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Short Communication

16S rRNA gene-based identification of microbiota associated with the parthenogenetic troglobiont sand fly *Deanemyia maruaga* (Diptera, Psychodidae) from central Amazon, Brazil

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Abstract

Bacteria associated with the parthenogenetic troglobiont sand fly *Deanemyia maruaga* were characterized by sequencing cloned 16S rDNA PCR products. Eleven novel partial 16S rDNA sequences, with varying degrees of similarity to Actinobacteria, were identified. None of the sequences identified had homology to those known from parthenogenesis-inducing bacteria.

Key words: Actinobacteria, bat guano, parthenogensis, sand flies, troglobiont.

Sand flies are dioecious, holometabolous insects responsible for transmission of *Leishmania* species, bacteria and viruses to humans and non-human animals (Rangel and Lainson, 2011). The ground-dwelling larvae feed on decaying organic matter present in the forest soil, animal burrows and rock crevices (Rangel and Lainson, 2011). In contrast to most known sand fly species, the non-vector *Deanemyia maruaga* is a parthenogenetic troglobiont (Alves *et al.*, 2008). It has been described from only a single cave in the central Amazon, Brazil, within which it completes its entire life-cycle (Alves *et al.*, 2008). Under laboratory conditions, *D. maruaga* has been shown to be autogenous, *i.e.* it does not require a bloodmeal for egg development, which implies that feeding, sufficient for reproduction, only occurs during the larval stages. Only female adults have so far been captured, suggesting that this species is parthenogenetic (Alves *et al.*, 2008, 2011). Parthenogenesis has been described in most insect orders and occurs by a variety of mechanisms (Normark and Kirkendall, 2009), but has only previously been reported for one other sand fly species, *Pintomyia mamedei* (Brazil and Oliveira, 1999). Recently, the occurrence of parthenogenesis in insects has been associated with the presence of various maternally-inherited bacteria (Hagimori *et al.*, 2006; Werren *et al.*, 2008; Zchori-Fein and Perlman, 2004). Here, we describe the bacteria associated with *D. maruaga* using the culture-independent method of sequencing cloned 16S rDNA PCR products.

The larvae and adults of *D. maruaga* were collected from the Refúgio do Maruaga cave, located in the municipality of Presidente Figueiredo, Amazonas state, Brazil (02°03'02" S, 59°57'48" W). Two collections were carried out in 2008/2009: one in the rainy season (March) and another in the dry season (November). In total, approximately 40 kg of guano were collected and immature sand flies were separated in the laboratory using the flotation method (Hanson, 1961). Adults of *D. maruaga* were collected using 10 CDC light traps distributed along the cave 5 m apart from each other, and approximately 2 m high. The insects were surface sterilized in 70% ethanol and then rinsed in sterile phosphate buffered saline (PBS) (Lindh *et al.*, 2008; Volf *et al.*, 2002). To detect and identify the bacteria that may be present in the sand fly larvae and adults, DNA was isolated, and a 586 bp partial fragment was amplified by touchdown PCR, cloned and sequenced as described else-

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where (Muyzer *et al.*, 1993; Muyzer *et al.*, 1998). Nucleotide sequences corresponding to 16S rRNA gene fragments have been deposited in GenBank under accession numbers JN040717 to JN040727.

Phylogenetic analyses were carried out with maximum likelihood (ML) method performed in PHYML (Guindon *et al.*, 2010). The sequences were checked for chimeras using Greengenes NAST server alignment tool (DeSantis *et al.*, 2006) and Bellerophon 3 (Huber *et al.*, 2004). None of the sequences were chimeric.

A total of 11 clones were successfully sequenced, yielding 11 unique partial 16S rRNA gene sequences, isolated from four pooled samples of the larvae of *D. maruaga* (Table 1 and Figure 1). No 16S rDNA PCR products were obtained from the adults of *D. maruaga*, even though the positive controls amplified the expected product.

