Wolbachia in leafcutter ants: a widespread symbiont that may induce male killing or incompatible matings

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Abstract

Wolbachia is a maternally inherited bacterium that manipulates host reproduction by inducing cytoplasmic incompatibility (CI), parthenogenesis or male killing (MK). Here, we report on a screening of seven leafcutter ant species of the genera *Atta* and *Acromyrmex*. Using *Wolbachia*-specific polymerase chain reaction (PCR) primers we show that all species are infected, usually by double A + B strain infections. For *Acromyrmex echinatior* and *A. octospinosus*, a screening across all castes shows that gynes (prospective queens) have higher infection rates than workers and males. The low infection rate of workers suggests that workers lose their infection during development. This we interpret as adaptive, because a heritable symbiont does not benefit from being present in sterile workers. Both CI and MK could potentially account for the low infection rate of males. Formal theoretical models show greater support for the MK scenario in the free living species *A. echinatior* and *A. octospinosus* but indicate that *Wolbachia* in the social parasite *A. insinuator* may cause CI, supporting a scenario of sympatric speciation of the social parasite. We conclude that *Wolbachia* represents a previously unrecognized source of reproductive conflict in leafcutter ant colonies.

Introduction

Wolbachia is a maternally transmitted bacterium that may manipulate the reproduction of its arthropod host (O'Neill *et al*., 1997; Werren, 1997; Stouthamer *et al*., 1999). It is known to induce parthenogenesis in parasitoid wasps (Stouthamer, 1997), feminize genetic males in isopods (Rigaud, 1997), and selectively kill male offspring in *Acraea* butterflies, a beetle and a fruit fly (Jiggins *et al*., 1998, 2000; Hurst *et al*., 1999, 2000; Fialho & Stevens, 2000). In this way, *Wolbachia* forces its host to produce broods of mostly female offspring, the sex which maximizes its transmission because of its exclusively maternal inheritance. In addition, it may cause reproductive incompatibilities between host strains ('cytoplasmic incompatibility (CI)'), whereby *Wolbachia* in males sterilizes uninfected females upon mating (Rousset & Raymond, 1991; Hoffmann & Turelli, 1997). Such harm to uninfected hosts reduces local competition and can benefit related *Wolbachia* clones present in neighbouring females (Frank, 1997). Incompatibility may be expressed in different forms: in diploids it causes failed broods (Hoffmann & Turelli, 1997), but in haplodiploids such as *Nasonia* it leads to the production of males only (Saul, 1961; Breeuwer & Werren, 1993; Reed & Werren, 1995; Perrot-Minnot *et al*., 1996).

From these studies, it is apparent that *Wolbachia* infections may profoundly influence host reproductive patterns. Perhaps the most important effect is its ability to affect the host's sex ratio, now documented in many solitary insects (e.g. Saul, 1961; Jiggins *et al*., 1998, 2000; Hurst *et al*., 1999, 2000; Fialho & Stevens, 2000). As yet, however, it remains unknown whether *Wolbachia* has a similar influence on the sex ratio in social insects such as ants, bees or wasps (Wenseleers *et al*., 1998; Chapuisat &

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Keller, 1999). Sex ratio biasing in haplodiploid social insects is an intriguing possibility, given that female biased sex ratios are commonly observed. Current explanations for this mostly centre on the Trivers & Hare (1976) queen-worker conflict theory (reviewed by Bourke & Franks, 1995; Crozier & Pamilo, 1996; Queller & Strassmann, 1998; Chapuisat & Keller, 1999; Sundström & Boomsma 2001). Although this model has been highly successful (e.g. Mueller, 1991; Queller *et al*., 1993; Sundström, 1994; Sundström *et al*., 1996), it critically assumes that sex ratios follow the interests of nuclear genes. The first analysis of *Wolbachia* infections across ant species, Wenseleers *et al*. (1998) showed that 25 out of 50 Indonesian ant species were infected by *Wolbachia*. Therefore, it may be inadequate to neglect the interests of maternally transmitted elements and their potential sex-ratio biasing effects. Recently, Jeyaprakash & Hoy (2000) also documented a high incidence of *Wolbachia* in ants.

