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A survey of *Wolbachia*, *Spiroplasma* and other bacteria in parthenogenetic and non-parthenogenetic phasmid (Phasmatodea) species

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Key words. *Wolbachia*, *Spiroplasma*, bacterial endosymbionts, parthenogenesis, phasmids, phasmid microbiota

Abstract. The ecological and genetic mechanisms that determine Phasmatodea reproductive biology are poorly understood. The order includes standard sexual species, but also many others that display distinct types of parthenogenesis (tychoparthenogenesis, automixis, apomixis, etc.), or both systems facultatively. In a preliminary survey, we analysed *Wolbachia* and *Spiroplasma* infection in 244 individuals from 28 species and 24 genera of stick insects by bacterial *16S rRNA* gene amplification. Our main aim was to determine whether some of the bacterial endosymbionts involved in distinct reproductive alterations in other arthropods, including parthenogenesis and male killing, are present in phasmids. We found no *Wolbachia* infection in any of the phasmid species analysed, but confirmed the presence of *Spiroplasma* in some sexual, mixed and asexual species. Phylogenetic analysis identified these bacterial strains as belonging to the Ixodetis clade. Other bacteria genera were also detected. The possible role of these bacteria in Phasmatodea biology is discussed.

INTRODUCTION

Parthenogenesis is a very common phenomenon in most animal groups, a reproductive mode that limits genetic recombination. Parthenogenetic females can, in principle, transfer all their genes to their offspring, while a bisexual female transmits only half of them because her chromosome number is reduced during meiosis. This can be interpreted as meaning that the representation of parthenogenetic female genes will double in the next generation, although this is controversial (Suomalainen et al., 1987). However, this could explain why the bacterial endosymbiont *Wolbachia* manipulates host reproduction in some cases (Werren et al., 2008). Given its almost complete maternal transmission, inducing parthenogenesis, clearly helps its own transmission, accompanied by the "correct" host genes, i.e. those ensuring parthenogenesis.

According to different estimates, *Wolbachia* affects around 40% of arthropod species. These Alphaproteobacteria mainly parasitize the reproductive tissues (eggs and testes) of their hosts, whose reproduction can be altered through male killing, feminization, parthenogenesis and cytoplasmic incompatibility (Werren et al., 2008; Brucker & Bordenstein, 2012; Zug & Hammerstein, 2012). *Wolbachia*-induced parthenogenesis has been documented in mites, thrips and several members of the order Hymenoptera (Pannebakker et al., 2004; Werren et al., 2008; Kremer et al., 2009; Watanabe et al., 2013), although other bacterial genera, such as *Rickettsia* and *Cardinium*, also seem able to induce parthenogenesis in Hymenoptera (Rabeling & Kronauer, 2013).

The order Phasmatodea has interesting biological and ecological characteristics. It comprises more than 2,500 species, some of which – the stick insects – bear a striking resemblance to branches or leaves. Parthenogenesis occurs in a number of phasmids (Bedford, 1978) and is particularly well documented in the genera *Bacillus* and *Timema* (Trewick et al., 2008; Schwander & Crespi, 2009), as well as occurring in *Sipyloidea*, *Carausius*, *Clitumnus* and many other genera (Suomalainen et al., 1987; Lacadena, 1996) (Table 1). There have been no reports of parthenogenesis induced by bacteria in phasmids, but a survey of the literature suggests this may primarily be because this possibility has not been explored empirically.

On the other hand, *Spiroplasma phylum* (Firmicutes) is another bacterial endosymbiont that can be considered one of the most important taxa because of its wide host range. It appears mainly in insects, but is occasionally found in other invertebrates (Haselkorn, 2010). This bacterial genus has been detected among the gastric flora of many arthropod species. Its association with intestinal epithelial cells appears to produce no adverse effects, and the genus is therefore considered to be commensal. However, under other circumstances, members of the genus are described as pathogens. The transition to pathogenicity may be linked to the ability to cross the barrier of the insect gut (Haselkorn, 2010) to reach the haemolymph, ovaries, salivary glands or hypodermis (Regassa & Gasparich, 2006). They have been characterised as pathogenic bacteria in shrimps, crabs and bees, in which they cause high levels of mortality (Haselkorn, 2010). In certain hosts infection

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Table 1. Species of the Phasmatodea order examined in this study. * Sex unknown (nymphs or juvenile individuals). Phasmid species and individuals analysed by PCR for the *16S rDNA* of *Wolbachia* sp., *Spiroplasma* sp. or eubacteria (number of infected individuals in brackets). Characterisation of the PCR products by sequencing and BLAST alignment. Source: samples kindly donated by J. Herranz-Barrera (Madrid, Spain) and A. Sevilla-Esquinas (Madrid) (1), P. Ruíz-Mínguez (Madrid) (2), J. Rodríguez-García (Villagarcía de Arosa, Spain) (3), and A. Fernández-Ramos (Madrid) (4).

