



## Short Communication

New *Spiroplasma* in parasitic *Leptus* mites and their *Agathemera* walking stick hosts from ArgentinaEmily DiBlasi<sup>a,c</sup>, Solon Morse<sup>a</sup>, Jason R. Mayberry<sup>a</sup>, Luciano J. Avila<sup>b</sup>, Mariana Morando<sup>b</sup>, Katharina Dittmar<sup>a,\*</sup><sup>a</sup> Department of Biological Sciences, SUNY at Buffalo, Buffalo, NY, United States<sup>b</sup> CONICET-CENPAT, Boulevard Almirante Brown 2825, U9120ACF, Puerto Madryn, Chubut, Argentina<sup>c</sup> Graduate Program of Ecology, Evolution and Behavior, SUNY at Buffalo, NY, United States

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## ABSTRACT

Here we report the presence of *Spiroplasma* 16S rRNA in populations of two parasitic *Leptus* mites (*Leptus sayi*; *Leptus lomani*) and their *Agathemera* walking stick hosts. In walking sticks *Spiroplasma*s were detected in the gut, as well as muscle-tissues, but not in eggs. Throughout Argentina 15.4% of *L. sayi* populations and 14.3% of *L. lomani* populations surveyed screened positive for *Spiroplasma*. Phylogenetic analyses (ML, BCMC) place all sequences within the *Ixodetis* group. Most sequences form a well-supported sister subclade to the rest of *Ixodetis*. We briefly discuss the role of *Leptus* mites in the natural transmission of *Spiroplasma*.

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## 1. Introduction

*Spiroplasma*s are a group of small, wall-less, helical bacteria (Gasparich, 2010), some of which are motile. Records place them mostly in association with insects, although sporadic finds are known from other invertebrates and plants (Gasparich, 2010). This distribution may be the result of sampling bias.

Taxonomically, *Spiroplasma*s are defined as part of the Mycoplasmales–Entomoplasmatales lineage of Mollicutes (Gasparich et al., 2004). Recent phylogenetic studies based on 16S rRNA resulted in the classification of four major clades within the monophyletic Entomoplasmatales, namely: the *Mycoides–Entomoplasmataceae* (ME) clade, the *Apis* clade, the *Citri–Chrysopicola–Mirum* (CCM) clade, and the *Ixodetis* clade (Gasparich, 2010; Regassa and Gasparich, 2006). Each of these clades represents hosts from a diverse range of invertebrates and plants. So far, no general co-evolutionary patterns between hosts and *Spiroplasma* can be discerned. Horizontal transfers seem to be frequent, and recent experiments have shown the potential of mites to act as vectors for *Spiroplasma poulsonii* across *Drosophila* species (Jaenike et al., 2007).

*Spiroplasma* interactions with their invertebrate hosts may be mutualistic or pathogenic (Gasparich, 2010; Haselkorn et al., 2009). Commensalism in the midgut seems to be the prevailing interaction (Regassa and Gasparich, 2006). Pathogenicity is assumed to be linked to the ability to cross the gut barrier, and reach the hemolymph or the reproductive system (Regassa and Gasparich, 2006). Hemolymph-invading *Spiroplasma*s can kill their hosts (e.g., *Spiroplasma melliferum* and *Spiroplasma apis*), whereas transovarially transmitted *Spiroplasma* may act as male-killers (e.g., *S. poulsonii*) (Regassa and Gasparich, 2006).

The study of biodiversity and distribution of *Spiroplasma* among invertebrates is continually advancing. Yet most studies still only concentrate on insects, neglecting other invertebrates. Furthermore, no study has explored naturally occurring organismal intersections, such as arthropod parasites and their insect hosts, which may facilitate bacterial exchanges.