Unfortunately, as yet, nothing is known about the effect of *Wolbachia* on ant reproduction and no data are available on the specific occurrence of *Wolbachia* across the sexes or female castes. The aim of the present study is to provide the first detailed screening of *Wolbachia* of a specific tribe, the *Atta* and *Acromyrmex* leafcutter ants, and to report on species-, population-, colony- and caste-specific infection patterns. Our study includes six free-living species and one social parasite, *A insinuator*, and shows that multiple *Wolbachia* infections are present in all of them. In addition, we find that (1) *Wolbachia* is present in higher frequencies in gynes (prospective queens) than in males in both *A. echinatior* and *A. octospinosus* and (2) workers probably lose their infection during development. We interpret these results in the light of current evolutionary theory on the maintenance of *Wolbachia* infections and show that both findings provide important clues as to what effects *Wolbachia* may have on leafcutter ant reproduction.

Materials and methods

Sampling and DNA extraction

Workers and sexuals of seven attine ant species were collected and preserved in 99% ethanol (Table 1). The social parasite *A. insinuator* has retained a caste of small workers ('minors'), but these could not be discriminated on morphological grounds from the minor workers of its host, *A. echinatior* (Schultz *et al*., 1998). To avoid identification errors, we, therefore, included only major and medium sized workers of *A. octospinosus* and *A. echinatior*; no workers of *A. insinuator* were tested. To minimize the risk of cross-contamination, the pre-extraction treatment and all DNA extraction procedures were performed under sterile conditions under a laminar flow hood. After taking them out of their collection tubes, ants were externally sterilized by immersion in 70% ethanol, followed by two rinses in double distilled water and

Table 1 *Wolbachia* infection rates in leafcutter ants. *N* and *n* = total number of colonies and individuals screened; A, B and AB = proportion of individuals harbouring A, B or double A + B strain *Wolbachia* infections; a, b and ab = proportion of colonies harbouring A, B or double A + B strain infections. Gynes are winged, prospective queens sampled together with the males before their mating flights. Proportions do not necessarily add up to 1.00 because part of the individuals/colonies are uninfected.

					Infected individuals (%)*			Infected colonies (%)		
Genus/species	Population (year of collection)	Caste	*n*	N	A	B	AB	a	b	ab
Acromyrmex	Total	Workers	602	48	0.35	0.002	0.3	0.77	0	0.21
		Males	63	17	0.26	0	0.24			
		Gynes	52	21	0.6	0	0.17			
A. echinatior	Gamboa, Panama (1993/1994/1996)	Workers	315	26	0.33	0	0.02	0.92	0	0.08
		Males	43	10	0.59	0	0			
		Gynes	25	11	0.91	0	0			
A. insinuator	Gamboa, Panama (1993/1994/1996)	Males	12	5	0.2	0	0.73	0.2	0	0.8
		Gynes	13	4	0.5	0	0.5			
A. octospinosus	Gamboa, Panama (1993/1994/1996)	Workers	287	22	0.37	0.003	0.03	0.73	0	0.23
		Males	8	2	0	0	0			
		Gynes	14	6	0.47	0	0			
Atta	Total	Workers	889	67	0.31	0.07	0.1	0.22	0.24	0.3
A. cephalotes	El Llano, Panama (1996)	Workers	181	12	0	0.45	0	0	0.83	0
	Gamboa, Panama (1994/1996/2000)	Workers	133	10	0.49	0.04	0.09	0.22	0	0.78
	Trinidad (1995)	Workers	16	2	0.17	0	0	0.5	0	0
A. colombica	Gamboa, Panama (1992/2000)	Workers	202	16	0.25	0.07	0.29	0.19	0.19	0.44
A. sexdens	Puerto Caimito, Panama (1996/2000)	Workers	342	26	0.58	0.04	0.07	0.31	0.12	0.35
A. texana	Austin, Texas (1995)	Workers	15	1	0.2	0	0	1	0	0

*Intracolony infection rates are calculated based on infected colonies only.

exposure for 5 min to UV radiation (250 nm). Whole ants were ground after freezing in liquid nitrogen; subsequently 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.