Species	Individuals analysed	Sex	Reproduction	*Wolbachia* sp.	*Spiroplasma* sp.	Eubacteria	Origin	Source
Achrioptera fallax	3	3 *	Sexual	0	0	–	Madagascar	2
Diapherodes gigantea	2	1 * 1♂	Sexual	0	1	*Serratia marcescens* (1)	Antilles-Grenada	2
Diapherodes venustula	3	1 ♀ 2 ♂	Sexual	0	0	–	Cuba	2,3
Entoria nuda	12	9 ♀ 3 ♂	Parthenogenetic / Sexual	0	3	*Lactococcus lactis* (1)	Japan	1,2
Epidares nolimetangere	3	3 ♂	Sexual	0	0	–	Borneo	2
Eurycantha calcarata	4	1 ♀ 1 ♂ 2*	Parthenogenetic / Sexual	0	0	*Lactococcus* sp. (1) *Enterobacter ludwigii* (1)	New Guinea	2
Extatosoma tiaratum	9	3 ♀ 6 ♂	Parthenogenetic / Sexual	0	0	–	Australia	4
Hypocyrtus scythrus	8	8*	Sexual	0	0	–	–	4
Leptynia montana	19	11 ♀ 8 ♂	Sexual	0	1	*Methylobacterium thiocyanatum* (2) *Nevskia ramosa* (1)	Spain	1
Lonchoides sp. n. (Negros Is.)	1	1 ♂	Sexual	0	0	–	Philippines	1
Medauroidea extradentata	23	17 ♀ 5 ♂ 1*	Facultatively parthenogenetic / Sexual	0	0	*Lactococcus lactis* (1) *Cupriavidus* sp. (1)	Vietnam	1
Mnesilochus latifemur	7	4 ♀ 3 ♂	Sexual	0	0	–	Malaysia	3
Neohirasea maerens	15	14 ♀ 1 ♂	Facultatively parthenogenetic / Sexual	0	1	*Rahnella aquatilis* (1) *Serratia* sp. (1)	Vietnam	1
Parapachymorpha zomproi	1	1 ♀	Parthenogenetic / Sexual	0	0	–	Thailand	3
Periphetes forcipatus	4	4 *	Sexual	0	0	*Brevibacterium* sp. (1)	Indonesia	2
Peruphasma schulteii	18	7 ♀ 11 *	Sexual	0	0	–	Peru	1,2,4
Phaenopharos khaoyaiensis	17	17 ♀	Parthenogenetic	0	0	*Cupriavidus metallidurans* (1)	Thailand	1
Pharnacia ponderosa	2	2 *	Sexual	0	0	*Serratia marcescens* (1)	Philippines	2
Phyllium giganteum	4	4 ♀	Parthenogenetic	0	0	–	Malaysia	2
Phyllium jacobsoni	1	1 ♂	Sexual	0	0	–	Java	2
Phyllium philippinium	12	11 ♀ 1 ♂	Parthenogenetic / Sexual	0	0	–	Philippines	2
Phyllium westwoodii	16	16 *	Parthenogenetic / Sexual	0	0	*Serratia* sp. (2) *Cupriavidus* sp. (1)	Thailand	2
Ramulus artemis	19	19♀	Parthenogenetic	0	11	–	Vietnam	1,2
Sipyloidea sipylus	1	1♀	Parthenogenetic	0	1	–	Madagascar	1
Sungaya inexpectata	12	8 ♀ 4 ♂	Parthenogenetic / Sexual	0	1	*Enterobacter* sp. (1)	Philippines	1,2
Tirachoidea biceps	3	1 ♀ 2 ♂	Sexual	0	0	*Serratia liquefaciens* (1)	Java	2
Trachyareaton carmelae	12	2♀ 10 *	Sexual	0	0	–	Philippines	1,2,4
Trachyareaton sp. n. (Aurora Prov., Luzon)	13	13 *	–	0	0	–	Philippines	2
Total	244	173		0	19	19		

with these bacteria may impair reproduction: the bacterium is transmitted maternally, inducing the selective elimination of male progeny. This phenotype is called male killing (Regassa & Gasparich, 2006). The most widely studied example is *S. poulsonii*, which was isolated from neotropical species of *Drosophila willistoni* (Sturtevant) (Williamson et al., 1999), in which, in the most extreme case, all the male offspring of infected females are eliminated. Other instances of male killing have been detected in strains of *Spiroplasma* that infect *D. melanogaster* Meigen (Montenegro et al., 2005), *Danaus chrysippus* (L.) (Lepidoptera: Danaidae) (Jiggins et al., 2000) and *Adalia bipunctata* (L.) (Coleoptera: Coccinelidae) (Hurst et al., 1999a), among others. In cases without male killing, males and females can both be infected with no obvious change in the phenotype (Haselkorn, 2010). In natural populations of *Drosophila*, an 85% infection rate of non-male-killing *Spiroplasma* has been noted (Watts et al., 2009).

In phasmids, *Spiroplasma* has been described in two Argentinian populations of *Agathemera* spp., as well as in their parasitic mites of the genus *Leptus* (Leptidae) (DiBlasi et al., 2011), although their phenotypic effects have not been associated with male killing. In addition, this endosymbiont has been identified in the strictly parthenogenetic *Ramulus artemis* (Westwood) and in the sexual *Pharnacia ponderosa* Stål (Shelomi et al., 2013), with unknown phenotypic effects.

