# Molecular Phylogeny of the Genus *Dactylopius* (Hemiptera: Dactylopiidae) and Identification of the Symbiotic Bacteria

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**ABSTRACT** Phylogenetic analyses, from polymerase chain reaction (PCR)-amplified 12S rRNA and 18S rRNA gene sequences from cochineal insects of the genus *Dactylopius* present in Mexico, showed that *D. ceylonicus*, *D. confusus*, and *D. opuntiae* are closely related. *D. coccus* constitutes a separate clade, and *D. tomentosus* is the most distantly related. Bacterial 16S rRNA sequences from all the *Dactylopius* species sampled showed a common β-proteobacteria, related to *Azoarcus*, also found in eggs and in bacteriocytes in *D. coccus*. We propose the name "*Candidatus* Dactylopiibacterium carminicum" for this endosymbiont. Other bacterial sequences recovered from the samples were close to those from soil or plant associated bacteria, like *Massilia*, *Herbaspirillum*, *Acinetobacter*, *Mesorhizobium*, and *Sphingomonas*, suggesting a possible horizontal transmission from Cactaceae plant sap to *Dactylopius* spp. during feeding. This is the first molecular analysis of *Dactylopius* species and of their associated bacteria.

**RESUMEN** Análisis filogenéticos de secuencias amplificadas mediante PCR de los genes 12S rARN y 18S rARN de insectos cochinillas del género *Dactylopius* presentes en México, mostraron que *D. ceylonicus*, *D. confusus* y *D. opuntiae* están cercanamente relacionados. *Dactylopius coccus* constituye un clado separado y *D. tomentosus* es el más alejado. Las secuencias del 16S rARN de bacterias de las especies de *Dactylopius* revelaron una β-Proteobacteria común, relacionada a *Azoarcus*, también encontrada en huevecillos y en bacteriocitos de *D. coccus*. Proponemos el nombre de "*Candidatus* Dactylopiibacterium carminicum" para este endosimbionte. Otras secuencias bacterianas recuperadas de las muestras fueron cercanas a bacterias del suelo o asociadas a plantas, como *Massilia*, *Herbaspirillum*, *Acinetobacter*, *Mesorhizobium* y *Sphingomonas*, sugiriendo que estas bacterias fueron transferidas de manera horizontal de la savia de las cactáceas a *Dactylopius* spp. durante la alimentación. Este es el primer análisis molecular de especies *Dactylopius* y de sus bacterias asociadas.

**KEY WORDS** Coccoidea, scale insects, systematics, endosymbiont, bacteriocytes

*Dactylopius* (Costa) is a genus of insects commonly known as cochineals that belongs to the family Dactylopiidae (Signoret) from the super family Coccoidea (scale insects) within the order Hemiptera. *Dactylopius* insects feed on Cactaceae plants from the genera *Opuntia* and *Nopalea* (Pérez-Guerra and Kosztarab 1992). Both the insects and their host plants are native to the Americas (Pérez-Guerra and Kosztarab 1992, Chávez-Moreno et al. 2009). Dactylopiidae has only one genus that includes nine described species (De Lotto 1974, Pérez-Guerra and Kosztarab 1992). Five of these species have been reported to be present in Mexico: *D. ceylonicus*, *D. confusus*, *D. opuntiae*, *D. coccus*, and *D. tomentosus* (Portillo and Vigueras 2006, Chávez-Moreno et al. 2009). *Dactylopius* spp. produce carminic acid, which is used by the insects for protection against predators (Eisner et al. 1994). It is used as a red dye for the production of cosmetics, drugs, food, and textiles. *D. coccus* has been preferentially used for carminic acid extraction because of its pigment quality and higher acid content (Hernández-Hernández et al. 2005). There are reports of its use in America since the 10th century (Portillo 2005, Chávez-Moreno et al. 2009). Currently, *D. coccus* is considered the only commercially important species in this genus, and it has undergone a domestication process. This species depends on human care for dispersion and reproduction (Pérez-Guerra and Kosztarab 1992). *Dactylopius* spp. have also been used as a biological control agent against

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invasive cactus in Africa and Australia (Moran and Zimmermann 1984).