PCR amplification

Two major *Wolbachia* groups, termed A and B (Werren *et al*., 1995), have been described from insects. Based on GenBank-deposited 16S rDNA *Wolbachia* sequences we developed primer pairs which selectively amplify both groups, and tested their specificity using PROBECHECK (Maidak *et al*., 1999). Relative to previously available primers (Werren *et al*., 1995), the advantage of these primers is that they amplify a greater diversity of A and B group *Wolbachia*. The general forward 16SWOLBF1 5′-AGT CCT GGC TAA CTC CGT GCC A-3′ combined with specific reverse primers 16SWOLBRA1 5′-GGG ATT RGC TTA GCC TCG CGA C-3′ and 16SWOLBRB1 5′-TAG CTT AGG CTT GCG CAC CTT G-3′ selectively amplify a 783-bp stretch of the gene encoding A and B *Wolbachia* SSU ribosomal RNA, respectively. To make sure that the absence of infection was not the result of the presence of polymerase chain reaction (PCR) inhibiting substances or poor DNA quality, we performed two types of PCR controls. The microsatellite primers Etta6 5′-CTG AAC TTC GCC CAG CG-3′ and Etta5 5′-CAG CTC TCG TAG AAG AGT-3′ (Fjerdingstad *et al*., 1998) were used to co-amplify a microsatellite locus in each reaction as an internal control to insure the quality of each DNA extract. This locus has been isolated from *Atta colombica* (Fjerdingstad *et al*., 1998*)*, but amplifies also from other *Acromyrmex* and *Atta* species (Bekkevold *et al*., 1999a; Boomsma *et al*., 1999). Samples for which we failed to amplify the ±250 bp microsat band were excluded from the analysis. In addition, we tested whether *Wolbachia* DNA, when added in small amount, could be amplified from negative samples. For this, we ran PCRs on negative samples with 10% infected gyne template added. Because of the excess of host DNA relative to symbiont DNA, this test is more stringent than the microsatellite co-amplification test.

PCR amplification reactions were carried out in 15 μL reaction mixtures. Final primer concentrations were 0.667 μM of each 16S primer and 0.333 μM of each microsatellite primer for the amplification of the A-*Wolbachia* specific 16S rDNA fragment and 0.333 μM of each 16S primer and 0.667 μM of each microsatellite primer for the amplification of the B-*Wolbachia* specific fragment. Reaction mixtures contained 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 μL of the crude DNA extract, 0.3 U of Taq DNA polymerase (AmpliTaq, Perkin Elmer Cetus) and 1 × PCR buffer specified by the manufacturer. PCR was performed with an initial denaturation at 95 °C for 3 min, followed by 35 cycles consisting of 95 °C for 30 s, 58 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 10 min. 10 μL of this 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. Samples of the ant *Gnamptogenys menadensis* (Sulawesi), containing an A strain *Wolbachia* (Wenseleers & Billen, 2000), and the gall wasp *Diplolepis rosae* (Aix-en-Provence, France), containing a B strain *Wolbachia* (Schilthuizen & Stouthamer, 1998), were included as positive controls in every amplification.

Statistical procedures

The statistical significance of differences in infection rates was assessed using a generalized linear model (*GLZ*). The infection status of each individual (0 or 1) was entered as the dependent variable and we used a binomial error structure and logit link function. The factors tested were genus, species, population and colony, nested in a hierarchical design. All statistical analyses were carried out using Statistica 5.5 (Statsoft, 1995).

Results

Patterns of infection

Inter-genus and Inter-specific comparisons

All investigated species harbour both A and B strain *Wolbachia*, except for the single analysed nest of *A. texana* which had only an A strain infection. However, the infection frequencies of workers vary considerably between genera and species, as indicated by the significances of the fitted *GLZ* model. At the genus level, *Atta* species have higer B *Wolbachia* infection frequencies than *Acromyrmex* species (*GLZ*, $P < 0.001$, Table 1), but no genus-level differences were observed for A *Wolbachia*. Within the genera, species differed significantly both in their A and B *Wolbachia* infections (*GLZ*, $P < 0.001$ for A and B *Wolbachia*, Table 1). *Acromyrmex echinatior* and *A. octospinosus* harbour B *Wolbachia* in very low frequencies, whereas this type of infection predominates in *A. insinuator* (Table 1). Lastly, there was significant inter-colony variance in infection rate within populations for both *Atta* and *Acromyrmex* species (*GLZ*, $P < 0.001$ for both A and B *Wolbachia*).

Intraspecific comparison across populations

In *A. cephalotes*, there is a significant variation in infection patterns between the three sampled populations (*GLZ*, $P < 0.001$ for both A and B *Wolbachia*, Table 1), and intra-colonial infection rates vary significantly within each population (*GLZ*, $P < 0.001$ for both A and B *Wolbachia*). The Trinidad population only harbours an A strain *Wolbachia*, whereas the El Llano population is exclusively infected with a B strain, and the Gamboa population has both infection types plus double

infections (Table 1). It cannot be excluded, however, that some of these differences reflect unrecognized cryptic species, a phenomenon that was earlier shown to be common in Panamanian *Acromyrmex* (*A. insinuator* and *A. echinatior* both used to be classified as *A. octospinosus*, Schultz *et al*., 1998).

