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Cloning and sequencing of *wsp* encoding gene fragments reveals a diversity of co-infecting *Wolbachia* strains in *Acromyrmex* leafcutter ants

S. Van Borm,[a,*] T. Wenseleers,[a,b] J. Billen,[a] and J.J. Boomsma[c]

[a] *Zoological Institute, Laboratory of Entomology, Katholieke Universiteit Leuven, Naamsestraat 59, B-3000 Leuven, Belgium*
[b] *Department of Animal and Plant Sciences, Sheffield University, Western Bank, Sheffield S10 2TN, UK*
[c] *Zoological Institute, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark*

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Abstract

By sequencing part of the *wsp* gene of a series of clones, we detected an unusually high diversity of nine *Wolbachia* strains in queens of three species of leafcutter ants. Up to four strains co-occurred in a single ant. Most strains occurred in two clusters (*InvA* and *InvB*), but the social parasite *Acromyrmex insinuator* hosted two additional infections. The multiple *Wolbachia* strains may influence the expression of reproductive conflicts in leafcutter ants, but the expected turnover of infections may make the cumulative effects on host ant reproduction complex. The additional *Wolbachia* infections of the social parasite *A. insinuator* were almost certainly acquired by horizontal transmission, but may have facilitated reproductive isolation from its closely related host.

1. Introduction

Wolbachia pipientis is a *Rickettsia*-like α-proteobacterium that infects a wide range of arthropods (O'Neill et al., 1997; Werren, 1997). It is known to selfishly enhance its matrilineal transmission through various alterations of host reproduction, including feminisation (reviewed by Rigaud, 1997), thelytokous parthenogenesis (reviewed by Stouthamer, 1997), male-killing (MK, reviewed by Stevens et al., 2001) and cytoplasmic incompatibility (CI, reviewed in Hoffmann and Turelli, 1997). Recently, fecundity enhancing effects have also been reported (Dedeine et al., 2001). *Wolbachia* therefore covers the entire range of endosymbiotic interactions, from parasitism to mutualism. Recent phylogenetic studies, based on 16S rDNA and faster evolving protein-encoding genes (*wsp*, *ftsZ*, and *GroE*) indicate that *Wolbachia* contains at least four major clades. Two are found in arthropods and are designated A and B (Werren et al., 1995b; Zhou et al., 1998). The other divisions (C and D) are found in nematodes (Bandi et al., 1998). A 16S rDNA sequence in a collembolan suggests a possible fifth group E (Vandekerckhove et al., 1999). In insects, *Wolbachia* is a widespread and common endosymbiont. PCR-based screenings have shown that 16% of a large sample of neotropical insects are *Wolbachia* infected (Werren et al., 1995a), and similar figures have been found in a sample of palearctic (West et al., 1998) and nearctic insects (Werren and Windsor, 2000). Using an ultra-sensitive assay, figures up to 76% have been obtained for nearctic arthropods (Jeyaprakash and Hoy, 2000).

Previous work on ants has shown that the majority of species screened so far carry *Wolbachia* infections: 25 out of 50 (50%) Indo-Australian ants (Wenseleers et al., 1998), 10 out of 10 (100%) Nearctic ants (Jeyaprakash and Hoy, 2000), and 7 out of 7 (100%) Panamanian leafcutter ants (Van Borm et al., 2001) have now been shown to harbour *Wolbachia*. The high incidence of *Wolbachia* in ants might be due to the more favourable conditions for invasion and maintenance in haplodiploid social hosts compared to solitary hosts (Wenseleers et al., 1998). For leafcutter and wood ants, *Wolbachia* has also been shown to be present at or near to fixation (Van Borm et al., 2001; Wenseleers et al., 2001). Specific PCR has

* Corresponding author. Fax: +32-16-324575.
E-mail address: Steven.Vanborm@bio.kuleuven.ac.be (S. Van Borm).