Here we report on *Spiroplasma* detected by exploratory PCR screens in understudied parasitic *Leptus* mites (Leptidae) and their walking stick hosts (*Agathemera* spp.) from Argentina. *Leptus* mites are protelean parasites, which are ectoparasitic on arthropods during the larval stages (Southcott, 1991). Adults are mostly unknown, although some are predators. Evolutionary studies have placed *Leptus* within the Erythraeoidea as part of the larger Parasitengona complex (Dusbabek and Bukva, 1991; Welbourn, 1991). *Leptus* has been classified as both a potential biological control agent for agricultural and invasive arthropod pests, and as a vector for pathogen transmission (Welbourn and Jenner, 1983). In Argentina, *Leptus*

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larvae often associate with *Agathemera* walking sticks. Host associations to other insects are very likely, but understudied. *Agathemera* is the sole genus of the suborder Agathemeroidea in Phasmida, and has undergone a radiation along both flanks of the Southern Cone Andean Cordillera (Camousseight, 2005). They occupy diverse habitats, feeding on plants from the genera *Citrus*, *Larrea*, *Rhus*, *Hoffmannseggia*, *Acaena*, and *Lecointea* (unpublished results).

In this project we explore the phylogenetic placement of the new *Spiroplasma* genotypes in relation to previously defined clades, and discuss the potential role of *Leptus* mites in *Spiroplasma* transmission.

