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Wolbachia infection in the tribe Naupactini (Coleoptera, Curculionidae): association between thelytokous parthenogenesis and infection status

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Abstract

Several parthenogenetic species of broad-nosed weevils exist, some of them of economic importance because of their pest status. Screening of the maternally inherited *Wolbachia* bacterium in 29 weevils of the tribe Naupactini, using multilocus sequence typing allowed us to assess a significant correlation between asexuality and infection, and suggests an involvement of *Wolbachia* in the origin of this reproductive mode. The nine *Wolbachia* strains retrieved from the Naupactini belong to the B supergroup. Phylogenetic analysis of these strains, along with other 23 strains obtained from arthropods and nematodes, supports previous hypotheses that horizontal transfer of *Wolbachia* amongst species from unrelated taxa has been pervasive.

Keywords: Curculionidae, Naupactini, *Wolbachia*, thelytokous parthenogenesis, multilocus sequence typing (MLST).

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Introduction

In parthenogenetic organisms the development of egg cells occurs without fertilization. Despite the twofold cost associated with sexual reproduction (Maynard Smith, 1978), it is not expected that parthenogenetic taxa survive in the long term (Normark, 2003). Therefore, the study of parthenogenesis may yield some insights into a great evolutionary puzzle: the superiority of sexuality over asexuality, known as 'the paradox of sex' (Maynard Smith, 1978).

Parthenogenesis is fairly common amongst plants and some animal taxa (Bell, 1982; Suomalainen *et al*., 1987; Koivisto & Braig, 2003; Majerus, 2003; de Meeûs *et al*., 2007), for example several insect orders (eg Kelly, 1913; Suomalainen, 1962; Lokki *et al*., 1976; Went, 1982; Hoffman *et al*., 2008). Within Coleoptera, the family Curculionidae encompasses a huge number of asexual lineages, distributed in three different subfamilies: Scolytinae (bark beetles), Listroderinae and Entiminae (broad-nosed weevils) (Suomalainen, 1962; Suomalainen *et al*., 1976; Smith & Virkki, 1978; Lokki & Saura, 1980; Normark, 2003).

The parthenogenesis of broad-nosed weevils is thelytokous and apomictic (Suomalainen, 1969). Consequently, progeny results in a group of females genetically identical to their mother (Vepsälainen & Järvinen, 1979). Despite the absence of meiosis, asexual weevils have demonstrated a great colonizing ability of new environments, probably resulting from the potential to start a new population from a single colonist (parthenogenetic female) at any stage of development (Gerritsen, 1980; Bell, 1982; Lanteri & Normark, 1995; Peck *et al*., 1998).

Within the subfamily Entiminae, the tribe Otiorhynchini includes several well-studied parthenogenetic species, particularly those of the genus *Otiorhynchus* (Suomalainen, 1961, 1962, 1969; Stenberg *et al*., 1997, 2000, 2003; Stenberg & Lundmark, 2004; Lundmark & Saura, 2006). On the contrary, the reproductive biology of the Naupactini is poorly known. There is no direct evidence of parthenogenesis, except for *Naupactus cervinus*,

Naupactus leucoloma, *Naupactus peregrinus*, *Pantomorus ruizi* and *Aramigus tessellatus* (Buchanan, 1939; Sanderson, 1973; Normark, 1996; Marvaldi, 1998). However, Lanteri & Normark (1995) proposed a list of about 30 presumably parthenogenetic species of Naupactini, on the basis of female-biased sex ratios (ie males either absent or scarce in part of their geographical range). Most of these species are native to the prairies and steppes of South America (central Argentina, southern Brazil and Uruguay), although several have been able to colonize other continents along with different crops, becoming serious pests of agriculture (Lanteri & Normark, 1995).

The mechanisms that originated parthenogenesis in broad-nosed weevils are still under discussion and a multiplicity of factors has been invoked (Tomiuk *et al*., 1994). Polyploidy and hybridization are both usually associated with this reproductive mode (Suomalainen, 1969; Saura *et al*., 1993; Tomiuk *et al*., 1994). However, the infection with the parthenogenesis inductor *Wolbachia pipientis* (hereafter '*Wolbachia*') brings another possibility, ie infectious origin of parthenogenesis.