Many insects harbor symbiotic bacteria in their guts or as endosymbionts inside specialized insect cells called bacteriocytes (Baumann 2005). Bacterial endosymbionts in the Hemiptera provide nutrients to insects with a limited diet such as phloem sap and blood that are deficient in essential amino acids and vitamins (Baumann 2005, Moran 2006). Some endosymbionts also synthesize bioactive compounds that can be used by insect hosts as defense against predators, parasites, and pathogenic microorganisms (Moran 2006). As endosymbionts are vertically transmitted, their DNA sequences can be used to trace insect phylogenies (Baumann 2005). Within the Coccoidea, endosymbionts have been found in the families Pseudococcidae (Thao et al. 2002), Diaspididae, and Margarodidae (Gruwell et al. 2007). The diversity of endosymbionts in *Dactylopius* spp. has not been reported, except for bacteria from the genus *Wolbachia* present in *Dactylopius* sp. eggs (Pankewitz et al. 2007).

The current identification and taxonomy of *Dactylopius* spp. has been based on morphological characters (De Lotto 1974, Pérez-Guerra and Kosztarab 1992), and Rodríguez et al. (2001) published a phylogeny of *Dactylopius* spp. on this basis. Until now, there have been no molecular phylogenies of the genus. There is only one phylogeny based on 18S rRNA sequences of the Coccoidea, which places Dactylopiidae close to clade E1 from the Eriococcidae (Cook et al. 2002). The aims of this work were to sequence and analyze mitochondrial and nuclear ribosomal genes from *Dactylopius* spp. to assess the phylogenetic relationships between the five species present in Mexico and to determine the symbiont bacteria species present in these insects.

## Materials and Methods

**Insect Sampling.** Specimens from five different *Dactylopius* species were collected from different regions in Mexico. Adult females were slide-mounted to allow identification according to descriptions given by De Lotto (1974) and Pérez-Guerra and Kosztarab (1992). Vouchers were deposited in the Colección Nacional de Insectos of the Instituto de Biología or in Centro de Ciencias Genómicas of the Universidad Nacional Autónoma de México. Specimens were collected from the following states (voucher numbers are indicated in parentheses): *D. coccus* from Oaxaca (DTY-ChM-101) and Morelos (Campo Carmín) (CCG-Sham-1), *D. confusus* from Tlaxcala (DTY-ChM-132), *D. ceylonicus* from Mexico state (DTY-ChM-110), *D. opuntiae* from Michoacán (DTY-ChM-106) and Querétaro (CCG-Cacau-2), and additionally we obtained *D. opuntiae* from Brazil, Pernambuco state (CCG-Cacau-8); all of these were parasitizing *Opuntia ficus-indica* L. Miller plants. *Dactylopius tomentosus* (DTY-ChM-190) was collected from Hidalgo on *Cylindropuntia tunicata* (Lehmann) Knuth. *Parasaissetia* sp. (Hemiptera: Coccidae) insects (CCG-AL-8) were collected in Morelos state on *Jacaranda mimosifolia* (D. Don). Gene sequences derived from this Coccidae were considered an outgroup in the phylogenetic analyses.

**DNA Extraction, Amplification, and Sequencing.** Female specimens from each insect species, freshly collected or frozen (−20°C), were superficially cleaned, removing the white wax, washed, and vortexed several times with ethanol and rinsed with sterile distilled water. DNA was extracted from whole insects with DNeasy Blood and Tissue Kit (Qiagen Hilden, Germany). A sample represented one specimen in the case of *D. coccus* and two to four specimens from the other species. Two DNA samples were analyzed from each species. DNA was used as a template in PCR reactions using primers F-12S-2: 5′-AAGAGTGACGGGCRATTTGTACATA-3′ and R-12S-2: 5′-GTGCCAGCAGTWGCGGTTA-3′ for insect mitochondrial 12S rRNA gene (Thao et al. 2004), primers 2880: 5′-CTGGTTGATCCTGCCAGTAG-3′ (Tautz et al. 1988) and B-: 5′-CCGCGGCTGCTGGCACCAGA-3′ (von Dohlen and Moran 1995) for insect nuclear 18S rRNA gene, and primers fD1: 5′-AGAGTTTGATCCTGGCTCAG-3′ and rD1: 5′-AAGGAGGTGATCCAGCC-3′ for bacterial 16S rRNA gene (Weisburg et al. 1991). Eggs and bacteriocytes were dissected from individual *D. coccus* females and washed several times with phosphate-buffered saline (PBS: 120 mM NaCl, 7 mM $Na_2HPO_4$, 3 mM $NaH_2PO_4$, [pH 7.4]), and DNA was extracted and used in PCR reactions as described above. Primers that specifically amplify a fragment from the 16S rRNA gene of END1 and closely related β-proteobacteria were designed (Beta428 F: 5′-GTGAATATCCGAAGCCGATGAC-3′, Beta1205R: 5′-GGCTTGGCAACCCTCTGTACCG-3′). Primers to identify clone O1 and related α-Proteobacteria were also designed (Alpha141 F: 5′-ACGGAAGAAAGTAGATATACGC-3′ and Alpha944R: 5′-ACCTGTTATGCTCCAACTAAAT-3′). These were used in addition to fD1 and rD1 primers with DNA of *Dactylopius* spp.