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demonstrated that both the A and B subgroup *Wolbachia* occur in ants (Jeyaprakash and Hoy, 2000; Shoemaker et al., 2000; Van Borm et al., 2001), but until now it has remained unclear how many distinct strains they represent. Sex-specific infection patterns and theoretical models are consistent with the *Wolbachia* strains in three species of Panamanian *Acromyrmex* leafcutter ants being either of the cytoplasmatic incompatibility (CI) type or of the "Male-killing" (MK) type (Van Borm et al., 2001). Detailed genetic characterisation of the strains involved may strengthen this conclusion if they cluster with strains that have these effects. These inferences are important because ants, in contrast to many other arthropods, can normally not be reared across generations in the laboratory, so that experimental crosses of infected and uninfected lineages are not available as tools to demonstrate the phenotypic effect of *Wolbachia* infections.

In the present study, we determine the number and the phylogenetic identity of *Wolbachia* strains present in the three sympatric Panamanian leafcutter ant species, *Acromyrmex octospinosus*, *Acromyrmex echinatior* and *Acromyrmex insinuator*. *A. octospinosus* is a common and widespread ant species in Panama, while *A. echinatior* and *A. insinuator* are rarer and more patchily distributed (J.J.B., unpubl. data). *A. insinuator* is a social parasite and can only successfully reproduce within *A. echinatior* colonies and not in colonies of the sympatric *A. octospinosus* (Bekkevold and Boomsma, 2000; Schultz et al., 1998). To characterise the *Wolbachia* strains that are present, we amplified, cloned and sequenced the gene that encodes the *Wolbachia* cell surface protein *wsp*. In a previous study, using strain specific primers, it was shown that both A and B strains were present (Van Borm et al., 2001). Using the present approach we can now assess the full diversity of strains that are present and accurately place them within the *Wolbachia* phylogeny.

2. Methods

2.1. Sampling and DNA extraction

Gynes (winged prospective queens) of *A. octospinosus*, *A. echinatior* and *A. insinuator* were collected in Gamboa, Panama, and preserved in 95% ethanol in April 2000. To minimise the risk of cross-contamination, the pre-extraction treatment and all DNA extraction procedures were performed under sterile conditions in a laminar flow hood. After taking them out of their collection tubes, five gynes (prospective queens; each from a different colony) of each species were externally sterilised by immersion in 70% ethanol, followed by two rinses in double distilled water and exposure to 250 nm UV radiation for 15 min. The entire ants were subsequently ground after freezing in liquid nitrogen, after which their DNA was extracted by 3 h incubation at 55 °C and 20 min boiling in 500 μl of a 10% Biorad Chelex 100 resin solution. The samples were centrifuged and stored at −20 °C until use.

2.2. General PCR amplification

General primers *wsp*81F 5′TGG TCC AAT AAG TGA TGA AGA AAC 3′ and *wsp*691R 5′ AAA AAT TAA ACG CTA CTC CA 3′ (Braig et al., 1998) were used to amplify a 586–617 bp stretch of the gene encoding the *W. pipientis* surface protein *wsp*. PCR amplification was carried out according to the protocol of Pintureau et al. (2000). After amplification, 10 μl of the reaction mixture was electrophoresed with a 100 bp DNA ladder size standard (GibcoBRL) on 1.5% agarose minigels. DNA bands were visualized by ethidium bromide staining. A specimen of the ant *Gnamptogenys menadensis* (Sulawesi) known to contain an A strain *Wolbachia* (Wenseleers and Billen, 2000) was included as a positive control in every amplification. For each species, we selected one *Wolbachia* positive gyne for cloning and sequencing of the PCR product.

2.3. Cloning

PCR amplification products were ligated without further purification into a pCR 2.1-TOPO vector (Invitrogen Topo TA Cloning Kit). Subsequently the vectors were transformed in chemically competent *Escherichia coli* cells (Invitrogen Topo TA Cloning Kit), plated on selective agar plates containing ampicillin and incubated overnight at 37 °C. The resulting clones were suspended in 50 μl double distilled water. Positive transformants were determined by PCR, using primers M13F (5′ GTA AAA CGA CGG CCA G 3′) and M13R (5′ CAG GAA ACA GCT ATG AC 3′) provided by the manufacturer. PCR amplification reactions were carried out in 25 μl reaction mixtures, which contained 0.8 μM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 μl of the suspended clone, 0.3 U of Taq DNA polymerase (AmpliTaq, Perkin–Elmer Cetus) and PCR buffer specified by the manufacturer. PCR was performed with an initial denaturation at 97 °C for 5 min, followed by 30 cycles consisting of 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min, and a final 10 min extension at 72 °C.