Wolbachia and *Spiroplasma* are inherited endosymbionts that can have various influences on their hosts, ranging from mutualistic to parasitic effects, potentially affecting their reproduction and evolution. Both bacteria are transmitted maternally from infected females to their offspring and are not incompatible with each other (Duron et al., 2008, Martinez-Rodriguez et al., 2013). Driving host reproduction leads to an increase in the number of infected females, even at the expense of males, improving the fitness of the bacterium and its transmission between individuals within the population (Haselkorn, 2010).

Stick insect species exhibit a wide range of reproductive mechanisms, some of which are characterised by the absence of males and are therefore compatible with the involvement of these bacteria or of others with similar effects. We explored this possibility in a broad survey of species of phasmids using a polymerase chain reaction (PCR) / DNA sequencing approach.

MATERIAL AND METHODS

Obtaining DNA and PCR characterisation

We obtained data from 244 individuals representing 28 species and 24 genera of the order Phasmatodea. Insects were collected in 2012 and 2013 from distinct captive populations of different geographic origin, all of them naturalised in Spain (Table 1). These individuals were kindly donated for this study, as recognised in Table 1, and preserved in absolute ethanol at –20°C until analysed.

Genomic DNA was obtained in different ways, depending on the size of the organism: (1) large individuals – from an abdominal fragment containing the gonads; (2) medium-sized adults – from the abdomen; and (3) small adults, nymphs and juveniles of reduced size – from the whole body (except for the head, in order to exclude eye pigments, which reduce the quality of the DNA), as detailed in Zabal-Aguirre et al. (2010) and Martinez-Rodriguez et al. (2013). DNA samples were standardised at a final concentration of 50 ng/µl using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA).

Preliminary analyses were performed to check the quality of the DNA samples. This enabled us to confirm that these were not fragmented, allowing further microbial detection (see below): (i) for each sample, a 2% agarose electrophoretic gel with 2 µl of sample was run at 70 V, and (ii) a PCR of the cytochrome oxidase I (*COI*) mitochondrial gene, and 0.6 mM of each primer (numbered as 1 in Table 2) were used in PCR reactions performed in a final volume of 50 µl (1 × buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 U *Taq* polymerase and 2.0 µl (100 ng) of DNA). Reagents were supplied by BIOTAQ (Bioline Reagents Ltd, London, UK). A Techne TC-512 thermocycler was programmed to give an initial denaturation step at 94°C for 10 min, followed by 36 cycles of denaturation at 94°C for 30 s, an annealing step at 54°C for 45 s, an elongation step at 72°C for 90 s, and a single final elongation cycle at 70°C for 10 min.

Wolbachia infection in these phasmids was checked by PCR detection of the *16S rRNA* sequence from this bacterium (Table 2, primers nº 2). When amplification was not detected by electrophoresis or the negative controls produced a band, the resulting products were re-amplified with the same primers to test for possible false-negatives due to low-level infection or contamination, respectively. Reactions were performed in a final volume of 50 µl, containing 1 × buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.6

Table 2. PCR primers used to amplify the cytochrome oxidase I (*COI*) mitochondrial gene (1) in order to check the quality of the DNAs examined, or to detect possible bacterial endosymbionts in the phasmid species studied: *16S rDNA* (2) and *wsp* (primers 3) from *Wolbachia*, *16S rDNA* from *Spiroplasma* (4) and from eubacteria (5).

Primers		Sequence (5'–3')	Size	Reference
1	C1-J-2195	TTGATTTTTTGGTCATCCAGAAGT	753 bp	Simon et al., 1994
	TL2-N-3014	TCCAATGCACTAATCTGCCATATTA		
2	16S_F	TTGTAGCTTGCTATGGTATAACT	1400 bp	Zabal-Aguirre et al., 2010
	16S_R	ACTGCTACCTTGTTACGACTT		
3	Wsp_F1	GTCCAATARSTGATGARGAAAC	603 bp	Baldo et al., 2005, 2006
	Wsp_R1	CYGCACCAAYAGYRCTRTAAA		
4	MGSO	TGCACCATCTGTCACTCTGTTAACCTC	500 bp	van Kuppeveld et al., 1992
	HA-IN-1	GCTCAACCCCTAACCGCC		Hurst et al., 1999b
5	9.27F	GAGTTTG(AC)TCCTGGCTCAG	1492 bp	Lane, 1991
	1492.1512R	ACGG(CT)TACCTTGTTACGACTT		

mM of each primer, 1.25 U of BIOTAQ polymerase (Bioline), and 2 µl of standardized DNA template solution from each individual insect analysed (100 ng). A Techne TC-512 thermocycler was programmed for 94°C for 2 min, followed by 37 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 90 s, and a single final elongation cycle at 70°C for 10 min (see Zabal-Aguirre et al., 2010).

To verify our results, a further *Wolbachia* detection system was developed: PCR of the *wsp* gene of *Wolbachia* (Table 2, primers nº 3) was performed in a final volume of 40 µl containing 1 × buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer, 0.5 U of BIOTAQ polymerase (Bioline), and 2 µl of standardised DNA template solution from each individual (100 ng). Techne TC-512 thermocycler conditions were here 94°C for 2 min, followed by 37 cycles of 94°C for 30 s, 58°C for 1 min and 72°C for 90 s, followed by a final elongation cycle at 72°C for 10 min.