PCR protocols were performed as described by Weisburg et al. (1991) and Thao et al. (2004), except for the 18S rRNA gene that was amplified using the procedures described by Cook et al. (2002). The following protocol was used with Beta428 F–Beta1205R and Alpha141 F–Alpha944R primers: an initial denaturation at 94°C for 3 min, followed by 33 cycles of amplification (1 min at 94°C, 1 min at 57 or 52°C, respectively, and 1 min at 72°C), and a final extension step of 5 min at 72°C. The amplified products were 1,500 (fD1 and rD1), 460 (F-12S-2 and R-12S-2), 620 (2880 and B-), 800 (Beta428 F and Beta1205R), and 825 bp (Alpha141 F and Alpha944R). PCR products were cloned and individual plasmid clones were sequenced in Macrogen (Seoul, Korea).

**Bacterial Culture Conditions.** *Dactylopius coccus* were superficially cleaned, and macerate extracts were plated in (LB) (Sambrook and Russell 2001) and (PY) medium (Noel et al. 1984) and grown for 21 d at 28°C.

**Phylogenetic Analyses.** Nucleotide sequences were compared using the GenBank database Blastn, and

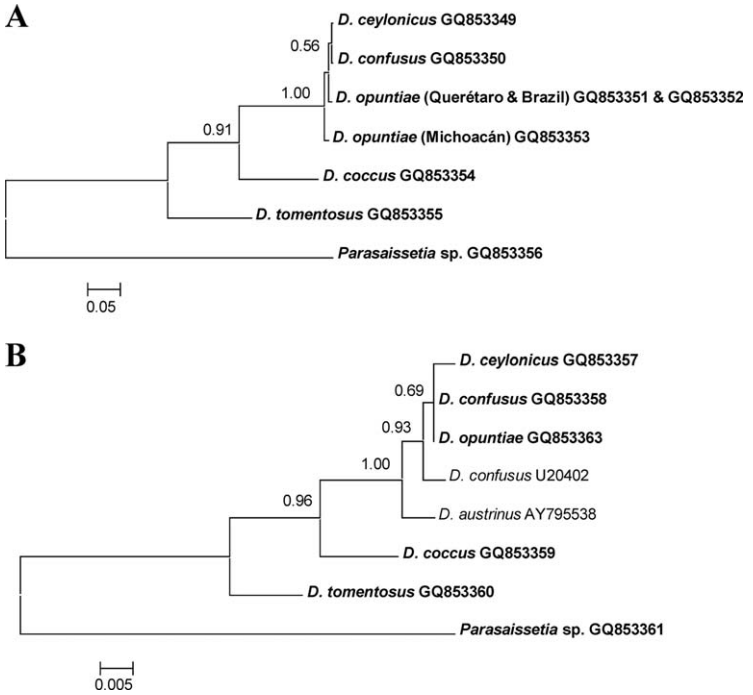


**Fig. 1.** Phylogenetic trees of 12S rRNA gene sequences (426 bp) (A) and 18S rRNA gene sequences (584 bp) (B) obtained from different *Dactylopius* species. Sequences from this work are in bold. Accession numbers are shown after scientific names. The tree was inferred with the Bayesian method using MrBayes under model GTR+I+G. Numerical values at each node indicate posterior probabilities. *Parasaissetia* sp. gene sequences were used as outgroups.

sequences from closely related organisms were retrieved. Sequence alignments were performed with CLUSTAL W (Thompson et al. 1994) and manually edited. Phylogenies were constructed with the Bayesian method using MrBayes (Huelsenbeck and Ronquist 2001). The best model of sequence evolution for each gene was selected using the MrAIC Perl script written by J.A.A. Nylander (http://www.abc.SE/≈nylander/). In all cases, the selected model was GTR+I+G. *Candidatus* Sulcia muelleri 16S rRNA gene sequence was used as an outgroup; this is an endosymbiotic flavobacterium of many species of the suborder Auchenorrhyncha of Hemiptera (Moran et al. 2005).

