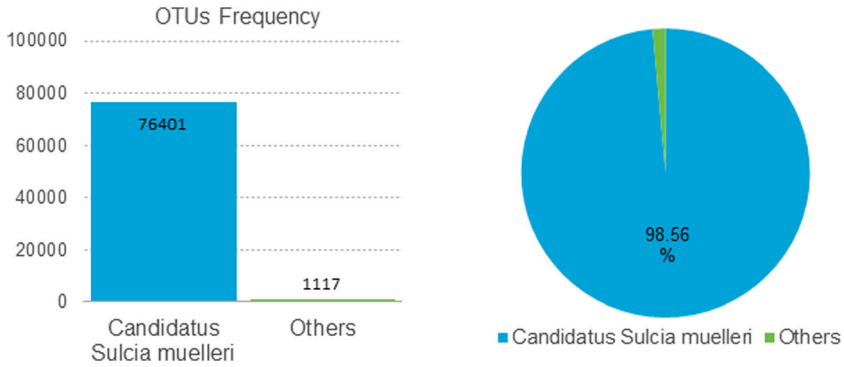

Fig. 3 Frequency of operational taxonomic units (OTUs) obtained by a 16S rDNA metagenetic analysis from DNA obtained from bacteriomes of *D. maidis*. Others refer to contaminants microorganisms

the metaribosomic analysis of the bacteriome revealed only the presence at a high frequency of *Sulcia* symbiont.

Morphology is additionally an important feature to characterize endosymbionts. In general, primary symbionts are distinguished by their unusually large cell size and pleomorphic shape (Baumann 2005; Takiya et al. 2006). Bacteria within bacteriomes of *D. maidis* had morphological characteristics typical of obligate endosymbionts and particularly they were similar to other *Sulcia* symbionts already described in the deltocephalines *N. cincticeps* (Noda et al. 2012), *Macrosteles laevis* (Kobialka et al. 2015a), and *Deltocephalus pulicaris* (Kobialka et al. 2015b). The morphology of the bacteria inhabiting bacteriomes of *D. maidis* was also similar to the "a-symbiont" present in the syncytium of the mycetome of *H. communis* described earlier by Chang and Musgrave (1972) and *Graphocephala coccinea* (Hemiptera: Jassidae) by Kaiser (1980). In species of Cicadellidae, co-primary symbionts were found residing in different bacteriocytes (Noda et al. 2012; Ishii et al. 2013; Szklarzewicz et al. 2016) or co-residing in the same bacteriocyte (Michalik et al. 2014). In agreement with our molecular results, we found that the syncytial zone of bacteriomes of *D. maidis* and the cytoplasm of uninucleate bacteriocytes harbored bacteria that look like *Sulcia*. Furthermore, the fact that symbionts appearing in micrographs as if they were leaving the syncytial zone of bacteriomes of *D. maidis*, apparently migrating to the posterior pole of oocytes and entering through follicular cells, suggests that they are vertically transmitted from mother to offspring, which is the typical mode of transmission of obligate symbionts (Buchner 1965; Moran et al. 2003, 2005; Sacchi et al. 2008; Michalik et al. 2009; Szklarzewicz et al. 2016).