## 2. Materials and methods

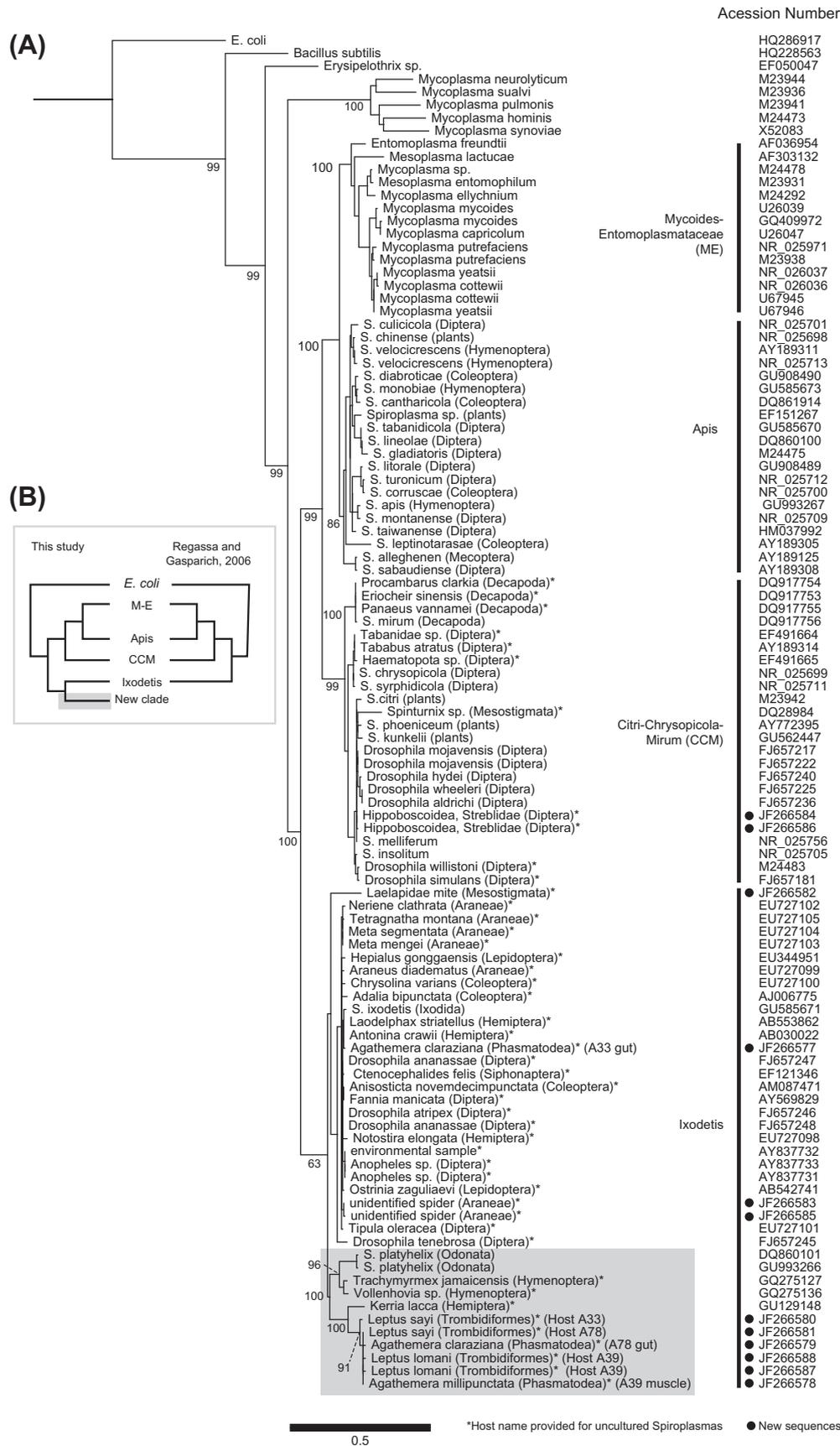
Larval mites and their *Agathemera* hosts (five species) were collected from 20 populations throughout Argentina (Fig. 1D). The average number of mites per walking stick was six. Current taxonomic keys identified the mites as *Leptus lomani* and *Leptus sayi*, resulting in new records for Argentina (Southcott, 1991; Welbourn, 1991). Walking sticks were identified using the taxonomic keys of Camousseight (1995, 2005), and hosts keyed to *Agathemera crassa*, *A. millipunctata*, *A. claraziana*, *A. luteola*, and one as of yet unidentified *Agathemera* species. Genomic DNA was extracted from ethanol preserved (96%) single mites, as well as walking stick gut, muscle tissue and eggs (if available) using the Qiagen Tissue kit (Qiagen). Genomic 16S rRNA was amplified using proofreading polymerase (Invitrogen, Accuprime), general primers and protocols previously described (Fukatsu and Nikoh, 1998). Positive samples were cloned using a TopoTA kit (Invitrogen). Twelve colonies per sample were screened for *Spiroplasma* presence. Sequences were aligned with MAFFT (Kato and Toh, 2008) under the L-INS-i criterion (1594 bp length). Model selection was performed using the Akaike Information Criterion (AIC) as implemented in jModelTest 0.1.1 (Posada and Buckley, 2004), and resulted in the GTR + I + G model. Phylogenetic analyses were conducted using Maximum Likelihood (ML), and Bayesian (BI) approaches together with 70 other *Spiroplasma* sequences from GenBank, representing all known clades (Fig. 2A). As in previous analyses of broad scope (Heres and Lightner, 2010), *Escherichia coli* was chosen as the outgroup. ML and BI analyses were performed through the CIPRES science gateway using the RAxML (Stamatakis, 2006) and MrBayes (Ronquist and Huelsenbeck, 2003) tools respectively (Miller et al., 2009). The default parameters in RAxML and the implementation of the GTR + I + G model were used to obtain the best-scoring ML tree of a thorough ML and rapid bootstrap analysis. The Bayesian analyses were performed with 8 MCMC chains implemented under the following settings: nruns = 2, nst = 6, rates = propinv, ngen = 10,000,000, samplefreq = 1000. Trees before convergence were discarded as burn in and the final consensus tree with posterior probabilities was generated from the remaining trees. Because of congruent topologies in the pertinent clades, we present only one phylogeny (ML).

## 3. Results and discussion

In this preliminary survey, 15.4% of the populations of Argentine *L. sayi*, and 14.3% of *L. lomani* were positive for *Spiroplasma*. For positive mites, *Agathemera* hosts also yielded *Spiroplasma* DNA, from either gut content, or muscle tissues (Fig. 2A). Sequence identity of all but one *Spiroplasma* detected in mites and their host species is within 98.2%; these sequences cluster in a well-supported subclade within the *Ixodetes* clade (Fig. 2A). Together with sequences from dragonflies, ants and a scale insect, they form a sister group to the rest of the *Ixodetes* clade. The only fully



**Fig. 1.** Overview and distribution of Argentine *Spiroplasma* hosts. (A) *Agathemera claraziana* walking stick with ectoparasitic mites (*Leptus sayi*) attached on the abdomen near the junction with the mesothorax. (B) Enlargement of the attached mites. (C) Overview of a *Leptus* mite attached to an *Agathemera* leg. (D) Map of the distribution of *Agathemera*-*Leptus* samples across Argentine provinces. Shaded shapes indicate samples where *Spiroplasma* was found.