## Results

**Phylogenetic Analyses.** Six clones from each *Dactylopius* species were analyzed (three clones per sample for each gene analyzed). Sequences obtained from a single insect species were >99% identical, and only one clone from each species was used for phylogenetic analyses. Phylogenetic trees obtained from the mitochondrial 12S rRNA and nuclear 18S rRNA genes are shown in Fig. 1A and B. A tree generated with concatenated sequences from both genes was similar (data not shown). Trees were congruent and showed that *D. ceylonicus*, *D. confusus*, and *D. opuntiae* clustered together with an identity of 99%. 12S rRNA gene sequences from *D. opuntiae* from three different geographic regions were very similar (>98.9% of identity), and 18S rRNA gene sequences from *D. confusus* and from the three *D. opuntiae* were 100% identical. Two 18S rRNA gene sequences were retrieved from NCBI GenBank corresponding to *D. confusus* [U20402, collected from Arizona (von Dohlen and Moran 1995)] and *D. austrinus* [AY795538, collected from Australia (Cook and Gullan 2004)]. The *D. confusus* sequence is similar to those reported here for *D. ceylonicus*, *D. confusus*, and *D. opuntiae* (99% of identity), and *D. austrinus* sequence was close to this cluster. *D. coccus* was separated, and *D. tomentosus* was the most distantly related.

**Identification of Symbiotic Bacteria.** The analyses of 16S rRNA gene sequences indicated that different bacteria were found inside *Dactylopius.* The abundance of clones found in each species and the percentage of identity to the closest NCBI match are shown in Table 1.

*Dactylopius* species had different associated bacteria; however, the only universally associated one was a β-proteobacteria (named here as END1) and was highly conserved with almost identical 16S rRNA gene sequences. END1 was first identified with 16S rRNA universal primers (fD1 and rD1) in all the individuals collected from *D. opuntiae*, including the ones collected from Brazil, and in *D. coccus* and *D. tomentosus* (Table 1). Subsequently, with primers specific to END1 (Beta428 F and Beta1205R), it was also found in *D. ceylonicus* and *D. confusus*, as well as in eggs and

**Table 1. Assignment and abundance (percentage of clones) of bacteria in *Dactylopius* species using 16S rRNA gene sequences obtained with universal primers rD1 and fD1**

| *Dactylopius* species | β-Proteobacteria | | | α-Proteobacteria | | | γ-Proteobacteria | Number of analyzed clones |
|---|---|---|---|---|---|---|---|---|
| | Soil bacteria (END1) | *Massilia* sp.(N6) | *Herbaspirillum* sp. (E7) | *Porcellio scaber* symbiont (O1) | *Sphingomonas insulae* (E1) | *Mesorhizobium* sp. (E4) | *Acinetobacter* sp. (N8) | |
| *D. ceylonicus* | 0[a] | 29 | 43 | 0 | 14 | 14 | 0 | 7 |
| *D. confusus* | 0[a] | 88 | 0 | 0 | 0 | 0 | 12 | 8 |
| *D. opuntiae* (Michoacán) | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 2 |
| *D. opuntiae* (Querétaro) | 63 | 0 | 0 | 37 | 0 | 0 | 0 | 27 |
| *D. opuntiae* (Brazil) | 43 | 0 | 0 | 57 | 0 | 0 | 0 | 7 |
| *D. coccus* | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| *D. tomentosus* | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |

The closest NCBI match is shown. Name of each clone is shown in parentheses.

[a] END1 symbiont was not found when universal 16S rRNA gene primers (rD1 and fD1) were used, but it was amplified by PCR when specific primers for β-proteobacteria were used (Beta428F and Beta1205R).

bacteriocytes of *D. coccus*. The sequence identity of END1 from the different *Dactylopius* species was >99.5%. The sequence had 95% identity to a soil isolate (AB024934) that was erroneously assigned to *Sphingomonas* sp. A1 (α-proteobacteria) and corresponds to a sequence of β-proteobacteria closely related to *Azoarcus* sp. (Fig. 2). END1 sequence also presents 95% identity to *Uliginosibacterium gangwonense*, an aerobic bacteria isolated from wetland peat samples (Weon et al. 2008). No cultured isolates were obtained from *D.*