Phylogenetic analysis of the 16S rDNA sequences recovered from the larvae of *D. maruaga* (Figure 1) showed that ten of our sequences grouped in the genus *Mycobacterium*, mainly from the nonpathogenic *M. terrae* complex (Torkko *et al.*, 1998). The remaining sequence (JN040723), also had similarity, albeit relatively low, to previously reported sequences from diverse environmental sources: soil samples (Yarwood *et al.*, 2009), water supplies (Le Dantec *et al.*, 2002), and rhizospheres (Burke *et al.*, 2008; Mirete *et al.*, 2007), respectively. It forms an independent and taxonomically unassigned lineage affiliated with Actinobacteria, also containing several other sequences previously deposited in GenBank obtained from uncultured environmental rhizosphere and soil samples.

The bacteria we found in the larvae of *D. maruaga* are very distinct from the microbiota previously described

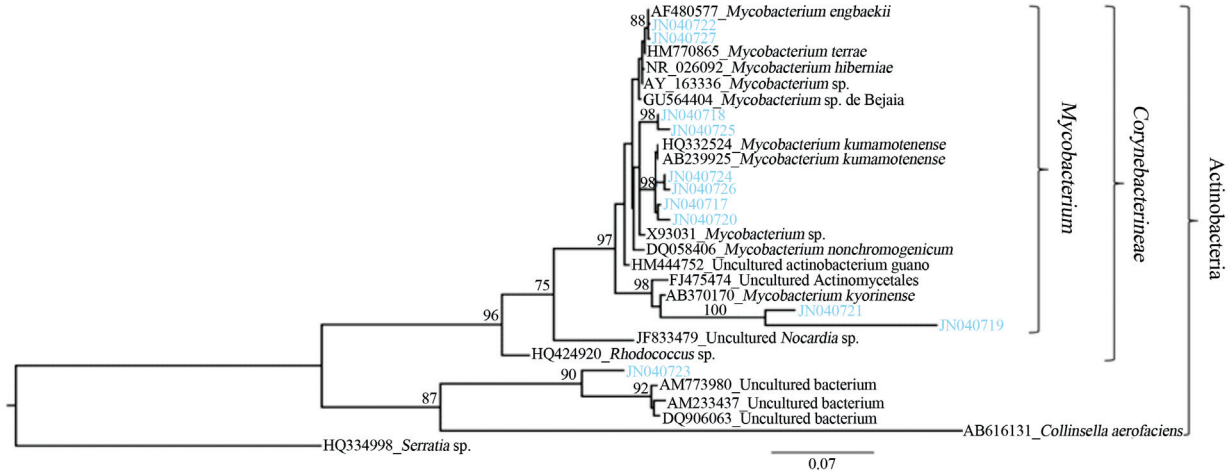

Figure 1 - Molecular Phylogenetic analysis by Maximum Likelihood method of the 16S rRNA gene sequences derived from larvae of the sand fly *Deanemyia maruaga*. The evolutionary history was inferred by using the Maximum Likelihood method performed in PHYML (Guindon *et al.*, 2010), applying a general time-reversible model of molecular evolution, with among-site variation using a four-category discrete approximation of a Γ distribution with a portion of invariable sites. Branch support was calculated with SH-like aLRT. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences: the 11 novel sequences reported here from *D. maruaga* (shown in blue), and 19 sequences with the first two hits previously deposited in GenBank (shown in black). There were a total of 416 positions in the final dataset.

Table 1 - The 11 novel partial16S rRNA gene sequences affiliated to the Class Actinobacteria isolated from larvae of the sand fly *Deanemyia maruaga*.