Spiroplasma infection was tested for the presence of the *16S* r*RNA* gene by PCR using specific primers as shown in Table 2 (primers nº 4). The possible presence of other bacteria in the individual insects as studied here was also checked by PCR of their *16S* r*RNA* sequences using universal primers for eubacteria (Table 2, primers nº 5). For these amplifications, reactions were conducted in a final volume of 50 µl containing the appropriate 1 × buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 mM of each primer, 1.25 U of BIOTAQ polymerase (Bioline) and 2.0 µl of the standardised DNA template solution (100 ng). Techne TC-512 thermocycler conditions were initially 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 54°C for 1 min, 72°C for 90 s, and a final elongation cycle of 72°C for 10 min (Martinez-Rodriguez et al., 2013 for details).

The amplification was checked electrophoretically in all cases: 10 µl of each PCR product were run at 70 V in a 2% agarose gel containing 0.5 mg/ml of ethidium bromide with a track reserved for a 1-kb DNA size marker (Biotools, Madrid, Spain), before visualising using a UV transilluminator (Uvitec UVIdoc HD2, Cambridge, UK).

All PCR reactions included the appropriate controls. As positive controls for *Wolbachia*, *Spiroplasma* and eubacteria, DNA from previously characterised infected individuals of *Chorthippus parallelus* (Zetterstedt) (Orthoptera: Acrididae) was used (Martinez-Rodriguez et al., 2013). For the negative controls, no DNA was included in the PCR reaction mix. All amplifications were made at least twice.

PCR product purification, sequencing and characterisation

PCR-amplified sequences from the *16S* r*RNA* gene of *Spiroplasma* and eubacteria were purified with the ExoSAP-IT kit supplied by GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, USA). Resulting products were automatically sequenced by STABVIDA (http://stabvida.com/, Caparica, Portugal). The genus and taxon were assigned (when possible) with BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/) using the consensus sequences in the databases of the National Center for Biotechnology Information (NCBI). The new sequences as here obtained have been registered in Genbank under accession numbers KJ685895 to KJ685899.

Sequence analyses, alignment and an evolutionary model

Phylogenetic analyses were based on the available *Spiroplasma* sp. *16S* r*RNA* nucleotide sequences. A preliminary manual analysis of the chromatograms was performed with DNAstar Lasergene Core Suite (http://www.dnastar.com) software. ClustalW software (Larkin et al., 2007) was used to align the sequences obtained and those registered from other arthropods. In all cases we found sufficient homology to enable further phylogenetic inference.

The on-line ALTER tool (Glez-Pena et al., 2010) was used to convert the data formats when they differed. Text files were manually edited with notepad++ software (http://notepad-plus-plus.org/). jModeltest software (Posada, 2008) was used to select the appropriate nucleotide substitution model with the Akaike information criterion (AIC) (Akaike, 1973, 1974). The model selected was the $GTR+G+I$ variant of the General Time Reversible (GTR) model described by Tavaré (1986), which considers distinct probabilities for each base substitution on the assumption that nucleotide base frequencies may differ.

Escherichia coli was used as the outgroup to root the tree. Figtree software (http://tree.bio.ed.ac.uk/software/figtree/) was employed to visualise and edit the phylogenetic trees.

We assessed the phylogenetic reconstruction by the method of maximum likelihood (ML) (Schmidt & von Haeseler, 2009; Vargas & Zardoya, 2012), as described by Felsenstein (1981), using RAxML (Randomized Axelerated Maximum Likelihood) v 7.0.4 (Stamatakis, 2006) implementing $GTR+G+I$, as described above. Bayesian inference (BI) (Vargas & Zardoya, 2012; Yang & Rannala, 2012) was also used for the same sequences with the MrBayes 3.2 program (Ronquist & Huelsenbeck, 2003) simultaneously conducting two analyses by the Monte Carlo method (MCMC), each one with four Markov chains, for a minimum of 10^6 generations until reaching convergence, defined as a standard deviation of split frequencies <0.01.

Only clades with a posterior probability >0.95 (Huelsenbeck & Rannala, 2004) were considered to be statistically supported.

RESULTS

None of the 244 phasmid individuals analysed showed *Wolbachia* infection (see Table 1). To be certain of this negative result, positive controls (as described in the Material and Methods section) and primers for two *Wolbachia*