This obligate intracellular bacterium, infecting 17–75% of arthropods (Werren *et al*., 1995a; West *et al*., 1998; Jeyaprakash & Hoy, 2000; Werren & Windsor, 2000; Jiggins *et al*., 2001) and 90% of filarial nematodes (Bandi *et al*., 1998), not only causes thelytokous parthenogenesis but also other reproductive alterations such as cytoplasmic incompatibility, embryonic male killing, larval male killing and feminization of genetic males (Engelstädter & Hurst, 2009). These modifications of the host reproduction give a selective advantage to the bacterium (Turelli, 1994) that becomes widespread through the host populations, increasing its frequency. Although maternal transmission is the primary mode for *Wolbachia* carriers, horizontal transfer amongst unrelated arthropod species is extensive, as it is demonstrated by the pandemic distribution of this microorganism (Braig *et al*., 2002).

Interestingly, *Wolbachia* infection has been recorded for three parthenogenetic Entiminae: *Aramigus tessellatus* (Naupactini; Werren *et al*., 1995b), *Cathormiocerus britannicus* (Trachyphloeini; Piper *et al*., 2001) and *Otiorhynchus sulcatus* (Otiorhynchini) (Son *et al*., 2008). In this contribution we approach the hypothesis of infectious parthenogenesis, through the assessment of the association between parthenogenetic reproduction and *Wolbachia* infection, in 29 species of Naupactini. To reach this goal, we have analysed:

1 The status of *Wolbachia* infection in parthenogenetic, presumably parthenogenetic and sexual species of broad-nosed weevils;

2 The phylogenetic relationships of the *Wolbachia* strains identified in this weevil tribe by means of multilocus sequence typing (MLST).

Results

Correlation between Wolbachia *infection status and reproductive mode in Naupactini tribe*

We positively diagnosed *Wolbachia* infection for 21 parthenogenetic or presumed to be parthenogenetic Naupactini species through the amplification of a fragment of *c.* 800 bp in length of the *16S rDNA* gene (Table 1). All the individuals tested within the same species were infected.

To confirm our diagnosis, eight random *16S rDNA* and *ftsZ* amplified fragments from *Naupactus cervinus* were sequenced. Identical sequences were obtained for each gene (GenBank accession nos GQ402143 and GQ402144, respectively). BLAST homology searches indicated that the sequence of *16S rDNA* from *N. cervinus* shared 99.0% nucleotide identity with the *16S rDNA* encoded by a *Wolbachia* strain from *Hishimonus sellatus* (Hemiptera: Cicadellidae) (GenBank accession no. AB073731.1, W. Mitsuhashi, T. Saiki, W. Wei, H. Kawakita and M. Sato, unpubl. data). Similar percentages for nucleotide identity were obtained for the *ftsZ* sequences between *Wolbachia* from *N. cervinus* and those obtained from GenBank (data not shown).

Diagnosis of *Wolbachia* infection (ie no amplification of the *16S rDNA* fragment) was negative for seven Naupactini sexual species and only one presumably parthenogenetic species (Table 1). All the samples assayed were positive for insect *COI* amplification, validating PCR diagnostic results.

A significant association ($P = 0.000$) was obtained between *Wolbachia* infection status and reproductive mode (ie parthenogenetic reproduction or presumably parthenogenetic reproduction vs. sexual reproduction).

Allelic variation at MLST loci and strain diversity

From the whole set of host species, a total of nine strains was characterized by MLST (Table 2). There were three *ftsZ* alleles, three *gatB* alleles, three *coxA* alleles, eight *fbpA* alleles and four *hcpA* alleles (Table 2). Out of the 21 infected species, seven share the strain *wNau1*, three the strain *wNau7* and seven have a unique strain (ie *wNau2*, *wNau3*, *wNau4*, *wNau5*, *wNau6*, *wNau8* and *wNau9*). In the remaining four species, the strains could not be fully characterized (Table 2), but if the set of sequenced alleles agreed with one of the sequence types (STs) already determined, we assigned the same strain (and then, the same ST) with a question mark.