Sex and caste specific infection patterns in Acromyrmex

Males and workers of both *A. octospinosus* and *A. echinatior* have lower A-*Wolbachia* infection rates than gynes (*GLZ*, $P < 0.001$ for both males vs. gynes and workers vs. gynes in each of the species, Table 1). Inferred absence of infection was never the result of the presence of PCR inhibiting substances, because both the host microsatellite DNA and *Wolbachia* DNA, when added in small amount, could be amplified from these samples. In contrast to the situation in *A. octospinosus* and *A. echinatior*, no significant difference in *Wolbachia* infections between males and gynes was observed in the social parasite *A. insinuator* (*GLZ*, $P > 0.05$ for both A and B *Wolbachia*, Table 1). Although the latter species has retained a small worker caste, these were not tested, as unambiguous morphological criteria to separate these workers from host workers were not available at the time of this study. Interestingly, in both *A. echinatior* and *A. octospinosus*, sexuals never carry a B strain *Wolbachia* infection and only very few workers do so. This contrasts with the situation in the social parasite *A. insinuator*, where double infections in both males and gynes are frequent.

Theoretical explanations for the observed infection patterns

In *A. insinuator*, both sexes were infected equally and the transmission efficiency approached 100% (96% infected males; 100% infected gynes; Table 1). This is a typical figure for *Wolbachia* causing CI (Hoffmann & Turelli, 1997). In *A. echinatior* and *A. octospinosus*, on the other hand, gynes had a higher infection rate than both workers and males (Table 1; not a single infected male was found in *A. octospinosus*). The low infection rate of workers is easy to explain by curing (see Discussion), but the lower infection rate of males compared with gynes is less obvious to interpret. The aim of this section is to show theoretically that two *Wolbachia* phenotypes, CI (Saul, 1961; Breeuwer & Werren, 1993; Reed & Werren, 1995; Perrot-Minnot *et al*., 1996) and male killing (MK, Fialho & Stevens, 2000; Hurst *et al*., 2000; Jiggins *et al*., 2000) could both cause overproduction of uninfected males, but that only the latter mechanism is fully consistent with the observed infection patterns. Feminization (Rigaud, 1997) and parthenogenesis induction (Stouthamer, 1997) are also known to cause differences in infection rate between the sexes, but can be excluded on *a priori* grounds because (1) feminization in haplodiploid social insects would result in haploid, sterile queens and (2) both *Atta* and *Acromyrmex* are known to reproduce only sexually (Fjerdingstad *et al*., 1998; Bekkevold *et al*., 1999; Boomsma *et al*., 1999; Ortius-Lechner *et al*., 2000; further arguments on this point are given in Wenseleers & Billen, 2000).

Theoretical explanations for the overproduction of uninfected males based on CI and MK have the following characteristics. When *Wolbachia* kills males, only uninfected males are expected to survive and should thus be found in excess compared with uninfected females. CI, on the other hand, would cause uninfected males to arise in broods of infected queens, because segregation – loss of infection from mother to offspring – makes a fraction of the eggs incompatible with sperm from infected males. In *Nasonia* wasps such eggs have been shown to develop into males (Saul, 1961; Breeuwer & Werren, 1993; Reed & Werren, 1995; Perrot-Minnot *et al*., 1996; but see Vavre *et al*., 2000), and CI thus also predicts an excess production of uninfected males in ants. Below we present two simple models of the respective epidemiological dynamics of MK and CI *Wolbachia* in ants and show that only MK can adequately explain the observed infection rates in free-living *Acromyrmex* ants. We will focus our discussion of the model results primarily on *A. echinatior*, but the same conclusions hold for *A. octospinosus* (although the sample size was lower for the latter species).

Male killing

Freeland & McCabe (1997; eqn 26) showed that a MK *Wolbachia* is maintained in a population when

$$C > \frac{1}{F(1-\mu)} \qquad (1.1)$$

where C is the increase in fitness of females in the brood of an infected mother, F is the relative fecundity of an infected queen and μ is the proportional loss of the symbiont during transmission from mother to offspring. This inefficiency of transmission can be estimated from the percentage of infected *A. echinatior* gynes produced by infected colonies, i.e. $\mu = 1-0.91 = 9\%$ (Table 1). Under the conservative assumption that *Wolbachia* has a near zero physiological cost to its host ($F \cong 1$), eqn. (1.1) implies that $C > 1.099$ for a MK *Wolbachia* has to be maintained in the population. This means that the death of nestmate males should give developing gynes of the same cohort a survival benefit of at least 10%. This survival benefit may either arise because at least 10% more gyne-potential larvae actually develop into adult gynes instead of workers, or because the increased availability of resources gives the same number of gynes a survival benefit of at least 10% during the later colony founding stage. This minimum estimate of the required fitness benefit parameter C is realistic, given the typically strong competition among developing sibs in ant colonies, and the considerably more restrictive estimate of C for MK *Wolbachia* in the butterfly *Acraea encedana* ($C = 1.8$, Jiggins *et al*., 2000).