2.4. RFLP

To distinguish distinct clones, restriction fragment length polymorphisms were determined by incubating 10 μl of the M13-PCR product, 0.5 μl of 10× buffer provided by the manufacturer and 1 μl of restriction enzyme *Eco*RI or *Hin*dIII (MBI Fermentas) at 37 °C for 4 h. Of each resulting RFLP type (a group of identical clones representing a single *Wolbachia* strain), one was sequenced from the purified M13-PCR product.

2.5. Sequencing

DNA was purified from the M13-PCR product (GFX PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech). Sequencing reactions contained 4 pmol of each IRD (infrared-dye)-labeled M13 primer, 5 U of SequiTherm ExcelII DNA polymerase (Epicentre Technologies) and buffer prescribed by the manufacturer. The PCR reaction was performed with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 15 s and 70 °C for 1 min, and a final 10 min extension at 70 °C. Subsequently, reactions were transferred to a LI-COR automated sequencer for PAGE.

2.6. Phylogenetic analysis

The partial *wsp* gene sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) followed by manual refinements taking translation into account. A 50 bp region (positions 533–583) corresponding to the third hyper-variable region of the gene (Braig et al., 1998) could not be aligned with certainty and was deleted from the analysis. The resulting data set included 555 nucleotide sites (of which 177 were variable). Sequences were subsequently aligned to a subset of previously published *Wolbachia* sequences from other insects and arthropods (24 sequences found in GenBank). A distance matrix was calculated with *MEGA* version 2.0, using a Kimura two-parameter distance metric and pairwise deletion of insertions and deletions (Kumar et al., 2001). From this matrix, a neighbour-joining tree was produced. Bootstrap analysis, using 1000 pseudoreplicates, was used to test the reliability of the clades in the phylogeny. In addition, we performed a maximum parsimony analysis, also using *MEGA* version 2.0 (Kumar et al., 2001), bootstrapped with 500 pseudoreplicates. Finally, *PHYLIP* 3.57c (Felsenstein, 1995) was used for a maximum likelihood analysis, using a bootstrap analysis with 500 pseudoreplicates. The trees were rooted using the A *Wolbachia* clade as the sister group of the B *Wolbachia* clade. The phylogenetic organisation of insect-associated *Wolbachia* in two subgroups, A and B, has been confirmed in *ftsZ* (Werren et al., 1995b), *wsp* (Zhou et al., 1998) and *GroE* (Masui et al., 1997) sequencing studies.

3. Results

3.1. Cloning and sequencing

We picked up nine clones with an insert of the expected size in a single gyne of *A. insinuator*, 25 clones in a gyne of *A. octospinosus*, and 13 clones in an *A. echinatior* gyne, adding up to a total of 47 positive transformants. Among these clones, we found three distinct RFLP types for *A. insinuator*, four types for *A. octospinosus* and three for *A. echinatior*. We interpreted each RFLP type as a group of identical clones representing a single *W. pipientis* strain, and determined the partial *wsp* sequence for one clone belonging to each type. The sequences of two *A. echinatior* clones were almost identical (sequence divergence 0.29%). We assumed that these differences were likely due to polymerase errors, and used only the consensus sequence of these two ('*A. echinatior* Bcons' in Fig. 1 and Table 1). The sequences of the remaining eight RFLP types were considerably more different (sequence divergence >1%, Table 1) or resided in different host species, resulting in a total of nine recognised and distinct *Wolbachia* strains: three in *A. insinuator*, four in *A. octospinosus*, and two in *A. echinatior*. Their *wsp* sequences were submitted to the GenBank database and are available under the accession numbers given in Fig. 1.