Phylogenetic analysis of Wolbachia *strains for Naupactini species*

The phylogenetic analyses for concatenated MLST loci revealed that the nine *Wolbachia* strains that infected the

Table 1. Naupactini species diversity

Naupactini species	Reproductive mode	Individuals assayed	Infection status
Aramigus conirostris	Parthenogenesis	1	✓
Aramigus tessellatus morph.* *pallidus*	Parthenogenesis	2	✓
Aramigus tessellatus morph.* *tessellatus*	Parthenogenesis	2	✓
Atrichonotus sordidus	Parthenogenesis	1	×
Atrichonotus taeniatulus	Parthenogenesis	1	✓
Enoploplactus lizeri	Sexuality	1	×
Enoploplactus sulfureovitattus	Sexuality	1	×
Eurymetopus fallax	Parthenogenesis	1	✓
Eurymetopus globosus	Parthenogenesis	1	✓
Mimographus ocellatus	Presumed parthenogenesis	1	✓
Naupactus ambiguus	Presumed parthenogenesis	3	✓
Naupactus cervinus	Parthenogenesis	20	✓
Naupactus cinereidorsum	Sexuality	5	×
Naupactus condecoratus	Presumed parthenogenesis	1	✓
Naupactus cyphoides	Parthenogenesis	1	✓
Naupactus dissimilis	Presumed parthenogenesis	1	✓
Naupactus dissimulator	Sexuality	5	×
Naupactus leucoloma	Parthenogenesis	3	✓
Naupactus minor	Parthenogenesis	3	✓
Naupactus peregrinus	Parthenogenesis	1	✓
Naupactus purpureoviolaceus	Presumed parthenogenesis	1	✓
Naupactus tremolerasi	Presumed parthenogenesis	1	✓
Naupactus tucumanensis	Sexuality	1	×
Naupactus verecundus	Presumed parthenogenesis	1	✓
Naupactus versatilis	Sexuality	1	×
Naupactus xanthographus	Sexuality	3	×
Pantomorus auripes	Presumed parthenogenesis	3	✓
Pantomorus cinerosus	Presumed parthenogenesis	2	✓
Pantomorus viridisquamosus	Presumed parthenogenesis	2	✓

Naupactini species assayed to detect *Wolbachia* infection. A positive diagnosis is indicated by means of the symbol ✓ and a negative by ×.
Sampling locations can be seen in the Supporting Information. Reproductive modes are based on previously published information (see references in the Supporting Information). Infection status refers to all the individuals tested within a species.
*Morphotype: group of morphologically differentiated individuals of a species of unknown or of no taxonomic significance.

Table 2. *Wolbachia* strains diversity

Host (strain)	Strain	ST	*coxA*	*fbpA*	*ftsZ*	*gatB*	*hcpA*
Aramigus conirostris	*wNau1*	190	14	181	96	9	13
Aramigus tessellatus morph. *pallidus*	*wNau2*	191	106	183	96	9	15
Aramigus tessellatus morph. *tessellatus*	*wNau3*	193	14	183	96	9	15
Atrichonotus taeniatulus	*wNau1*	190	14	181	96	9	13
Eurymetopus fallax	*wNau1*	190	14	181	96	9	13
Eurymetopus globosus	*wNau1*	190	14	181	96	9	13
Mimographus ocellatus	*wNau4*	193	107	132	97	79	124
Naupactus ambiguus	*wNau1*	190	14	181	96	9	13
Naupactus cervinus	*wNau5*	194	14	9	11	127	126
Naupactus condecoratus	*wNau6*	**A**	14	185	96	?	?
Naupactus cyphoides	*?*	?	?	?	?	?	?
Naupactus dissimilis	*wNau7*	195	14	14	96	9	15
Naupactus leucoloma	*wNau7*	195	14	14	96	9	15
Naupactus minor	*wNau1*	190	14	181	96	9	13
Naupactus peregrinus	*wNau8*	**B**	14	184	?	9	15
Naupactus purpureoviolaceus	*wNau1?*	190?	14	181	?	9	13
Naupactus tremolerasi	*wNau1?*	190?	14	181	?	9	13
Naupactus verecundus	*wNau7*	195	14	14	96	9	15
Pantomorus auripes	*wNau3?*	193?	14	183	?	9	15
Pantomorus cinerosus	*wNau9*	196	14	182	96	9	15
Pantomorus viridisquamosus	*wNau1*	190	14	181	96	9	13