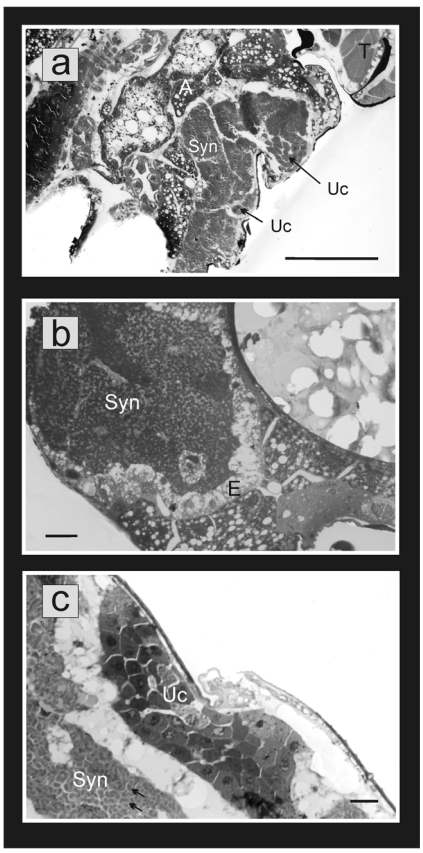

Fig. 4 Light micrographs of a bacteriome-like structure of *D. maidis*. **a** Localization in the abdomen and general appearance showing the syncytial tissue and the uninucleate cells. *Scale bar* = 0.25 mm; **b** detail of the monolayered epithelium and the syncytial tissue; **c** detail of irregular and large size organism in the syncytial zone (*arrows*) and uninucleate cells. *Scale bar* = 25 μm. *A*, abdomen; *T*, thorax; *E*, epithelium; *Syn*, syncytial tissue; *Uc*, uninucleate cells

Although *Sulcia* has frequently been found coexisting with a co-primary symbiont in Deltocephalinae species, this study suggest that it might be the only symbiont-associated bacteriome in *D. maidis*. The following can be regarded as evidences of this outcome. Firstly, Bennett and Moran (2013) mentioned that replacements and losses of symbionts sometimes occurred in phloem-feeding lineages. This is the case of the deltocephaline *Scaphoideus titanus*, a species lacking both *Sulcia* and *Nasuia* symbionts but possessing transovarially transmitted *Cardinium* (Bacteroidetes) and yeast-like symbionts instead, which are related to those found in certain lineages of planthoppers and aphids (Sacchi et al. 2008). Secondly, we failed to amplify any endosymbionts other than *Sulcia*. Lastly, the metaribosomic approach proved that *Sulcia* is likely the only microorganism inhabiting bacteriomes of *D. maidis*. Thus, we suggest that the dual symbiotic "*Sulcia*-betaproteobacteria" system might not be a rule for Deltocephalinae leafhoppers.

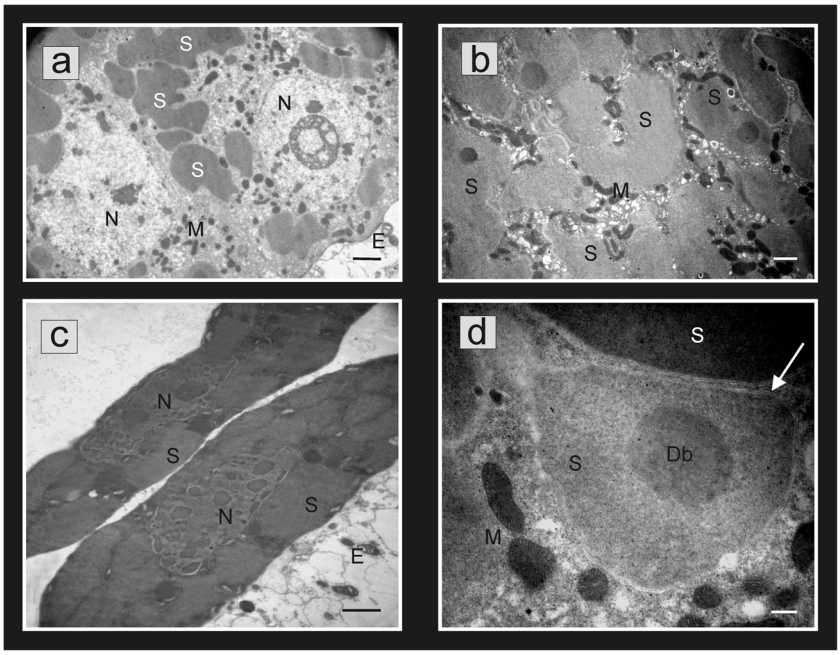

Fig. 5 Transmission electron microscopy of the bacteriome-like structures of *D. maidis*. **a**, **b** Syncytium with numerous symbionts and mitochondria. *Scale bar* = 2 μm; **c** uninucleate cells with symbionts. *Scale bar* = 2 μm; **d** endosymbiont showing a dense body and peripheral membranes (*arrow*). *Scale bar* = 0.4 μm. *N*, nucleus; *S*, symbiont; *E*, epithelium; *M*, mitochondrion; *Db*, dense body