The results of the neighbour-joining analysis are shown in Fig. 1. The trees based on maximum likelihood and parsimony analyses are not shown, but do not significantly deviate from these results, apart from some minor changes in the interior branching pattern within clusters *InvA* and *InvB*. Each of the two free-living (i.e. non-parasitic) species (*A. octospinosus* and *A. echinatior*) has one A *Wolbachia* strain and 1–3 B *Wolbachia* strains. The A *Wolbachia* of *A. echinatior* and *A. octospinosus* cluster together with other ant *Wolbachia* (*Solenopsis richteri* and *Solenopsis invicta*) in a well-supported monophyletic group. We propose to name this subgroup *InvA*, after the A *Wolbachia* of *S. invicta* which was the first sequence described in this group (Shoemaker et al., 2000). The social parasite (*A. insinuator*) *Wolbachia* A is different from this cluster and groups together with *Wolbachi* a from *Muscidifurax* parasitoid wasps (subgroup *InsA*). A similar infection-pattern was found for the *Wolbachia* B strains. One well-supported group of ant *Wolbachia* (for which we propose the designation *InvB*) consists of three *A. octospinosus Wolbachia*, one *A. echinatior Wolbachia*, one *A. insinuator Wolbachia*, and one *S. invicta* strain (which was the first to be described, Shoemaker et al., 2000). A second *Wolbachia* B strain of the social parasite *A. insinuator* is phylogenetically isolated from the *InvB* group and clusters together with a *Wolbachia* strain of the parasitoid wasp *T. nawai* (*Naw* subgroup, Arakaki et al., 2000). The Bootstrap support values of the parsimony analysis confirm the robustness of most *Acromyrmex Wolbachia* clusters, with values of 99, 97, and 99 for the clusters InvB, Naw, and InvA, respectively. Confirmation of the InsA cluster (59) was less good, but it remained unambiguous that this clade is differentiated from the InvA clade. Differences between several strains in the *InvB* cluster are rather small, but always well above 1.1% within the same host ant, i.e. above the

