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No sex in fungus-farming ants or their crops

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Asexual reproduction imposes evolutionary handicaps on asexual species, rendering them prone to extinction, because asexual reproduction generates novel genotypes and purges deleterious mutations at lower rates than sexual reproduction. Here, we report the first case of complete asexuality in ants, the fungus-growing ant *Mycocepurus smithii*, where queens reproduce asexually but workers are sterile, which is doubly enigmatic because the clonal colonies of *M. smithii* also depend on clonal fungi for food. Degenerate female mating anatomy, extensive field and laboratory surveys, and DNA fingerprinting implicate complete asexuality in this widespread ant species. Maternally inherited bacteria (e.g. *Wolbachia*, *Cardinium*) and the fungal cultivars can be ruled out as agents inducing asexuality. *M. smithii* societies of clonal females provide a unique system to test theories of parent–offspring conflict and reproductive policing in social insects. Asexuality of both ant farmer and fungal crop challenges traditional views proposing that sexual farmer ants outpace coevolving sexual crop pathogens, and thus compensate for vulnerabilities of their asexual crops. Either the double asexuality of both farmer and crop may permit the host to fully exploit advantages of asexuality for unknown reasons or frequent switching between crops (symbiont reassociation) generates novel ant–fungus combinations, which may compensate for any evolutionary handicaps of asexuality in *M. smithii*.

Keywords: asexual; fungus-growing ants; symbiosis; *Mycocepurus smithii*; *Wolbachia*; thelytoky

1. INTRODUCTION

The vast majority of eukaryotes reproduce sexually. Multicellular asexuals are rare, occur sporadically across the tree of life, and, with a few notable exceptions (Judson & Normark 1996; Butlin 2002), are thought to be short-lived descendants derived recently from sexual ancestors (Barton & Charlesworth 1998). Theory predicts asexuality is advantageous because asexual lineages should out-compete sexual ones by circumventing the costs of sex (e.g. cost of meiosis, mating effort and producing males), however asexuality is thought to be evolutionarily disadvantageous because it purges deleterious mutations and generates novel genotypes more slowly than sexual reproduction (Butlin 2002). However, the pervasiveness of sex among multicellular organisms suggests that the advantages outweigh the costs (Barton & Charlesworth 1998). The real evolutionary conundrum, therefore, is not the pervasiveness of sexual lineages, but the persistence of some asexual lineages over extended evolutionary time (Judson & Normark 1996; Herre *et al.* 1999).

Similar to all other fungus-growing ants in the strictly Neotropical tribe Attini, *Mycocepurus smithii* (Formicidae, Attini) obligately farms basidiomycete fungi for food (Mueller *et al.* 1998). *M. smithii* has one of the widest distributions of any fungus-growing ant, ranging from Mexico and the Caribbean to Argentina (Mackay *et al.* 2004; Fernández-Marín *et al.* 2005). Moreover, no

males have been found in extensive nest excavations of *M. smithii* from throughout the Americas (Rabeling 2004; Fernández-Marín *et al.* 2005; Rabeling *et al.* 2007), suggesting *M. smithii* may be parthenogenetic (Fernández-Marín *et al.* 2005; see electronic supplementary material). As in other Hymenoptera (Werren & Windsor 2000), asexuality in *M. smithii* could be caused by infection with endosymbionts such as *Wolbachia* bacteria (Stouthamer *et al.* 1999), or by the vertically transmitted exosymbiont (e.g. the fungal cultivar; Mueller 2002). Here, we test the hypothesis that *M. smithii* is asexual using genetic, morphological and experimental analyses.

2. MATERIAL AND METHODS

(a) Colony collections

All *M. smithii* colonies in this study were collected in March–April 2001, June 2002 and May 2003 in the Republic of Panama from five populations 50–150 km apart (Parque Soberanía, Sherman Forest Reserve, or the Colón Province). Colonies were maintained in the laboratory for up to four years and never produced males. Field surveys in Panama (100 nests; AGH & UGM), Guyana (5 nests; UGM), Ecuador (6 nests; AGH), Peru (20 nests; C. Rabeling 2004, personal communication), Argentina (7 nests; UGM), and Brazil (132 garden chambers from an unknown number of neighbouring nests; Rabeling 2004; Rabeling *et al.* 2007) failed to find any males in *M. smithii*, complementing Fernández-Marín's survey of 228 male-less *M. smithii* nests in Puerto Rico (Fernández-Marín *et al.* 2005). DNA samples were refrigerated in 95 per cent ethanol and extracted using Qiagen Dneasy kits.