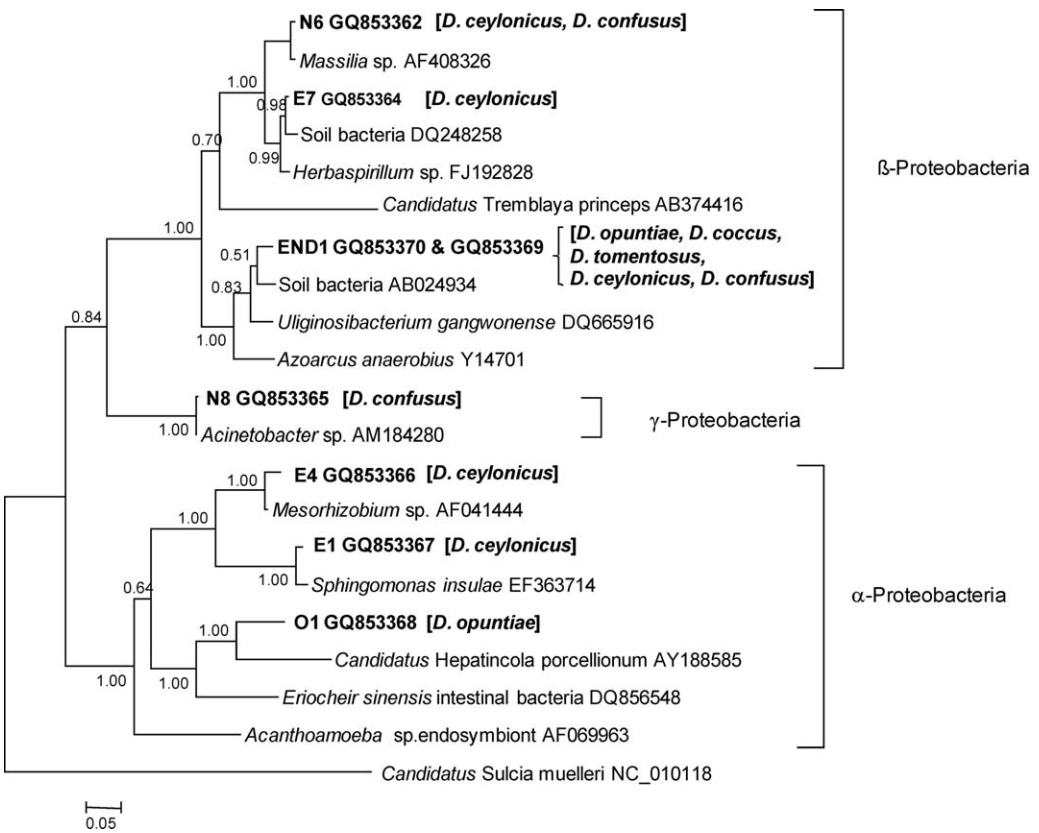


**Fig. 2.** Phylogenetic tree of 16S rRNA gene sequences of bacteria (1284 bp) obtained from different *Dactylopius* species. Sequences from this work are in bold. Other sequences from closely related organisms were included. Accession numbers are shown after scientific names. Host *Dactylopius* species are shown in brackets. The tree was inferred with the Bayesian method using MrBayes under model GTR+I+G. Numerical values at each node indicate posterior probabilities. *Candidatus* Sulcia muelleri 16S rRNA gene sequence was used as outgroup.

*coccus* female macerates plated in LB and PY medium (data not shown).

Additionally, 39% of the 16S ribosomal RNA clones obtained from the DNA samples of all *D. opuntiae* specimens presented 88% identity to *Candidatus* Hepatincola porcellionum, the extracellular symbiont of the hepatopancreas of *Porcellio scaber* (common woodlouse, Crustacea: Isopoda) (Wang et al. 2004) (clone O1 in Table 1 and Fig. 2), which belongs to the order Rickettsiales from the α-proteobacteria. Specific primers for O1 did not amplify DNA from other *Dactylopius* species.