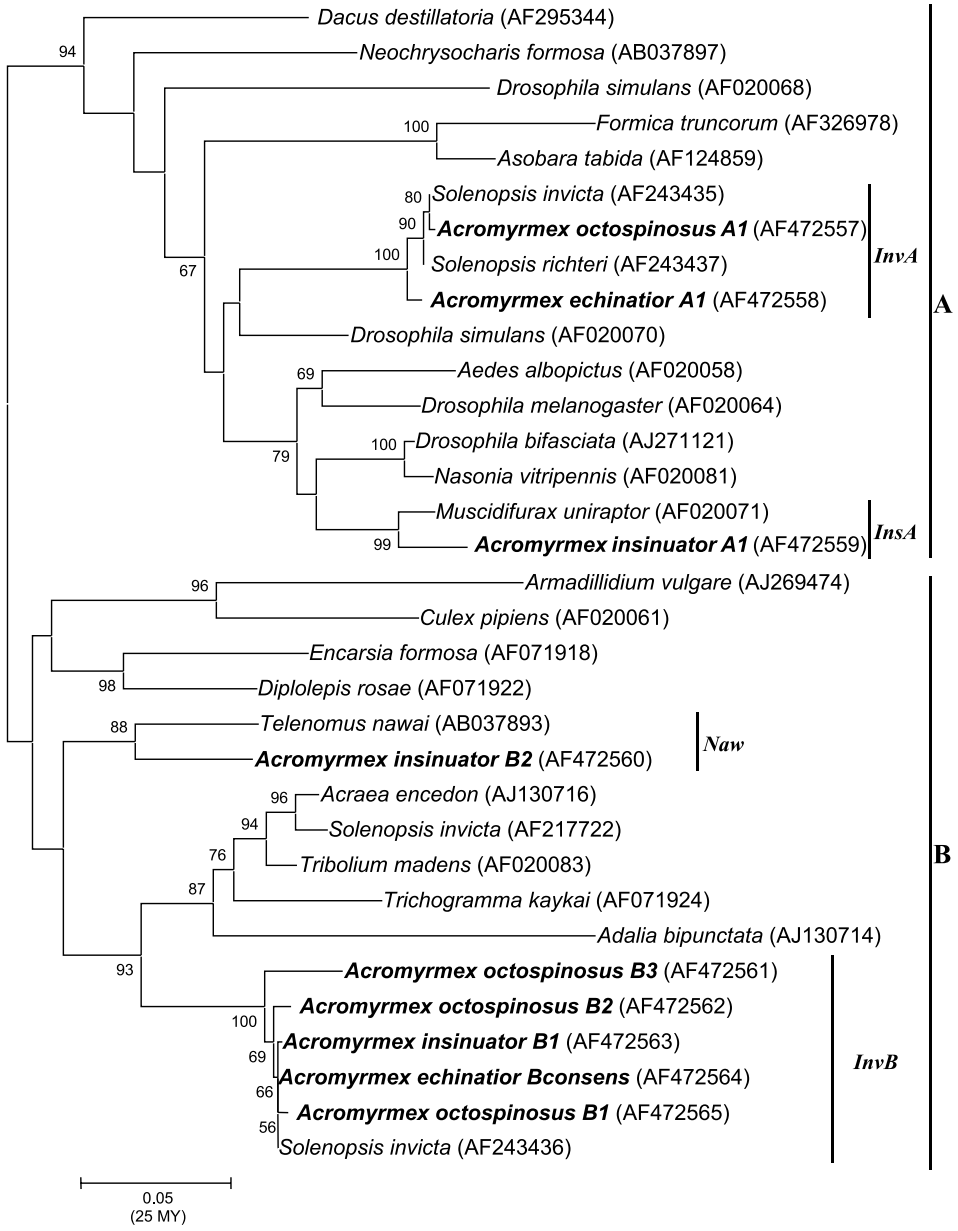

Fig. 1. Neighbour-joining tree for *Wolbachia* strains based on partial sequences of the *wsp* gene. Distances were calculated using the Kimura two-parameter model in *MEGA 2.0*. Bootstrap support values (1000 replicates) over 50% are shown above the branches. *Wolbachia* strains are identified by the host species from which they were isolated, followed by a GenBank accession number. Sequences generated in this study are indicated in bold. The bar represents a distance of 0.050 (about 25 MY if a *wsp* evolutionary rate of 0.2% per MY is assumed, Wenseleers et al., 2001).

expected cloning error rate for a 500–600 bp fragment. They therefore represent genuine strains and do not merely reflect sequencing errors.

4. Discussion

Previous work has documented the widespread occurrence of *Wolbachia* in ant species (Jeyaprakash and Hoy, 2000; Wenseleers et al., 1998) and has demonstrated a high prevalence of these symbionts in colonies of *Atta* and *Acromyrmex* leafcutter ants and *Formica* wood ants (Van Borm et al., 2001; Wenseleers et al., 2001). In the present study we demonstrate a highly complex diversity of strains occurring in single individuals of three *Acromyrmex* leafcutter ant hosts. Up to four different *Wolbachia* strains were detected in a single female (gyne), which to our knowledge is the highest number of co-infecting strains reported in a single insect so far. Multiple *Wolbachia* infections have been widely reported, but have as yet been restricted to either double (e.g., Breeuwer et al., 1992; Malloch et al., 2000; Rousset

Table 1
Percentages of *wsp* nucleotide sequence dissimilarities between different *Wolbachia* strains infecting Panamanian species of *Acromyrmex* leafcutter ants

	A. echinatior B consensus	*A.insinuator* B1	*A.insinuator* B2	*A.octospinosus* B1	*A.octospinosus* B2	*A.octospinosus* B3	*A. echinatior* A1	*A.insinuator* A1	*A.octospinosus* A1
A. echinatior Bconsensus	0.0								
A. insinuator B1	4.4	0.0							
A. insinuator B2	17.1	14.0	0.0						
A. octospinosus B1	4.5	0.6	14.4	0.0					
A. octospinosus B2	4.9	1.0	14.7	1.1	0.0				
A. octospinosus B3	7.6	3.8	17.3	4.0	3.7	0.0			
A. echinatior A1	22.5	20	21.3	20.4	20.0	19.3	0.0		
A. insinuator A1	25.1	21.7	23.4	23.0	23.4	22.5	14.5	0.0	
A. octospinosus A1	23.7	21.3	22.6	21.6	21.3	18.4	1.3	14.0	0.0