The fact that 59% of the investigated males of *A. echinatior* were infected could be explained if some of the males are resistant to the effects of the MK *Wolbachia*. If a proportion H of the infected males escape death, then the expected overall proportion of infected males in the population (M^+) is given by

$$M^+ = 0.59 = (1 - \mu) \qquad (1.2)$$

Rearrangement shows that H would need to be 0.648, implying that 35% of the infected males would be effectively killed. This hypothetical scenario of MK is thus consistent with the observed infection patterns in *A. Octospinosus* and *A. echinatior*.

Cytoplasmic incompatibility

A simple test for CI in social insects is to compare the segregation rate with the proportion of uninfected colonies in the population: incompatible matings should eliminate most uninfected queens during the founding stage, as they would be unable to produce any workers (Wenseleers *et al*., 1998). Even if part of the males (e.g. 41% in *A. echinatior*) are uninfected, most multiple mating queens (Bekkevold *et al*., 1999) would mate with at least some infected males. The *Wolbachia*-induced male offspring of these latter matings are a severe resource drain during the colony founding stage comparable with the production of diploid males that effectively kills inbred colonies of fire ants (Ross & Fletcher, 1985; Ross *et al*., 1993). In *A. echinatior*, 9% of the gynes produced were uninfected, whereas none of the 26 colonies screened were uninfected. This difference is consistent with the prediction based on CI (i.e. the elimination of uninfected queens in the founding stage), but is inconclusive as it remains within the limits of a mere sampling effect (Fisher exact test, $P > 0.05$).

A more decisive argument can be obtained from a theoretical exploration of the conditions required for a CI *Wolbachia* to persist in the population given the observed infection rates. As specific incompatibility models for social Hymenoptera have not been published, we developed such models here (Table 2). The model assumes that, as in *Nasonia*, incompatibility results in fertilization failure and the production of males only (Saul, 1961; Breeuwer & Werren, 1993; Reed & Werren, 1995; Perrot-Minnot *et al*., 1996). The difference with the *Nasonia* model (Vavre *et al*., 2000) is that incompatibly mated females (the UF × IM cross in Table 2) are assumed to die during the colony-founding stage, as a result of their inability or inefficiency to produce workers (the 'colony level' selection component mentioned in Wenseleers *et al*., 1998). An excess production of uninfected males would be realized only in the infected queen × infected male (IF × IM) cross, as these queens would survive past the colony founding stage and would produce some excess fraction of uninfected male offspring because of segregation (Table 2). The model assumes single mating, whereas leafcutter ants are known to mate multiple (e.g. Bekkevold *et al*., 1999; Fjerdingstad & Boomsma, 2000). However, this assumption can be relaxed to include multiple mating, provided that the total level of incompatibility is a linear function of the proportion of incompatible males mated with. We thus assume that the combined survival and productivity (S) of queens mated to infected males is a linear decreasing function of the segregation rate (μ), because segregation makes it increasingly difficult for these queens to produce worker daughters. Slight deviations from proportionality in this assumed $S = 1-\mu$ relationship do not change the conclusions of our model. The progeny produced by the different mating types (Table 2) can be used to calculate the infection frequency of males and females in the next generation (p'_m and p'_f). If IF, UF, IM and UM denote the number of infected and uninfected females and males produced by the current generation, then the frequency of infection in the next generation is given as $p'_f = IF/(IF + UF)$ and $p'_m = IM/(IM + UM)$. After filling in the terms given in Table 2, this set of equations gives both the invasion and equilibrium conditions of a CI *Wolbachia* infection. The equations are difficult to handle analytically, but can easily be solved numerically. Figure 1 shows the invasion conditions and polymorphic equilibria for a range of parameter values. The conclusion is that *Wolbachia* cannot persist in the population with the observed efficiencies of transmission. The actual

Table 2 Progeny produced by different mating types under a hypothetical scenario of cytoplasmic incompatibility (CI) (UF and IF = uninfected and infected females, UM and IM = uninfected and infected males, p_m and p_f = frequency of infected males and females, μ = vertical transmission inefficiency (segregation rate), f = proportion of eggs that the queen attempts to fertilize).