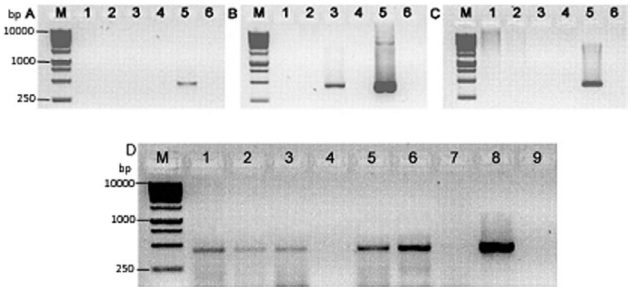

Fig. 1. Electrophoretic gels (A to D) showing the PCR amplification with primers for the *16S rDNA* of *Spiroplasma* sp. in single phasmid individuals of different stick insect species. M indicates the DNA size marker. A – Tracks 1–4, *Periphetes forcipatus* (no infection); track 5, *Ramulus artemis* (positive); track 6, *Phyllium jacobsoni* (no infection). B – Track 1: *Trachyaretaon carmelae* (negative); tracks 2–5: *Ramulus artemis* (positive in 3 and 5), and track 6, *Medauroidea extradentata* (negative). C – Track 1, *Phaenopharos khaoyensis* (negative); tracks 2 and 3: *Pharnacia ponderosa* (negative); track 4, *Phaenopharos khaoyensis* (negative); tracks 5 and 6: positive and negative controls, respectively. D – Track 1, *Diapherodes gigantea* (positive); track 2, *Sipyloidea sipylus* (positive); track 3, *Sungaya inexpectata* (positive); track 4, *Tirachoidea biceps* (negative); track 5, *Neohirasea maerens* (positive); track 6, *Sungaya inexpectata* (positive); track 7, *Phaenopharos khaoyaiensis* (negative); tracks 8 and 9, positive and negative controls, respectively.

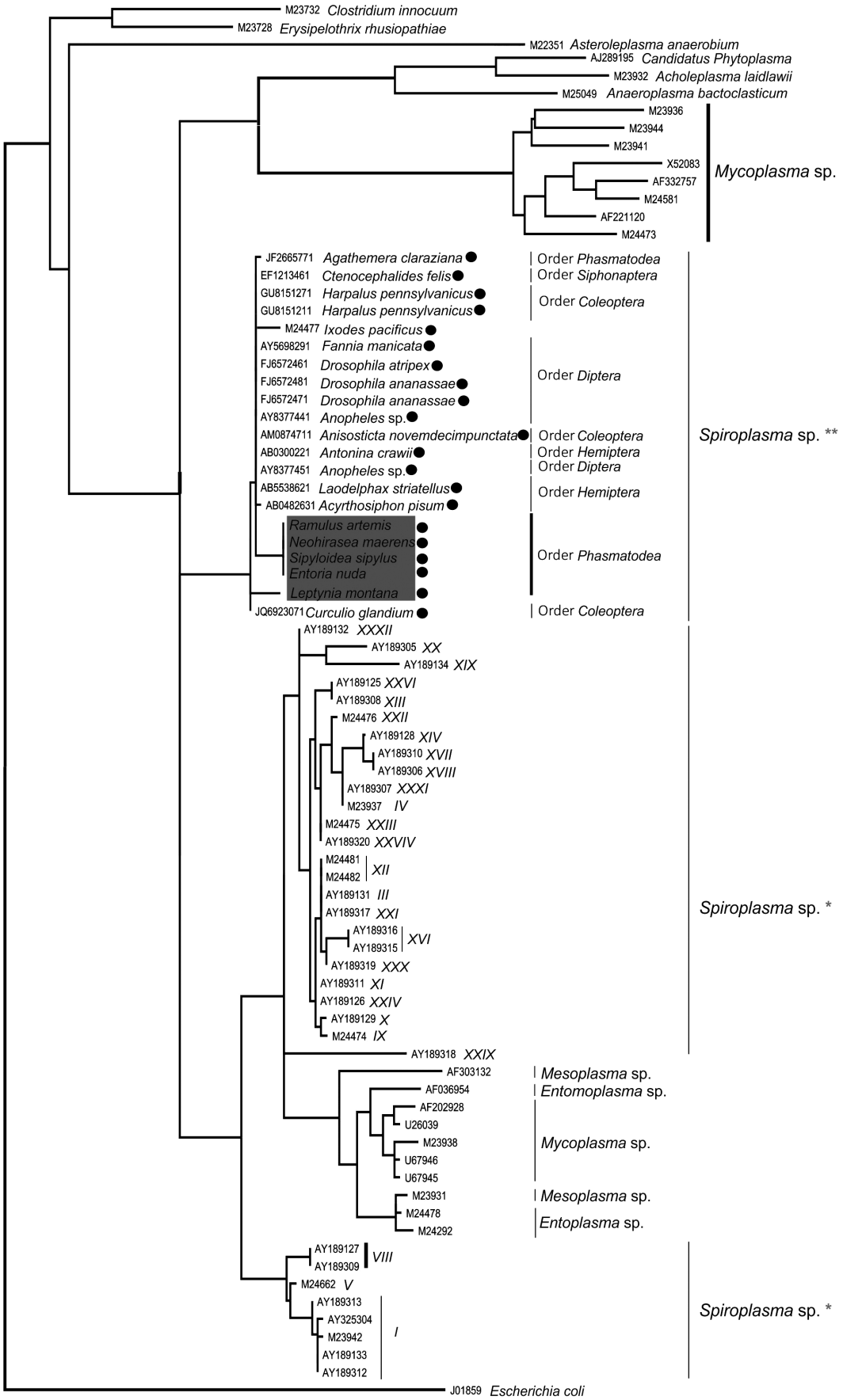

Fig. 2. *Spiroplasma* spp. phylogeny based on the *16S rDNA* gene using the ML approach, indicating the infected host species (●). Roman numbers refer to the serological classification system for *Spiroplasma*. * *Spiroplasma* sp. belonging to previously described clades. ** *Spiroplasma* sp.: new clade described in this study. Phasmid species used for this study infected by *Spiroplasma* sp. are shaded.