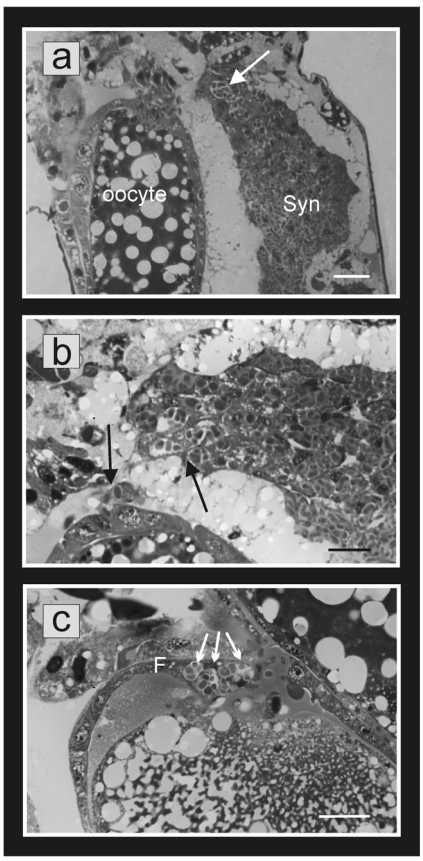

Fig. 6 Light micrographs showing different stages of the transovarial transmission of symbiotic bacteria in *D. maidis* (**a**) symbionts (*arrows*) in the terminal syncytial zone of the bacteriome. *Scale bar* = 50 μm; **b** detail of the symbionts in the terminal zone of the bacteriome and near the follicular cells of the terminal oocyte. *Scale bar* = 25 μm; **c** symbionts (*arrows*) enter the posterior pole of the oocyte through follicular cells. *Scale bar* = 50 μm. *F*, follicular cells, *Syn*, syncytium

Bennett and Moran (2013) found that *Sulcia* genomes characterized to date have a size ranging from 191 to 277 kb and differ in their genes for biosynthesis of seven or eight essential amino acids; for the remaining essential amino acids, insect hosts have acquired other lineage-specific symbionts (McCutcheon et al. 2009; McCutcheon and Moran 2010). Recently, Chang et al. (2015) identified the complete genome sequence of *Sulcia* ML strain from whole *D. maidis* specimens from Brazil. Since we found that *Sulcia* appears to be the only symbiont within bacteriomes of this species, we are currently planning to sequence its genome in order to carry out a comparative genetic analysis with other organisms including *Sulcia* found in *D. maidis* from Brazil by Chang et al. (2015). Furthermore, larger samples of *D. maidis* populations as well as other species of *Dalbulus* genus should be obtained in order to identify if other symbionts coexist with the primary symbiont *Sulcia*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Baumann P (2005) Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59:155–189

Baumann P, Moran NA, Baumann L (2006) Bacteriocyte-associated endosymbionts of insects. Prokaryotes 1:403–438

Bennett GM, Moran NA (2013) Small, smaller, smallest: the origins and evolution of ancient dual symbioses in a Phloem-feeding insect. Genome Biol Evol 5:1675–1688

Bennett GM, Abbà S, Kube M, Marzachì C (2016) Complete genome sequences of the obligate symbionts "*Candidatus* Sulcia muelleri" and "*Ca.* Nasuia deltocephalinicola" from the pestiferous leafhopper *Macrosteles quadripunctulatus* (Hemiptera: Cicadellidae). Genome Announc 4:e01604–e01615. doi:10.1128/genomeA.01604-15

Buchner P (1965) Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York

Carloni E, Carpane P, Paradell S, Laguna I, Giménez Pecci MP (2013) Presence of *Dalbulus maidis* (Hemiptera: Cicadellidae) and of *Spiroplasma kunkelii* in the temperate region of Argentina. J Econ Entomol 106:1574–1581