Each of these nine sequences represents a group of clones with an identical RFLP restriction pattern (accounting for a total of 47 clones). *A. echinatior* "B consensus" is the consensus sequence of two sequences which were only 0.29% different. The other sequences have higher dissimilarities or come from different host species.

and Solignac, 1995; Wenseleers et al., 1998; Werren et al., 1995a, b; Werren and Windsor, 2000) or more rarely, triple infections (Kondo et al., 2002; Vavre et al., 1999). A stable triple *Wolbachia* infection has also been created artificially by microinjection of an additional strain in double infected *Drosophila* (Rousset et al., 1999).

The *wsp* sequencing as performed here was able to amplify a greater diversity of strains than could be obtained from 16S rDNA amplification (Van Borm et al., 2001). The *wsp* gene was particularly effective in detecting the four different B strains, all belonging to the InvB subgroup, in *A. octospinosus* and *A. echinatior*, whereas none were found in these species using B group-specific 16S rDNA primers (Van Borm et al., 2001). It is likely that this is due to the greater stringency of the strain specific 16S rDNA primers used by Van Borm et al. (2001) relative to the general *wsp* primers adopted in this study. Indeed, similar differences in specificity between different primer sets have also been observed in other studies. For example, Zhou et al. (1998) could not amplify B group *Wolbachia* from *N. vitripennis* strains using *wsp* primers, even though these were known to be present based on *ftsZ* and 16S rDNA sequencing studies (Breeuwer et al., 1992; Werren et al., 1995b).

The multitude of infections demonstrated from *wsp* sequencing suggests that the *Acromyrmex* ants must have acquired their *Wolbachia* during a series of horizontal transmission events. Horizontal transmission is also evident from the fact that strains belonging to four different *Wolbachia* subgroups (InvA, InsA, Naw, and InvB) are present in three closely related *Acromyrmex* host species and, reversely, that closely related strains are present in distantly related host species (the InvA and InvB *Wolbachia* of *Acromyrmex* are both closely related to *Wolbachia* found in *Solenopsis* fire ants, Fig. 1). Earlier, horizontal transmission has been inferred from a lack of congruence between host and *Wolbachia* phylogenies (Schilthuizen and Stouthamer, 1997) and from documented transmissions in vivo (Huigens et al., 2000). Horizontal transmission may even occur across different insect orders (Van Meer and Stouthamer, 1999; Werren et al., 1995b). Parasitoids have been suggested to be a major route of horizontal transmission, and both transmission from host to parasitoid (Heath et al., 1999) and the reverse (Van Meer and Stouthamer, 1999) have been documented. As ants are known to host a wide range of parasitoids (Schmid-Hempel, 1998), it may be that they acquire their *Wolbachia* in a similar way.

The similarity between two of the strains found in *A. octospinosus* (InvA and InvB) and those found earlier in *Solenopsis* fire ants (Shoemaker et al., 2000) is intriguing. Possible explanations are that: (1) these *Wolbachia* strains are specialised to ant hosts (or more specifically,