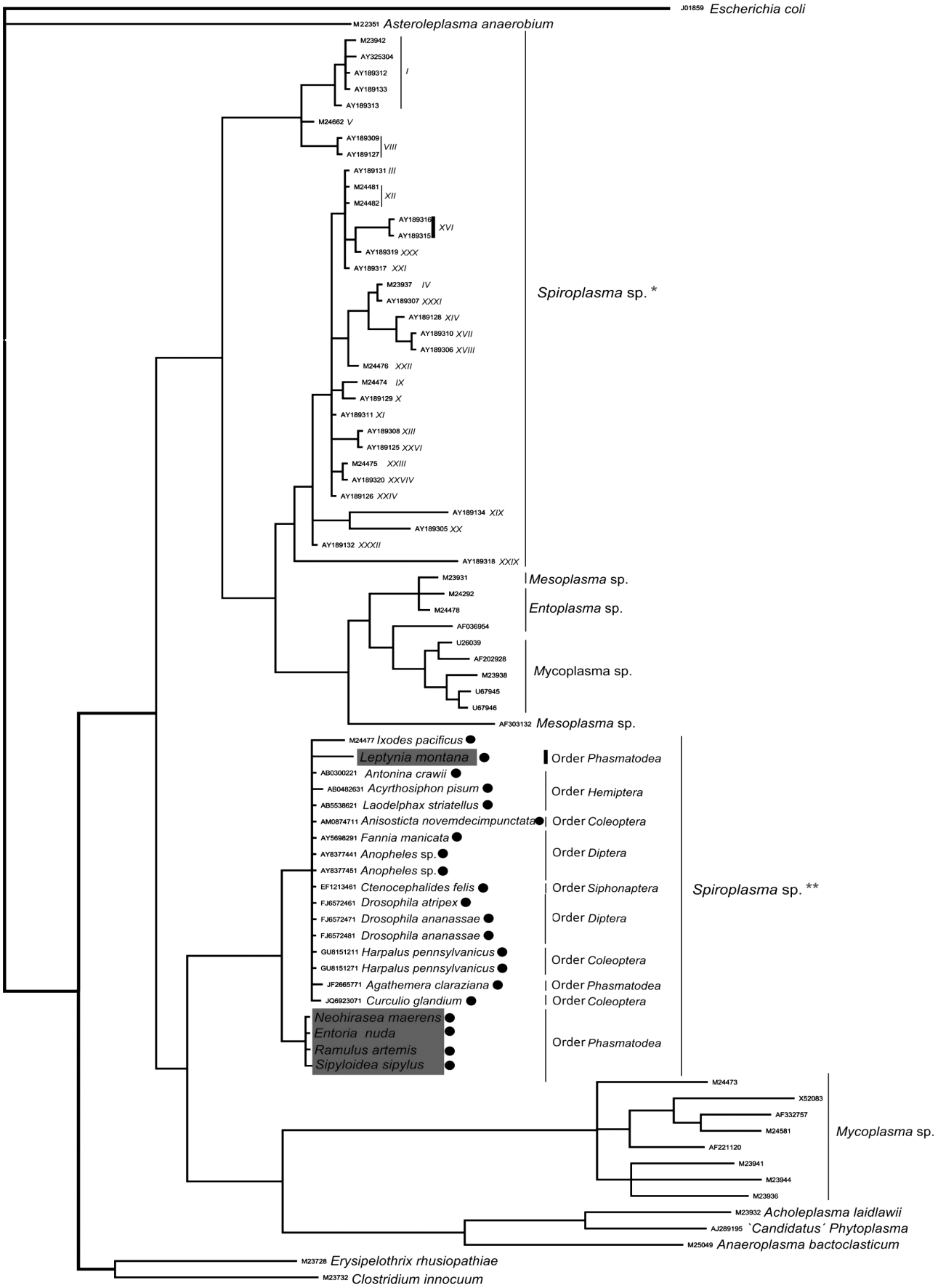

Fig. 3. *Spiroplasma* spp. phylogeny based on the *16S rDNA* gene, using the BI approach, indicating the infected host species (●). Roman numbers refer to the serological classification system for *Spiroplasma*. * *Spiroplasma* sp. belonging to previously described clades. ** *Spiroplasma* sp.: new clade described in this study. Phasmid species used for this study infected by *Spiroplasma* sp. are shaded.

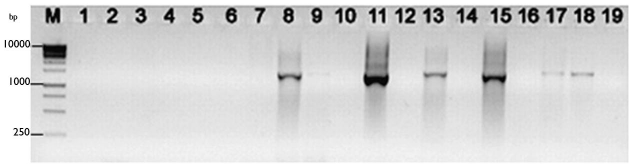

Fig. 4. Gel electrophoresis of the PCR products obtained after amplification with eubacterial universal primers for the *16S rDNA* gene of DNA from the phasmid species studied. Their sequencing and BLAST alignment assign them to the genus and/or bacterial species indicated below. M: DNA size marker. Tracks 1–7, *Phaenopharos khaoyensis*; tracks 8–9, *Pharnacia ponderosa*; track 10, *Sungaya inexpectata*; track 11, *Diapherodes gigantea*; track 12, *Sipyloidea sipylus*; track 13, *Phaenopharos khaoyensis*; track 14, *Tirachoidea biceps*; track 15, *Neohirasea maerens*; track 16, *Sungaya inexpectata*; track 17, *Phaenopharos khaoyensis*, and tracks 18 and 19 correspond to the positive and negative controls, respectively. The bands in tracks 8 and 11 correspond to *Serratia marcescens*; track 13 to *Enterobacter* sp.; track 15, *Serratia* sp. We were unable to determine the sequence from the bands in tracks 9 and 17 (*Phaenopharos khaoyensis*).

loci were used (Table 2). This enabled us to rule out false negatives, and the possibility of sequence variation in the sequences not recognized by a singular pair of primers.