Chang KP, Musgrave AJ (1972) Multiple symbiosis in a leafhopper, *Helochara communis* Fitch (Cicadellidae: Homoptera): envelopes, nucleoids and inclusions of the symbiotes. J Cell Sci 11:275–293

Chang H, Cho ST, Canale MC, Mugford ST, Lopes JR, Hogenhout SA, Kuo CH (2015) Complete genome sequence of "*Candidatus* Sulcia muelleri" ML, an obligate nutritional symbiont of maize leafhopper (*Dalbulus maidis*). Genome Announc 3:e01483–14. doi:10.1128/genomeA.01483–14

Darriba D, Taboada GL, Doallo R, Posada D (2012) Model Test 2: more models, new heuristics and parallel computing. Nat Methods 9:772

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72:5069–5072

Felsenstein J (1985) Confidence limits on phylogenies: an approach using bootstrap. Evolution 39:783–791

Galindo Miranda N (1994) Los micetomas, un análisis morfofisiológico de su interacción con los Cicadellidae (Homoptera). Folia Entomol Mex 92:1–8

Giménez Pecci MP, Laguna I, Ávila AO, de Remes Lenicov AMM, Virla E, Borgogno CF, Nome G, Paradell S (2002) Difusión del Corn Stunt Spiroplasma del maíz (*Spiroplasma kunkelii*) y del vector (*Dalbulus maidis*) en la República Argentina. Rev Fac Agron La Plata 105:1–8

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704

Ishii Y, Matsuura Y, Kakizawa S, Nikoh N, Fukatsua T (2013) Diversity of bacterial endosymbionts associated with *Macrosteles* leafhoppers vectoring phytopathogenic phytoplasmas. App Environ Microbiol 79:5013–5022

Kaiser B (1980) Licht- und elektronenmikroskopische unter-suchlung der symbioten von *Graphocephala coccinea* Forstier (Homoptera: Jassidae). J Insect Morphol Embryol 9:79–88

Kobialka M, Michalik A, Walczak M, Lz J, Szklarzewicz T (2015a) *Sulcia* symbiont of the leafhopper *Macrosteles laevis* (Ribaut, 1927) (Insecta: Hemiptera:Cicadellidae: Deltocephalinae) harbors *Arsenophonus* bacteria. Protoplasma 253:903–912

Kobialka M, Michalik A, Walczak M, Lz J, Szklarzewicz T (2015b) Symbiotic microorganisms of the leafhopper *Deltocephalus pulicaris* (Fallén, 1806) (Insecta: Hemiptera: Cicadellidae: Deltocephalinae): molecular characterization, ultrastructure and transovarial transmission. Pol J Entomol 84:289–304

Koga R, Bennett G, Cryan JR, Moran NA (2013) Evolutionary replacement of obligate symbionts in an ancient and diverse insect lineage. Environ Microbiol 15:2073–2081

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA et al (2007) ClustalW and ClustalX version 2. Bioinformatics 23:2947–2948

Marzorati M, Alma A, Sacchi L, Pajoro M et al (2006) A novel Bacteroidetes symbiont is localized in *Scaphoideus titanus*, the insect vector of Flavescence dorée in *Vitis vinifera*. Appl Environ Microbiol 72:1467–1475

McCutcheon JP, Moran NA (2007) Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci U S A 104:19392–19397

McCutcheon JP, Moran NA (2010) Functional convergence in reduced genomes of bacterial symbionts spanning 200 My of evolution. Genome Biol Evol 2:708–718

McCutcheon JP, McDonald BR, Moran NA (2009) Convergent evolution of metabolic roles in bacterial co-symbionts of insects. Proc Natl Acad Sci U S A 106:15394–15399

Michalik A, Jankowska W, Szklarzewicz T (2009) Ultrastructure and transovarial transmission of endosymbiotic microorganisms in *Conomelus anceps* and *Metcalfa pruinosa* (Insecta: Hemiptera: Fulgoromorpha). Folia Biol (Kraków) 57:131–137

Michalik A, Jankowska W, Kot M, Gołas A, Szklarzewicz T (2014) Symbiosis in the green leafhopper, *Cicadella viridis* (Hemiptera, Cicadellidae). Association in *statu nascendi*? Arthropod Struct Dev 43:579–587