				Progeny produced			
Mating type	Frequency	Proportion of failed fertilizations	Colony survival	Infected females	Uninfected females	Infected males	Uninfected males
IF × IM	$p_f p_m$	μ	$S = 1 - \mu$	$S(1 - \mu)f$	0	$S\,(1 - \mu)\,(1 - f)$	$S\mu$
IF × UM	$p_f(1 - p_m)$	0	1	$(1 - \mu)f$	μf	$(1 - \mu)\,(1 - f)$	$\mu(1 - f)$
UF × IM	$(1 - p_f)p_m$	1	0*	–	–	–	–
UF × UM	$(1 - p_f)\,(1 - p_m)$	0	1	0	F	0	$1 - f$

*Queens mated with an incompatible male fail to produce workers and thus cannot establish a colony.

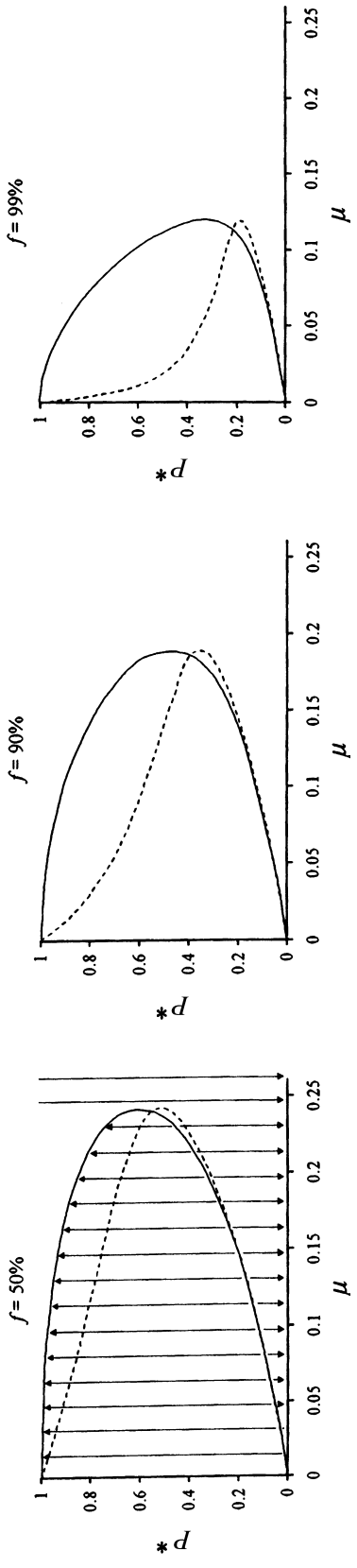

Fig. 1 The evolutionary dynamics of a hypothetical cytoplasmic incompatibility (CI) *Wolbachia* infection in social Hymenoptera. The invasion (lower curves) and equilibrium infection frequencies (upper curves) of males (dashed line) and gynes (full line) are plotted as a function of the transmission efficiency μ and the frequency of fertilization of queen eggs (f). The vector field in the left graph illustrates that for a *Wolbachia* to spread it must exceed a critical frequency: if present in a lower frequency *Wolbachia* disappears from the population, if present in higher frequency it spreads towards an equilibrium frequency. The equilibrium frequency is lower for males than for gynes, especially when the queen fertilizes most of her eggs, because the production of uninfected males in the infected queen × infected male (IF x IM) cross is caused purely by segregation.

efficiency of transmission is difficult to estimate under a scenario of CI, because the proportion of infected males and females produced also depends on the fertilization frequency, a parameter that we do not know. A lower estimate of the segregation rate, however, is simply given by (1-the proportion of infected gynes produced by infected colonies), i.e. 1−0.91 = 9% for *A. echinatior* and 0% for *A. insinuator* (Table 1). The perfect accuracy of transmission in the social parasite *A. insinuator* would allow a CI *Wolbachia* to spread (Fig. 1). For *A. echinatior*, however, the observed μ of 9% would only be sufficient to maintain an already established CI *Wolbachia* in the population, but is too large to allow initial spread. As shown in Fig. 1, an initial threshold frequency of approximately 10% would be needed to let the infection spread, a condition that is met only when effective population size is small ($N_e \leq 10$). Such population structure is highly impossible because reproductives of free-living *Acromyrmex* aggregate in mating swarms. We cannot exclude the possibility that the transmission accuracy was higher at the time of invasion and dropped since the original infection, but such scenario seems impossible, because the transmission rate is expected to increase and not decrease over evolutionary time (Poinsot & Merçot, 2001). We, therefore, conclude that a CI scenario is plausible for the social parasite *A. insinuator*, but not for *A. echinatior*, where MK explains the observed data best. Also the inferred segregation rate of *Wolbachia* in *A. octospinosus* ($\mu = 0.53$) is more compatible with the MK model than with the CI model.