Nineteen individuals belonging to the following species – *Neohirasea maerens* (Brunner von Wattenwyl), *Ramulus artemis*, *Leptynia montana* Scali, *Entoria nuda* Brunner von Wattenwyl, *Sungaya inexpectata* (Zompro), *Diapherodes gigantea* (Gmelin) and *Sipyloidea sipylus* (Westwood) showed PCR amplification using the primers for the *Spiroplasma* sp. *16S rRNA* gene (Fig. 1, Table 1). PCR products were automatically sequenced and sequences BLAST aligned up to the genus level. Sequences showing at least 97% of identity were considered operational taxonomic units (OTUs). 11 of the 19 analysed individuals of the strictly parthenogenetic *Ramulus artemis* and three of the 12 individuals of the occasionally parthenogenetic *Entoria nuda* proved to be infected by *Spiroplasma*. The other species only comprised one infected individual each (Table 1).

Phylogenetic reconstruction with these sequences based on ML and BI linked the strains detected with those previously described in phasmids (Gasparich et al., 2004; DiBlasi et al., 2011; Shelomi et al., 2013) (see * *Spiroplasma* sp. in Figs 2 and 3). It is of interest that our strains assign to a new and different *Spiroplasma* clade (** *Spiroplasma* sp. in Figs 2 and 3). This new clade (** *Spiroplasma* sp.) is further divided into two subclades (Fig. 3). One of these includes four of the phasmid species studied here: *R. artemis*, *N. maerens*, *E. nuda* and *S. sipylus*; the other subclade comprises various arthropods, including our *L. montana* and *Agathemera*.

The survey with universal *16S rDNA* PCR primers to identify other possible eubacterial endosymbionts infecting our phasmid species yielded 19 positive results. These PCR products were sequenced and BLAST-aligned. Again, using the minimum of 97% identity as the criterion for being considered an OTU, we were able to assign these sequences to different bacterial taxa (Fig. 4; Table 1).

DISCUSSION

The reproductive alterations induced by *Wolbachia* have been found in many organisms (Werren, 1997; Werren et al., 2008; Brucker & Bordenstein, 2012). However, to our knowledge, the possibility that this bacterial endosymbiont infects phasmids has not previously been explored, even though these arthropods are a well-known example of occasional parthenogenesis (thelytoky) (More, 1996), a phenomenon potentially induced by this bacterium (Simon et al., 2003).

In an attempt to evaluate the role played by *Wolbachia* in the reproduction of these organisms, we studied the incidence of this bacterial endosymbiont in phasmid species displaying different kinds of reproductive mode – from standard bisexual reproduction, to automictic or apomictic parthenogenesis and tychoparthenogenesis. However, in none of the species and individuals analysed was the presence of *Wolbachia* detected by the approaches here used. This makes it very unlikely, in our opinion, that this bacterium is generally involved in the reproductive systems of phasmids, although we cannot discount the possibility of it being involved in particular cases. The absence of *Wolbachia* infection from all these organisms is striking, given the high proportion of insect and arthropod species infected (Zug & Hammerstein, 2012). This by itself may be of evolutionary significance in this group of organisms.

On the other hand, we found *Spiroplasma* sp. in 7.7% of the individuals and 25% of the species analysed (Table 1). This bacterial endosymbiont of maternal transmission also induces reproductive alterations in several organisms. The preferential killing of male descendants is its most common effect, with a variable incidence (from 5 to 90% of infected females) depending on the taxon under consideration and other ecological and probably genetic aspects (Hurst & Jiggins, 2000; Hutchence et al., 2012; Ventura et al., 2012; Martin et al., 2013; Sanada-Morimura et al., 2013; Harumoto et al., 2014; Xie et al., 2014).

However, we have detected this bacterium in phasmid species apparently characterised by obligate sexual reproduction, like *Leptynia montana* and *Diapherodes gigantea*, in species with obligatory parthenogenesis, such as *Ramulus artemis* and *Sipyloidea sipylus*, and in *Neohirasea maerens* and *Entoria nuda*, which show occasional parthenogenesis. These preliminary results are promising and suggest the value of further research involving more individuals and populations, progeny analyses, experimental crosses between infected and uninfected individuals, and perhaps studies with previously infected individuals from parthenogenetic lineages treated with antibiotics.