Clones (accession number)	Most similar sequences from GenBank (accession number)	Similarity (%)
JN040717	*Mycobacterium kumamotense* (AB239925)	99
JN040718	*Mycobacterium nonchromogenicum* (DQ058406)	98
JN040719	*Mycobacterium* sp. de Bejaia (GU564404)	91
JN040720	*Mycobacterium kumamotenense* (AB239925)	99
JN040721	Uncultured Actinomycetales (FJ475474)	93
JN040722	*Mycobacterium engbaekii* (AF480577)	99
JN040723	Uncultured bacterium clone (DQ906063)	92
JN040724	*Mycobacterium kumamotonense* (HQ332524)	99
JN040725	*Mycobacterium nonchromogenicum* (DQ058406)	97
JN040726	*Mycobacterium kumamotenense* (AB239925)	98
JN040727	*Mycobacterium engbaekii* (AF480577)	99

from other sand fly species (Dillon *et al.*, 1996; Volf *et al.*, 2002; Gouveia *et al.*, 2008; Hillesland *et al.*, 2008). Most previous studies investigated adults of vector species, which feed on plant juices and vertebrate blood (Gouveia *et al.*, 2008). The bacteria found in these sand flies were predominantly Gram-negative members of the γ-Proteobacteria, and a few Gram-positive Firmicutes (Dillon *et al.*, 1996; Gouveia *et al.*, 2008; Hillesland *et al.*, 2008). In contrast, only Actinobacteria were found in the larvae of *D. maruaga*, and this class of bacteria, including mycobacteria, has not previously been associated with any species of sand fly.

Larvae of *D. maruaga* live within, and feed upon, bat guano deposited on the cave floor, and are likely to acquire their microbiota from the bat guano ingested during feeding. Seven of the sequences we report here had BLAST matches to two 16S rDNA sequences previously isolated from bat guano collected in a cave in Slovakia (Hill *et al.*, 2011), although another microbiological survey of bat guano did not report the occurrence of Actinobacteria (Konieczna *et al.*, 2007). Whether the Actinobacteria in the bat guano are derived from the bats (*i.e.* their defecated intestinal microbiome) or the cave environment (*i.e.* soil or rock walls) is unclear. Culture-dependent microbiological analysis of the gut content of bats has shown the presence of Actinobacteria from the genus *Corynebacterium* (Klite, 1965; Heard *et al.*, 1997). However, the presence of Actinobacteria has also been reported from rock wall surfaces and soil samples from other caves through culture-independent methods (Schabereiter-Gurtner *et al.*, 2002, 2004; Zhou *et al.*, 2007), indicating a possible environmental source for the Actinobacteria in bat guano. Bat guano is acidic, and would accordingly provide an ideal, nutrient-rich substrate for acidophilic Actinobacteria (Goodfellow and Williams, 1983).

The absence of amplification of bacterial DNA from the adults of *D. maruaga* is unexpected, but might be explained by the apparent absence of transtadial passage of bacteria during the larval to adult metamorphosis (Killick-Kendrick, 1979; Lindh *et al.*, 2008; Moll *et al.*, 2001) and the autogeny of *D. maruaga* (Alves *et al.*, 2011), eliminating the microbial exposures experienced by adults of other sand fly species associated with ingestion of plant juices and bloodfeeding on vertebrate hosts. Another possibility is that the density of bacteria associated with adults of *D. maruaga* is extremely low, several orders of magnitude lower than that of larvae, and therefore below the detection threshold of our PCR reaction conditions.

The universal eubacterial primers we used in our study should allow the detection of 16S rDNA fragments from the endosymbiotic bacteria associated with the occurrence of parthenogenesis in other insects (*e.g. Wolbachia*, *Rickettsia* and *Cardinium*) (Hagimori *et al.*, 2006; Werren *et al.*, 2008; Zchori-Fein and Perlman, 2004). Since we did not detect sequences from any of these or related bacteria in either the larvae or adults of *D. maruaga*, another mechanism is probably responsible for the parthenogenesis of this sand fly. The apparent absence of Actinobacteria from the adults of *D. maruaga* would seem to preclude the possibility that these bacteria cause parthenogenesis.

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