Allelic profiles and sequence types (ST) of *Wolbachia* strains *wNau*. For *wNau6* and *wNau8*, no STs could be assigned because the allelic profile was not complete. However, we included these strains in the multilocus sequence typing analysis because they have different alleles. We assigned bold capital letters to these strains.

Naupactini herein studied belong to the supergroup B (Fig. 1).

Overall, closely related *Wolbachia* strains are sometimes found in distantly related species of arthropods, whereas Naupactini species are infected with distantly related *Wolbachia* strains, suggesting the occurrence of horizontal transfer.

Evidence of horizontal transfer was also found in other Curculionidae and in other Coleoptera families, such as Chrysomelidae and Tenebrionidae (Fig. 1). For instance, the bark beetles *Xylosandrus germanus* and *Pityogenes chalcographus* harbour strains belonging to both supergroups A and B (Arthofer *et al*., 2009; Kawasaki *et al*., 2010), and the same is seen for the leaf beetles *Acromis sparsa* and *Chelymorpha alternans* (Baldo *et al*., 2006).

Discussion

Wolbachia *infection in Naupactini*

We provide the first report of *Wolbachia* infection for 19 Naupactini species and confirm the infection for two asexual lineages of *Aramigus tessellatus* (first report by Werren *et al*., 1995b), increasing the list of parthenogenetic Curculionidae infected by this reproductive parasite.

The nine strains of *Wolbachia* herein identified belong to the B supergroup, the same as those of other Entiminae (Werren *et al*., 1995b; Son *et al*., 2008). However, they are scattered across the phylogeny of the *Wolbachia* strains and do not form a monophyletic clade. This result supports previous hypotheses that horizontal transfer of *Wolbachia* amongst insect species from unrelated taxa has been pervasive (eg O'Neill *et al*., 1992; Werren *et al*., 1995b; Zhou *et al*., 1998). Possible agents for this transfer within the Naupactini could be parasitoids and mites (eg Werren & Bartos, 2001) commonly associated with these weevils (De Santis, 1948; Loiácono, 1982; Lanteri *et al*., 1998). A key factor for horizontal transfer of *Wolbachia* amongst distantly related hosts would be their adaptation to similar environments (Rodriguero, 2009).

Thelytokous parthenogenesis is one of the several disorders that *Wolbachia* causes in its host species in order to increase its frequency in their populations (Stouthamer *et al*., 1990, 1993). The significant correlation between the *Wolbachia* infection status and the presence of parthenogenesis supports the hypothesis that *Wolbachia* is involved in the origin of asexual reproduction in the species under study.

Similar results have been reported for the broad-nosed weevil genus *Cathormiocerus*, with one asexual species infected by *Wolbachia* and two sexual species uninfected (Piper *et al*., 2001). Within the *Aramigus tessellatus* species complex (Normark & Lanteri, 1998), there are also parthenogenetic infected lineages and sexual uninfected ones (unpublished result reported by Werren, Zhang & Guo and cited by Braig *et al*., 2002). However, in the Old World species *Otiorhynchus scaber*, *Wolbachia* has been detected in the sexual lineages, whereas it is absent in the asexual ones (Stenberg & Lundmark, 2004). For this reason, Stenberg & Lundmark (2004) ruled out the relationship between *Wolbachia* and parthenogenesis in this weevil species.