Other 16S rRNA gene clones were close to free-living bacteria, such as *Massilia* sp., *Herbaspirillum* sp., *Acinetobacter* sp., *Mesorhizobium* sp., and *Sphingomonas* sp. (Table 1; Fig. 2), with 98, 96, 99, 97, and 98% identity, respectively. These were obtained from *D. ceylonicus* and *D. confusus.*

## Discussion

Our phylogenetic results on the relationships of species in the cochineal genus *Dactylopius* differ from those reported by Rodríguez et al. (2001) based on morphological characters. They found that *D. austrinus*, *D. ceylonicus*, and *D. coccus* are more closely related between them and less related to *D. confusus* and *D. opuntiae.* The need to establish molecular phylogenies for *Dactylopius* has been recognized in several papers because conflicting results have been derived from morphological data (Portillo and Vigueras 2006).

*Dactylopius coccus* was domesticated and selected for producing high amounts of carminic acid. It has a large body size (females are 3–6 mm long) and presents a cover of white powdery wax instead of a white cottony wax with long filaments (Pérez-Guerra and Kosztarab 1992). The cottony wax cover protects the insects against desiccation and rain (Chávez-Moreno et al. 2009). Pérez-Guerra and Kosztarab (1992) considered the characteristic cover important for proposing *D. coccus* as the most primitive of the *Dactylopius* species; otherwise, this character could be a consequence of domestication.

In accordance with our results, *D. tomentosus* has been reported as the most distant species of the genus, because it has unique biological and morphological characteristics that differ considerably from other *Dactylopius* species (Mathenge et al. 2009). *Dactylopius tomentosus* host range is restricted to the subgenus *Cylindropuntia*, its egg incubation period is longer (17 d instead of minutes or hours), eggs are held on a mesh of waxy threads and remain attached to the female during the incubation period (in other *Dactylopius* species, eggs are not enclosed in a mesh and continue to hatch as more are laid), the size of female adults is smaller than in most of the other species (Mathenge et al. 2009), and its anal ring is obsolete (Pérez-Guerra and Kosztarab 1992, Rodríguez et al. 2001).

We found a characteristic set of bacteria from each *Dactylopius* species. Of special interest is END1, a β-proteobacteria found in all the *Dactylopius* species and in eggs and bacteriocytes of *D. coccus.* These findings suggest that END1 is a primary endosymbiont that could have been acquired before the radiation of this genus. Its location should be subsequently confirmed by in situ hybridization. Within the Coccoidea in the Pseudococcidae family, another β-proteobacteria primary endosymbiont, *Candidatus* Tremblaya princeps, has been reported (Thao et al. 2002), with 16S rRNA gene sequence 79% identical to that from END1 (Fig. 2).

We propose the designation *Candidatus* Dactylopiibacterium carminicum for the β-proteobacteria named here as END1, identified from the insects of all the species in the genus *Dactylopius.* This bacterium is thus far uncultured. It has unique sequences in the 16S rRNA gene at the following sites (homologs to *Escherichia coli* positions): (1) 69–96, GATTCAAGGGGCTTGCTCCTTGGGT, and (2) 461–476, GGTGAATATCCGAAGCC. The 16S rRNA gene has an average of G + C content of 54.76 mol%. *Dactylopiibacterium*: Dac.ty.lo.pi.i.bac.te'ri.um. N.L. n. *Dactylopius*, cochineal scientific genus name; L. neut. n. bacterium, N.L. neut. n. *Dactylopiibacterium*, a bacterium isolated from *Dactylopius* spp.; *caminicum*: car.mi.ni'cum. M.L. n. carminium, carmine; *carminicum*, belonging to carmine (red pigment) that is produced by all *Dactylopius* spp.

Clone O1 collected from *D. opuntiae* belongs to the order Rickettsiales. In this order, many intracellular symbionts and pathogens of eukaryotes have been found (Weinert et al. 2009). The sequence from O1 and other related sequences, however, did not group with any of the major clades of Rickettsia, meaning that they could represent new taxa within this group. In contrast to published data (Pankewitz et al. 2007), we did not find *Wolbachia* in *Dactylopius* spp.

The sequences from the clones close to free-living bacteria belong to soil or to plant associated bacteria. The location of some of these bacteria could be the gut, in which case its origin could plausibly be the sap that serves as food for the insects. It would be of ecological interest to explore this fact by analyzing the endophytic bacteria from the plants parasited by the insects. Their location inside the insect must be also determined.

Our description of *Dactylopius* spp. endosymbionts will be the basis for studying its role in the development and physiology of the insect. These bacteria could be implicated in providing amino acids, vitamins, or antimicrobial compounds to the host or in degrading plant toxic compounds, as occurs in other sap feeding insects.

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