Discussion

Leafcutter ant colonies are a prime example of a highly evolved symbiosis, involving a fungus that is reared as a crop (Mueller *et al*., 1998) and antibiotic producing actinomycete bacteria that protect the fungus garden from specialized fungal pathogens (Currie *et al*., 1999a,b). In the present study, we have shown that *Wolbachia* occurs universally in a sample of seven species of *Atta* and *Acromyrmex* leafcutter ants, further extending the complexity of the symbiotic interactions in this tribe of ants. In general, our results confirm the already documented wide distribution of *Wolbachia* in ants (Wenseleers *et al*., 1998; Jeyaprakash & Hoy, 2000). However, in contrast to prevailing patterns in previous screenings, we found that most species of leafcutter ants carry double (A plus B strain) *Wolbachia* infections. For *A. echinatior* and *A. octospinosus*, however, B *Wolbachia* were present only in minute fractions of the workers and none could be found in sexuals. A possible explanation for this pattern could be that these B *Wolbachia*-infected workers were workers of the social parasite *A. insinuator* (Schultz *et al*., 1998; Bekkevold & Boomsma, 2000) that were accidentally included in spite of our efforts to avoid such mix ups. The parasite is known to enter colonies of both *A. echinatior* (Bekkevold *et al*., 1999) and those of

A. octospinosus but to reproduce only in the colonies of the former host (J.J. Boomsma, unpublished results). Although only medium or large workers of *A. octospinosus* and *A. echinatior* were used for DNA extractions, a small percentage of misidentifications may have been possible as most recent fieldwork has indicated that the social parasite occasionally also produces larger workers (S. Sumner *et al.*, in prep.). Also, preliminary sequencing data indicate that the B *Wolbachia* in these few *A. octospinosus* and *A. echinatior* workers are more similar to the *Wolbachia* of *A. insinuator* sexuals than to the *Wolbachia* in their presumed mothers (S. Van Borm, unpublished results).

The most important conclusions of this study concern the caste and sex specific infection patterns in *A. echinatior* and *A. octospinosus* which showed that A strain *Wolbachia* infections occurred in higher frequency in gynes than in workers and males in both species (not a single infected male was found in *A. octospinosus*). Such differences could in theory also arise as artefacts if gynes contain more potentially infected tissue than workers or males. However, this explanation is unlikely given that (1) the detection efficiency was excellent in all castes and size classes, because *Wolbachia* could still be amplified from infected females and workers up to a template dilution of 1:1000; (2) PCRs on body parts of infected gynes and workers have shown that *Wolbachia* not only occurs in the ovaries, but also in other tissues (data not shown); (3) unmated *Acromyrmex* gynes do not have developed ovaries. Successful application of all PCR controls further confirmed that *Wolbachia* was absent from all inferred negative samples.

Using simple models it was shown that the large differences in infection rate between the sexes of the free-living species *A. octospinosus* and *A. echinatior* could only be accounted for by MK (in which case uninfected males experience better survival) and not by CI (which only causes a minor overproduction of uninfected males in *Nasonia,* Breeuwer & Werren, 1993). In addition, the observed vertical transmission efficiency was too low for CI *Wolbachia* to be maintained in the population. In *A. echinatior* and *A. octospinosus, Wolbachia* was transmitted with an efficiency of 91% and 49%, respectively. Such low transmission rates have never been seen in CI *Wolbachia* (Hoffmann & Turelli, 1997), but are rather typical for male killers (Hurst & Jiggins, 2000). To our knowledge, the evidence accumulated in this study thus represents the first evidence that *Wolbachia* is able to manipulate ant reproduction. However, the evidence is still indirect and further experimental work is needed.