Parthenogenetic reproduction is scattered across the tree of life (Normark, 2003). In the tribe Naupactini there is enough evidence to state that this reproductive mode originated independently several times (see Lanteri & Normark, 1995; Scataglini *et al*., 2005), although parthenogenetic species may gather in certain clades. Indeed, the majority of the parthenogenetic Naupactini belong to the *Pantomorus–Naupactus* complex (Scataglini *et al*., 2005), and within this group, they occur in certain phylogenetically derived genera such as *Aramigus*, *Atrichonotus* and *Eurymetopus* (Lanteri, 1984; Lanteri & O'Brien, 1990; Lanteri & Díaz, 1994) and certain species groups within genera (eg the *Pantomorus auripes* species group, *Pantomorus viridisquamosus* species group and *Naupactus leucoloma* species group; Lanteri & Loiácono, 1990; Lanteri, 1995; Lanteri & Marvaldi, 1995). Whereas the basal species of these clades are bisexual and non-infected by *Wolbachia* (eg *N. tucumanensis* within the *N. leucoloma* species group), the derived ones are parthenogenetic and infected (eg *N. leucoloma, N. peregrinus* and *N. minor*).

Some traits associated with parthenogenesis in Naupactini are winglessness (complete loss of flight), dull coloration, a striking simplification of the external morphology and polyploidy (Lanteri & Normark, 1995). The correlation between parthenogenesis and polyploidy has been demonstrated for several broad-nosed weevils from Europe (Smith & Virkki, 1978; Lokki & Saura, 1980; Suomalainen *et al*., 1987) and Japan (Takenouchi, 1983). Indeed, it has been proposed that polyploidy rather than parthenogenesis is the key factor that explains the expansion of *Otiorhynchus scaber* clones over new colonization areas of northern Europe (Stenberg *et al*., 2000; Stenberg *et al*., 2003; Lundmark & Saura, 2006).

Ploidies have been very scarcely studied in Naupactini. It is interesting however, that the only three parthenogenetic species whose ploidies are known are infected with *Wolbachia* [*Aramigus tessellatus* and *Naupactus peregrinus* are 3× and *Aramigus conirostris* is 5× (Sanderson, 1973; Normark, 1996)]. Certainly, *Wolbachia* may have played an important role in ensuring the viability of the eggs, and consequently, in saving clones from extinction. As odd-numbered polyploids are not able to sexually reproduce, parthenogenesis would be the only way to perpetuate the species.

