Short Communication

New Spiroplasma in parasitic Leptus mites and their Agathemera walking stick hosts from Argentina

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1. Introduction

Spiroplasmas 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, Spiroplasmas are defined as part of the Mycoplasmatales–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-Chrysopicolae-Mirum (CCM) clade, and the Ixodetes 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).

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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 Spiroplasmas 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 Ixodetes group. Most sequences form a well-supported sister subclade to the rest of Ixodetes. We briefly discuss the role of Leptus mites in the natural transmission of Spiroplasma.

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 Spiroplasmas 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 organisical 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 Jenning, 1983). In Argentina, Leptus...
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 Phasmina, 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 (Katoh 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, 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 Ixodetis clade (Fig. 2A). Together with sequences from dragonflies, ants and a scale insect, they form a sister group to the rest of the Ixodetis 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 mite. (C) Overview of a Leptus sayi 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.
(A) 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 Jenning (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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