Another interesting result was that workers were infected at a lower rate than gynes. Analogous results have recently been obtained in another ant species, *Formica truncorum* (T. Wenseleers, L. Sundström & J. Billen, unpublished results). In that study, it was shown that the low infection rate of workers is the result of infection clearance and that this boosts colony productivity and a similar explanation may apply in *Acromyrmex*. In fact, such infection clearance is expected to be adaptive not only for the host, but also for *Wolbachia*, because the symbiont is not transmitted through sterile workers. Costs of *Wolbachia* infection have also been shown in other studies (Min & Benzer, 1997; Fleury *et al*., 2000), adding further plausibility to this explanation. Proximate factors, such as regression of worker ovaries (Hölldobler & Wilson, 1990), exposure to high temperatures (Hoffmann *et al*., 1986; Stevens, 1989) or naturally occurring antibiotics (Stevens & Wicklow, 1992; Turelli & Hoffmann, 1995) might further contribute to loss of infection in ant workers. An alternative explanation for higher infection rates in gynes vs. workers might be that *Wolbachia* biases the development of female larvae in favour of gynes (Bourke & Ratnieks, 1999). However, it is hard to see how *Wolbachia* could influence caste development in *Acromyrmex*, given that gynes are much larger than workers and that female larvae would thus require significantly more food to develop as gynes. In addition, Bourke & Ratnieks (1999) scenario has been challenged by recent theoretical models showing that *Wolbachia* is not selected to manipulate the caste fate of individual larvae (Wenseleers *et al*., 2001). An effect of *Wolbachia* on caste development is therefore unlikely to explain the higher infection rate of gynes compared with workers.

Where the sex- and caste-specific infection patterns of *A. octospinosus* and *A. echinatior* provide indirect evidence for MK, this explanation does not apply to the third *Acromyrmex* species. The A *Wolbachia* of the social parasite *A. insinuator* was found to be transmitted with perfect accuracy from mother to offspring and did not occur in lower frequencies in males as compared with gynes. It thus is clear that this strain does not cause MK. An intriguing possibility is that the social parasite *A. insinuator* contains CI *Wolbachia*. This might explain how this incipient social parasite has evolved from its host *A. echinatior* by sympatric speciation. As argued by Schultz *et al*. (1998), *A. insinuator* is an unusually clear example of a phylogenetically close resemblance between a social parasite and its host, a phenomenon that is known as 'Emery's rule' (Buschinger, 1986; Baur *et al*., 1995, 1996). *Wolbachia*-induced post-mating reproductive isolation has been convincingly demonstrated in *Nasonia* (Bordenstein *et al*., 2001; for reviews see Hurst & Schilthuizen, 1998; Werren, 1998; Stouthamer *et al*., 1999; Rokas, 2000) and sympatric speciation has been predicted to be a logical implication of Emery's rule in social Hymenoptera (Buschinger, 1986).

The *Wolbachia* infections of leafcutter ants illustrate that there is ample scope for reproductive conflict because of single or multiple infections with this symbiont. Previously, other aspects of the leafcutter ant-fungus symbiosis have been suggested to be similarly prone to conflict. For example, it has been argued that the fungal symbiont might be selected to cause female biased sex

ratios, because the cultivated fungus is dispersed only by female foundresses and not by males (Bourke & Franks, 1995). However, this study is the first to provide circumstantial evidence that *Wolbachia* symbionts induce changes in ant reproduction. Whether this ultimately results in more female biased sex ratios remains to be seen, as few data on leafcutter ant sex ratios are currently available (Murakami *et al*., 2000). Sex ratio biasing in ants is eventually controlled by the sterile workers (Passera & Aron, 1996; Sundström *et al*., 1996; Chapuisat *et al*., 1997; reviewed by Bourke & Franks, 1995; Crozier & Pamilo, 1996; Queller & Strassmann, 1998; Chapuisat & Keller, 1999), who may compensate for MK or CI in their feeding regimes of larvae of different sex. This might result in realized sex ratios that are close to the relatedness asymmetry equilibria of workers, thus hiding conflictuous dynamics owing to single or multiple *Wolbachia* infections.

Note added in proof

In an additional PCR, we checked whether the nine workers of *A. octospinosus* and *A. echinatior* that were identified as carrying a B infection in the FtsZ-primed PCR were misidentified *A. insinuator* workers. We used the *A. insinuator* B *Wolbachia* strain-specific primer WinsB2F 5′-GAT GCA GGT GTA AGC AGG TAC TAC AC 3′ (designed using preliminary sequencing data) in combination with the general primer wsp691R 5′ AAA AAT TAA ACG CTA CTC CA 3′ to selectively amplify an approximately 480-bp stretch of the wsp gene from the DNA extracts. We were able to show that all these workers carried the *A. insinuator* specific B *Wolbachia* strain, and that they must have been unrecognized workers of the social parasite *A. insinuator*. This result does not change any of the conclusions reported in this paper and makes the differences in A and B infections among the three *Acromyrmex* species even more distinct. It means that all B and AB infections reported for *Acromyrmex* workers in Table 1 were *A. insinuator* workers.

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