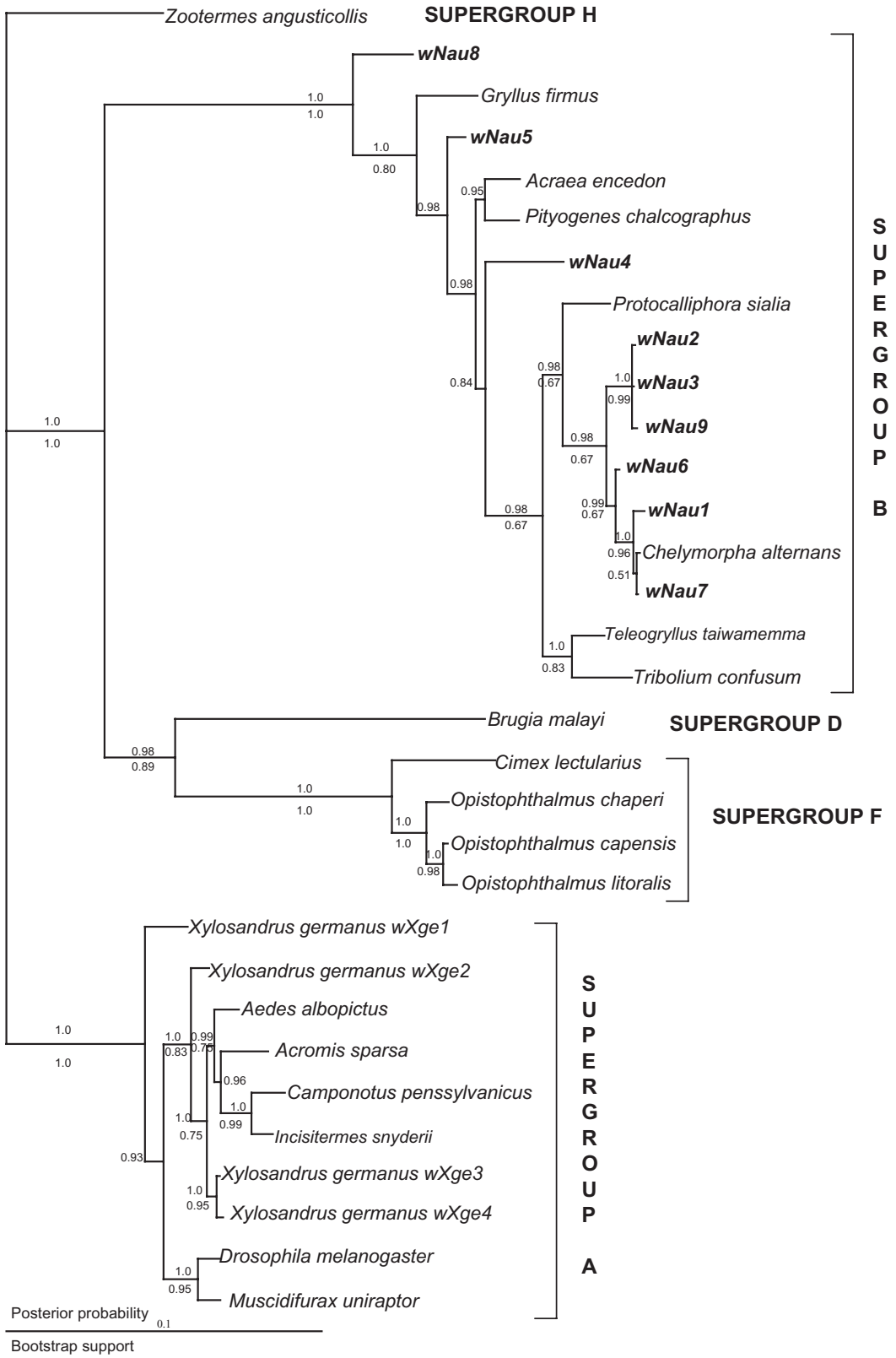

Figure 1. Horizontal transfer of *Wolbachia* in Naupactini. Phylogeny of *Wolbachia* strains found in Naupactini based on concatenated sequences of *Wolbachia* multilocus sequence typing sequences. Naupactini lineages are indicated in bold letters.

Werren (1997) stated that the lineages of *Wolbachia* could easily invade haplodiploid systems, being capable of inducing only automixis. Later, it was proved that *Wolbachia* induced apomixis in haplodiploid mites (Weeks & Breeuwer, 2001) and automictic parthenogenesis in a diplodiploid collembolan (Vandekerckhove *et al*., 1999). However, asexual Naupactini are diplodiploid and apomictic, as other Entiminae. If *Wolbachia* were involved in the origin of thelytokous parthenogenesis in this weevil tribe, as is suggested from our results, a great exception to *Wolbachia* parthenogenesis inductors could be confirmed.

Son *et al*. (2008) reported the effects and implications of an antibiotic treatment on parthenogenetic females of *Otiorhynchus sulcatus* infected with *Wolbachia*. They concluded that cure of the infection led to a significant decrease of larval hatching rates, whereas the numbers of eggs produced were similar in both treated and control females. An alternative hypothesis to explain the association between *Wolbachia* and thelytokous parthenogenesis may be the lesser ability of thelytokous weevils to rid themselves of *Wolbachia* infections once these happen. A curing experiment may shed light on the role that *Wolbachia* plays in the thelytokous parthenogenesis found in these beetles.