ants hosts of the subfamily Myrmicinae to which both *Acromyrmex* and *Solenopsis* belong), (2) they were transmitted directly between them by horizontal transmission or (3) they were transmitted from another insect, e.g., a parasitoid, carrying a common *Wolbachia* ancestor. Direct transmission or transmission via a parasitoid is unlikely, however, since *Acromyrmex* and *Solenopsis* occur together in only a very small part of their range in Brazil, whereas introduced fire ants in North America lack *Wolbachia* infections (possibly due to a bottleneck effect, Shoemaker et al., 2000) and have a distribution that does not overlap with the most northerly distributed *Acromyrmex* species in Mexico and Arizona (J.T. Longino, pers. comm.). Nevertheless, direct or indirect transmission of *Wolbachia* between fire ants and leafcutter ants in the southern part of their range cannot strictly be excluded, as a number of diapriid wasps and phorid flies belonging to the same genera are known to infect both *Solenopsis* spp. and *Acromyrmex* spp. (Schmid-Hempel, 1998 and references therein). However, adaptation to the physiology of a specific ant host seems more likely, and would be in agreement with the general pattern that other ants tend to be infected by a limited set of closely related *Wolbachia* strains (Wenseleers et al., 2001). Host specialisation has also been suggested for parthenogenesis inducing *Wolbachia* of *Trichogramma* wasps, where a single *wsp* clade occurs in nine different host species (Schilthuizen and Stouthamer, 1997), and for feminizing *Wolbachia* of isopods, where a similar clustering within the *Wolbachia* phylogeny has been shown (Bouchon et al., 1998).

Sometimes the *Acromyrmex* species also share closely related strains (the InvB strains in *echinatior*, *insinuator*, and *octospinosus* and the InvA strains in *echinatior* and *octospinosus*) and in this case it cannot unequivocally be inferred that the bacteria were inherited from a common ancestor or acquired by horizontal transmission, after speciation had occurred. If *Wolbachia* was present already before speciation, the estimated divergence time between the *Wolbachia* strains should accurately reflect the time of host speciation. For the InvB *insinuator* and *echinatior Wolbachia* the estimated divergence time based on *wsp* sequence differences is 1.04 MY (Fig. 1), which agrees reasonably well with the 2 MY divergence time inferred from mitochondrial DNA sequencing (S.R. Sumner et al., manuscript in prep.). Nevertheless, the co-occurrence of several InvB infections in *octospinosus* suggests that horizontal transmission must also have been important.

The highly complex pattern of *Wolbachia* infections in leafcutter ants revealed in this study significantly complicates inferences about the potential effects of these symbionts on their host's reproduction. Previously, the A strain *Wolbachia* of *A. octospinosus* and *A. echinatior* (the InvA strains identified here, Fig. 1) has been found to be more abundant in gynes than in males (Van Borm et al., 2001). This observation has been taken as indirect support for an effect on the host's sex ratio via partial male killing (Van Borm et al., 2001). The A strain infection of *A. insinuator* (the InsA strain identified here), on the other hand, has been found to be equally common in males and gynes (Van Borm et al., 2001), which precludes any effects on the host's sex ratio, and suggests that it may have other effects, such as cytoplasmic incompatibility (Van Borm et al., 2001) or enhanced fecundity (Dedeine et al., 2001). A hypothetical prediction about the phenotypes associated with the B strains is more difficult, because multiple B strains occur in each host, and no information exists on the prevalence of each of the strains. Nevertheless, a theoretical analysis by Frank (1998) has predicted that a class of multiply infected individuals can spread in a population of singly infected infividuals only when double infected males are incompatible with single infected females of both strain types. This strengthens the idea that some of the identified strains could be of the incompatibility type.

If future experiments confirm that some of the *A. Wolbachia* do cause incompatibility, then this could have important implications. For example, it could explain how the social parasite *A. insinuator* could have evolved from its host, *A. echinatior* (Bekkevold and Boomsma, 2000; Schultz et al., 1998) by sympatric speciation. The close phylogenetic affiliation of ant social parasites and their hosts (Schultz et al., 1998) is known as 'Emery's rule' (Baur et al., 1995, 1996; Buschinger, 1986) and while sympatric speciation is often invoked to explain this pattern (Baur et al., 1995, 1996; Buschinger, 1986), it hard to see how it could arise in the absence of a clear mechanism. Incompatibility between multiple *Wolbachia* types within a population could provide such a mechanism (Hurst and Schilthuizen, 1998; Stouthamer et al., 1999; Werren, 1998). Given that *A. insinuator* carries two additional, phylogenetically isolated,*Wolbachia* infections relative to its host, *A. echinatior* (Fig. 1) and that *Wolbachia*-induced speciation has now been well documented in *Nasonia* (Bordenstein et al., 2001), this scenario deserved further investigation.

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