A previous morphological study found *Spiroplasma* in the gut and certain muscle tissues of another stick insect, *Agathemera* spp. (Phasmatodea), but not in its eggs. This seems to rule out the possibility that this bacterium can induce the male-killing phenotype in these phasmids (DiBlasi et al., 2011). In our study, *Spiroplasma* was isolated from the abdomen, where the gonads (and the eggs in females) are located. This leads us to assume that the bacteria follow their standard maternal mode of transmission, the eggs

presumably also being infected. We found the infection in both males and females, which may rule out the possibility of male killing in these cases.Even so, we are reminded of certain cases in which this phenotype only affects a limited proportion of the descendants, as observed in natural Japanese populations of *Gastrolina depressa* Baly (Coleoptera: Chrysomelidae): male killing is absent from northern and southern populations, but is present in 50 to 80% of the females from the centre of the islands (Chang et al., 1991; Hurst & Jiggins, 2000).

Taxonomically, *Spiroplasma* is classified within the order Entomoplasmatales (Regassa & Gasparich, 2006), in the Mollicutes lineage (Gasparich et al., 2004). Recent phylogenetic analyses based on the *16S rRNA* gene classified this genus as non-monophyletic (Regassa & Gasparich, 2006). The phylogenetic characterisation of the *16S rRNA* sequences of the *Spiroplasma* detected here ascribes the strains found in *R. artemis*, *N. maerens*, *E. nuda* and *S. sipylus* to a new divergent clade, with the ML and BI approaches (Figs 2 and 3, respectively). They appear to be associated with a *16S rRNA* sequence previously described in a mite (GenBank: M24477), and classified in serogroup VI of *Spiroplasma* (Weisburg et al., 1989; Tully et al., 1995). This serogroup belongs to the *Ixodetis* clade, which includes the single lineage *S. ixodetis* and is at a considerable evolutionary distance from the other characterised *Spiroplasma* spp. (Regassa & Gasparich, 2006). Similar divergence is also displayed by the other known case of this microorganism infecting a phasmid (DiBlasi et al., 2011; Shelomi et al., 2013). This prevents a simple interpretation of the possible biological effects of *Spiroplasma* in these hosts. More data from other organisms infected by these strains will shed light on this specific clade and the phenotype induced in its hosts.

Spiroplasma strains similar to *S. ixodetis* have been associated with abnormal sex ratios in the butterfly, *D. chrysippus* and the ladybird beetle, *A. bipunctata* (Regassa & Gasparich, 2006). However, DiBlasi et al. (2011) did not find male killing induced by *Spiroplasma* in *Agathemera* spp. (Phasmatodea). In our case, we have no data that would justify the inference of a possible phenotypic effect of this bacterium in its hosts. As indicated above, further complex experiments (F_1 and F_2 crosses with infected and uninfected individuals, the use of antibiotics, etc.) are needed to clarify this matter.

Given the absence of correlation between our results with *Wolbachia* and *Spiroplasma* and the reproductive mode of the stick insects analysed, we complemented our study of the microbiota of these phasmids with a broad PCR-based survey of other eubacteria, in an attempt to detect other endosymbionts that might influence their reproductive biology. Insects are usually associated with microorganisms that contribute to their physiology (Mohr & Tebbe, 2006; Belda et al., 2011). However, our sequencing and BLAST comparison results indicate a relatively scarce microbial presence, with ~ 8.0% (19 out of 244) representativeness (Fig. 4), Proteobacteria and Firmicutes being the most commonly associated phyla (Fig. 5). This low representativeness may have several non-mutually exclusive explanations. Of these, we acknowledge that the captivity of the individuals studied here may have affected the bacterial diversity. In fact, this may have a significant influence in this kind of studies, as reported by Lo et al. (2006). Their non-natural diet was probably a major contributor to this, although our organisms did come from four distinct sources. Neither can we rule out the possibility that certain bacteria are insufficiently represented, which would make them difficult to detect by these methods. In any case, the bacterial taxa detected seem to be related to the nutritional function of their hosts, being microorganisms commonly associated with insects.

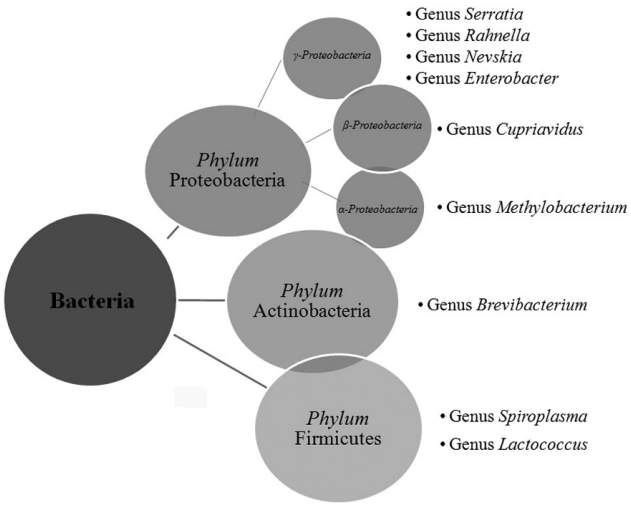

Fig. 5. The phylogeny of the bacterial endosymbionts found in Phasmatodea shows three main phyla: Proteobacteria (classes α, β and γ), Actinobacteria and Firmicutes. *Spiroplasma* spp. belongs to the latter phylum.

In summary, our results fail to reveal any definite association between bacterial infections and the reproductive modes of phasmids, more especially any clear link with the most common microorganisms involved here, *Wolbachia* and *Spiroplasma*. In the latter genus, however, further studies would ascertain its possible phenotypic or physiological effect on infected individuals and species.

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