Experimental procedures

Sampling

We tested 29 species of Naupactini for *Wolbachia* infection, 12 of them parthenogenetic (eg *N. leucoloma*, *N. peregrinus*, *A. tessellatus*), seven with sexual reproduction (eg *Naupactus xanthographus*, *Naupactus dissimulator*, *Enoploplactus lizeri*) and 10 presumably parthenogenetic (after Lanteri & Normark, 1995 and Guedes & Parra, 2004; eg *Mimographus ocellatus*, *Naupactus ambiguus*, *Naupactus tremolerasi*) (Table 1; see Table S1 for references on the reproductive mode).

Adult specimens were collected on native plants and soy bean crops in several locations from Argentina, Brazil and Uruguay during the summer season of 2004–07 (Table S1). Samples were obtained using a beating sheet (0.55 × 0.55 cm). Specimens were stored at −80 °C or 100% alcohol (at 4 °C) for molecular analyses.

PCR assay and sequencing

Total genomic DNA was extracted from adult weevils to screen for *Wolbachia* infection following the protocol of Reiss *et al*. (1995). Total genomic DNA from *Drosophila melanogaster* naturally infected with *Wolbachia* was used as a positive control. Negative controls consisted of samples lacking DNA template from insects and *D. melanogaster* treated with tetracyline. *D. melanogaster* DNA was kindly provided by Dr Scott O'Neill (Queensland University, Australia). All experiments were repeated at least twice.

Wolbachia infection was diagnosed in 29 Naupactini species (Table 1) through amplification of the *16S rDNA* gene, using the primers designed by O'Neill *et al*. (1992). This gene is very suitable for this purpose because it shows a conserved sequence across *Wolbachia* strains. *16SrDNA* and *ftsZ* genes were sequenced in one species (*N. cervinus*) to confirm the *Wolbachia* infection after comparison with databases through BLASTN sequence analyses (Altschul *et al*., 1990, 1997).

Considering the problem of intragenic and intergenic recombination detected by Baldo *et al*. (2005, 2006), we characterized the *Wolbachia* strains by means of full MLST through amplification and sequencing of the *cytochrome oxidase subunit I* (*coxA*), *fructose-bisphosphate aldolase* (*fbpA*), *cell division protein ftsZ* (*ftsZ*), *aspartyl/glutamyl-tRNA amidotransferase subunit B* (*gatB*) and *conserved hypothetical protein* (*hcpa*) fragments using the primers designed by Baldo *et al*. (2006). Primers S1718 and A2442, designed by Normark (1994) and specific for the weevil mitochondrial *cytochrome oxidase subunit I* (*COI*) gene, were used to assess the quality of the DNA extraction (see Scataglini *et al*. 2005 for details).

All amplifications were carried out in a 50 μl volume reaction with 50–100 ng of DNA used as template, 0.5 μm of each primer, 0.1 mM of each deoxynucleoside triphosphate, 3.0 mM MgCl2, 0.05 units of Taq polymerase and 1X buffer provided by Invitrogen (Carlsbad, CA, USA). The reactions were performed in a Gene Amp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) under the conditions specified by O'Neill *et al*. (1992) for the *16S rDNA* gene, Baldo *et al*. (2006) for the *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpa* genes and Scataglini *et al*. (2005) for the *COI* gene.

Double-stranded PCR products were separated by electrophoresis on a 1% agarose gel with Tris-acetate-EDTA (TAE) buffer containing 0.5 mg/ml of ethidium bromide. The bands were excised from the gel and the DNA was purified with a QUIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA). The DNA fragments were sequenced using an ABI-3730XL Automatic Sequencer (PE Applied Biosystems).

Data analysis

Statistical relationship between Wolbachia *and reproduction biology.* To assess the correlation between the reproductive mode of the host species and the *Wolbachia* infection status, we carried out a Fisher's Exact test (Fisher, 1922) using the software STATISTICA v. 7 (StatSoft, 2004).