**Fig. 2.** *Spiroplasma* phylogeny. (A) Best scoring ML phylogeny with bootstrap values based on the *Spiroplasma* 16S rRNA gene. (B) Overview of phylogenetic analysis from this study compared with Regassa and Gasparich's (2006).

characterized *Spiroplasma* in this subclade is *S. platyhelix*, a unique tightly coiled helical *Spiroplasma* isolated from dragonflies (Tully et al., 1995). Sequences are most similar (97%) to a sequence isolated from *Kerria lacca* (GU129148), a scale insect (Fig. 2A). Relationships of the *Ixodetis* clade to other previously characterized clades (ME, CCM, and *Apis*) mirrors results from previous analyses (Fig. 2B).

Simultaneous presence of the same strains in hosts and their mites suggests either independent acquisitions from some environmental source, and/or transfer between mites and hosts. One point of connection that could lead to independent acquisition by both walking sticks and their larval parasites are plants. *Agathemera* feed directly on plants, whereas adult *Leptus* mites have been observed as predators of plant pests. Many *Spiroplasma* are part of the gut fauna of insects, and plant surfaces with insect fecal matter may act as a bacterial reservoir. Thus, the same plant associated *Spiroplasma* could invade both organisms, provided a vertical transmission from adult to offspring in mites. Because whole mite specimens were used in this study, specific tissue affinities of *Spiroplasma* in mites remain unclear, and need to be further investigated.

From what we know about *Spiroplasma* biology, a scenario involving transmission between host and parasite seems more intuitive. Mites could be responsible for horizontally transferring *Spiroplasma* among walking stick hosts, without invoking a plant connection. *Leptus* larvae feed on hemolymph, which would facilitate uptake of *Spiroplasma* from infested hosts. Recent studies by Jaenike et al. (2007) show the potential of mites to transfer *S. poulsonii* between different *Drosophila* species in a laboratory setting.

One *Spiroplasma* strain isolated from the gut of an *A. claraziana* shows greater divergence from other strains of this study (92.6% identity), while still falling into the *Ixodetis* clade (Fig. 2a). This suggests the potential of multiple *Spiroplasma* associating with the same hosts, possibly using different mechanisms of invasion. Similar results have been found in a recent study of *Spiroplasma* associated to *Drosophila* (Haselkorn et al., 2009).

The presence of the same strains of *Spiroplasma* in gut and muscle tissues speaks to their potential in crossing the gut barrier, which is usually associated with pathogenicity and fitness effects; however, no apparent detrimental physical effects were observed on *Spiroplasma*-positive mites or hosts. The absence of *Spiroplasma* in eggs of otherwise positive female hosts make a transovarial transmission unlikely, which may preclude an endosymbiotic function (e.g., male killing) in the walking sticks.

Ultimately, more data are needed, and *Spiroplasma* need to be isolated (cultured) from these organisms to conclusively inform about their biology. Unfortunately, the ethanol preservation of the available specimens did not allow such study at this point. Detailed characterization of their serology and biology is especially important in the context of the proposed use of these mites, and *Spiroplasma*, as a biological control agent. Hackett et al. (1992) suggested the potential usefulness of genetically modified *Spiroplasma* in controlling the Colorado potato beetle (*Leptinotarsa decemlineata*). Likewise, Welbourn and Jennings (1983) hypothesized about the use of erythraeoid mites (e.g., *Leptus*) in controlling pest insects. However, *Leptus* mites are not very host specific, and infest many insect hosts (including all major orders known to be associated with *Spiroplasma*). If they have a natural association

with *Spiroplasma*, as suggested by our data, they may potentially disperse therapeutic *Spiroplasma* to insects other than the intended target organism, leading to undesirable side effects in the ecosystem.

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