Moran NA (1998) Bacteriocyte-associated symbionts of insects. Bioscience 48:295–304

Moran NA (2007) Symbiosis as an adaptive process and source of phenotypic complexity. Proc Natl Acad Sci U S A 104:8627–8633

Moran NA, Dale C, Dunbar H, Smith WA, Ochman H (2003) Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. Environ Microbiol 5:116–126

Moran NA, Tran P, Gerardo NM (2005) Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial Phylum Bacteroidetes. Appl Environ Microbiol 71:8802–8810

Nault LR (1990) Evolution of an insect pest: maize and the corn leafhopper, a case study. Maydica 35:165–175

Nault LR, Ammar D (1989) Leafhopper and planthopper transmission of plant virus. Annu Rev Entomol 34:503–529

Noda H (1977) Histological and histochemical observation of intracellular yeast-like symbiotes in the fat body of the small brown planthopper, *Laodelphax striatellus* (Homoptera: Delphacidae). Appl Entomol Zool 12:134–141

Noda H, Watanabe K, Kawai S, Yukuhiro F, Miyoshi T, Tomizawa M (2012) Bacteriome-associated endosymbionts of the green rice leafhopper *Nephotettix cincticeps* (Hemiptera: Cicadellidae). Appl Entomol Zool 47:217–225

Oliveira E, Waquil JM, Fernandes FT, Paiva E, Resende RO, Kitajima EW (1998) Enfezamento pàlido e enfezamento vermelho na cultura do milho no Brasil Central. Fitopatol Bras 23:45–47

Sacchi L, Genchi M, Clementi E, Bigliardi E, Avanzati AM, Pajoro M, Negri I, Marzorati M, Gonella E, Alma A (2008) Multiple symbiosis in the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae): details of transovarial transmission of *Cardinium* sp. and yeast-like endosymbionts. Tissue Cell 40:231–242

Sandstrom J, Moran NA (1999) How nutritionally imbalanced is phloem sap for aphids? Entomol Exp Appl 91:203–210

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci U S A 74:5463–5467

Summers CG, Newton AS, Opgenorth DC (2004) Overwintering of corn leafhopper, *Dalbulus maidis* (Homoptera: Cicadellidae), and *Spiroplasma kunkelii* (Mycoplasmatales: Spiroplasmataceae) in California's San Joaquin Valley. Environ Entomol 33:1644–1651

Szklarzewicz T, Grzywacz B, Szwedo J, Michalik A (2016) Bacterial symbionts of the leafhopper *Evacanthus interruptus* (Linnaeus, 1758) (Insecta: Hemiptera: Cicadellidae: Evacanthinae). Protoplasma 253: 379–391

Takiya D, Tran P, Dietrich C, Moran N (2006) Co-cladogenesis spanning three phyla: leafhoppers (Insecta: Hemiptera: Cicadellidae) and their dual bacterial symbionts. Mol Ecol 15:4175–4191

Talavera G, Castresana J (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol 56:564–577

Tsai JH, Perrier JL (1996) Morphology of the digestive and reproductive systems of *Dalbulus maidis* and *Graminella nigrifrons* (Homoptera: Cicadellidae). Fla Entomol 79:563–578

Urban JM, Cryan JR (2012) Two ancient bacterial endosymbionts have coevolved with the planthoppers (Insect: Hemiptera: Fulgoroidea). BMC Evol Biol 12:87

Virla E, Díaz C, Carpane P, Laguna I, Ramallo J, Gómez L, Giménez Pecci MP (2004) Evaluación preliminar de la disminución en la producción de maíz causada por el "Corn Stunt Spiroplasma" (CSS) en Tucumán, Argentina. Bol San Veg Plagas 30:403–413

Wangkeeree J, Miller T, Hanboonsong Y (2012) Candidates for symbiotic control of sugarcane white leaf disease. Appl Environ Microbiol 78:6804–6811

Wu D, Daugherty SC, Van Aken SE, Pai GH et al (2006) Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. Plos Biol 4:e188