Phylogenetic analysis. Standard chromatographic curves of forward and reverse sequences were edited using the program BIOEDIT (Hall, 1999). BLASTN sequence analyses were conducted to compare the sequences of the *Wolbachia* strains retrieved from the Naupactini assayed with those of other bacterial lineages.

Identical nucleotide sequences on a given locus for different strains were assigned to the same arbitrary allele number, after comparison with the *Wolbachia* MLST database (http://pubmlst.org/wolbachia). Then, each strain was characterized by the combination of the MLST numbers (ie allelic profile or ST). Strain and host information were deposited in the MLST database (see Table 2).

The terminal units for the phylogenetic analysis were 23 *Wolbachia* strains belonging to almost all known supergroups (A, B, D, F and H) retrieved from the *Wolbachia* MLST database (see Table S2) and the nine strains retrieved from the Naupactini species (see GenBank accession no. in Table S3). It was not

possible to amplify all MLST genes in all strains (Table 2), and in the *Naupactus cyphoides* strain we could not amplify any gene sequences.

Following Goloboff *et al*. (2009), we decided to include those strains with incomplete STs, because a larger taxonomic sampling compensates the effect of the missing entries in the phylogenetic analysis.

Our complete dataset includes 2079 aligned nucleotide positions. The *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpa* gene sequences were concatenated and aligned using the CLUSTALW algorithm (Thompson *et al*., 1994) and adjusted by eye. MRMODELTEST software v. 2.2 (Nylander, 2004) was used to infer the most appropriate model of molecular evolution, based on the Akaike information criterion, as suggested by Posada & Buckley (2004). The general time reversible (GTR) + Gamma distribution (G) model (Tavare, 1986; Yang, 1993) was selected as the best fit model of nucleotide substitution for the *coxA*, *ftsZ*, *gatB* and *hcpA* partitions and the general-time reversible with rate variation among sites and a proportion of invariant sites (Yang, 1994) was the best choice for the *fbpA* partition.

Bayesian phylogenetic analysis of the concatenated MLST sequences was applied through the 'metropolis-coupled Markov chain Monte Carlo' (MC^3) algorithm implemented in MRBAYES v. 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). A partitioned algorithm, using the program defaults, was applied to account for the heterogeneity amongst the five datasets. Two independent analyses were run with a random starting tree over 2000 000 generations, with a sample frequency of 100. The tree space was explored using four chains: one cold and three incrementally heated chains, with temperature (*T*) set to 0.20. The first 5000 trees were discarded as burn-in.

We applied several tests to assess stationarity of the cold Markov chain for all MRBAYES analyses implemented in TRACER (Rambaut & Drummond, 2007), and the online convergence program ARE WE THERE YET? (AWTY; Wilgenbusch *et al*., 2004; Nylander *et al*., 2008), in addition to the standard deviation of the split frequencies. All posterior samples of a run prior to the burn-in point were discarded. Remaining trees were taken into account to obtain a 50% majority-rule consensus tree and mean branch length estimates. The frequency of all bipartitions was estimated to assess the support of each node (Huelsenbeck & Ronquist, 2001).

Posterior probabilities are usually higher than maximum likelihood bootstrap values (Alfaro *et al*., 2003; Douady *et al*., 2003; Erixon *et al*., 2003). Consequently, we also assessed the clade stability through maximum parsimony bootstrap. This analysis was performed with TNT v. 1.1 (Goloboff *et al*., 2003) using 1000 replicates and 100 random-addition replicates per bootstrap replicate. A 50% majority-rule bootstrap was applied.

As the root for the overall tree of the *Wolbachia* genus is still undetermined (Casiraghi *et al*., 2005; Bordenstein *et al*., 2009), we did not include any outgroup. However, for analysing horizontal transfer, it is necessary to examine sister group relationships. Thus, we arbitrarily rooted the tree with supergroup H.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI 10.1111/j.1365-2583.2010.1018.x

Table S1. Naupactini species diversity.

Table S2. *Wolbachia* strains diversity.

Table S3. GenBank accession numbers.

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