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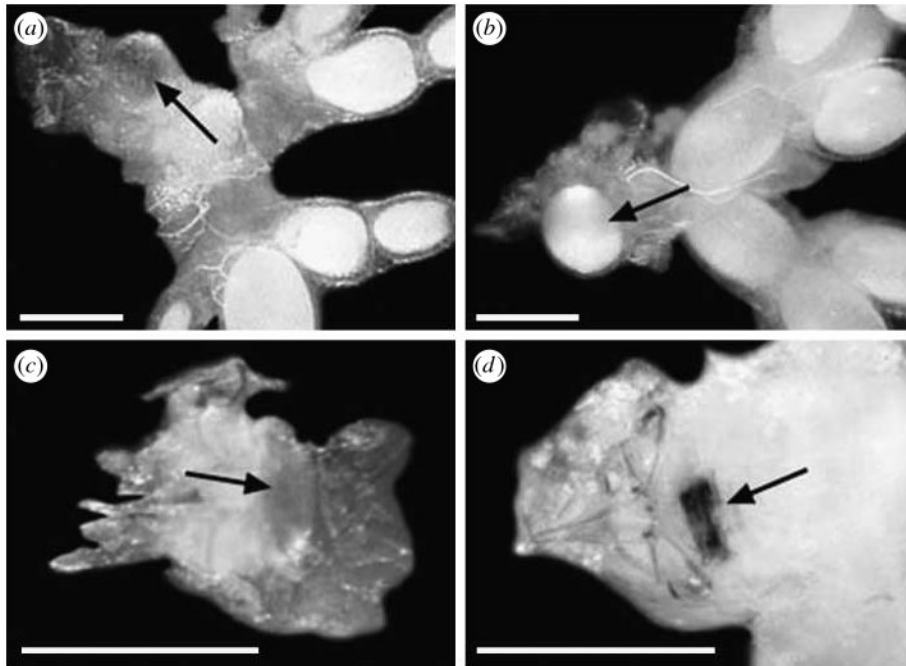


Figure 1. Reproductive tracts of *M. smithii* and *M. tardus* queens. (a) The spermatheca of *M. smithii* is translucent indicating that it is empty. (b) By contrast, a sperm-filled spermatheca the congener *M. tardus* appears opaque. (c) Fully developed ovaries in both species contain mature eggs and several yellow bodies indicating the queens were active egg layers. The mussel organ, an internal lock structure of the female mating apparatus, is degenerate and unsclerotized in *M. smithii* compared to (d) the sclerotized functional mussel organ of *M. tardus*. Scale bar, 0.25 mm.

(b) Genotyping: DNA extraction and microsatellite amplification

To test whether *M. smithii* offspring were clones of their mothers, we screened 14 microsatellite primer pairs developed for other fungus-growing ant genera (Villesen *et al.* 2002). Thirteen of these loci either did not amplify or were monomorphic, and thus uninformative. Using the single informative locus Cypho15–16 (two alleles; 150, 152 bp), we genotyped 66 *M. smithii* specimens, from 12 Panamanian colonies for which both queen and workers were available (queen and 4–10 offspring per colony). DNA was extracted from single whole workers and queens' abdomens. Microsatellite PCR products were run on an ABI 3100 automated sequencer and analysed using GENESCAN v. 3.7 and GENOTYPER v. 3.6. Microsatellite primer Cypho 15–16 amplified ant DNA under the following PCR conditions: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 40 s; 59°C for 40 s; and 72°C for 30 s; and 1 final extension cycle at 72°C for 15 min. Each 10 µl PCR reaction contained: 1X enzyme buffer (Promega); 3.75 mM MgCl₂ (Promega); 0.25 mM of each dNTP (Promega); 0.25 U Taq DNA polymerase (Promega); 1 µM of each primer; and 1 µl DNA template at approximately 50 ng µl⁻¹.

(c) Female reproductive tract

Colonies of *M. smithii* and *Mycocetopus tardus* were excavated and six resident non-winged queens of each species were dissected to inspect their reproductive organs and determine reproductive status. Mated, reproductively active queens are characterized by: (i) presence of sperm in the spermatheca (empty spermatheca appears translucent grey; sperm-filled spermatheca appears opaque white; figure 1), (ii) fully developed ovaries containing mature eggs in the ovarioles, and (iii) the presence of yellow bodies in the ovarioles. Yellow bodies (remnants of follicular epithelium) indicate that the ant had laid eggs.

(d) Molecular screens for endosymbiotic bacteria

We tested *M. smithii* for the presence of *Wolbachia*, *Cardinium* or other endosymbiotic bacteria by PCR (Holden *et al.* 1993; Zhou *et al.* 1998; Jeyaprakash & Hoy 2000; Zchori-Fein & Perlman 2004). DNA was extracted from individual workers or gyne abdomens for the survey with three *Wolbachia*-specific primers (Wenseleers & Billen 2000; Fournier *et al.* 2005; Percy *et al.* 2005), and from gyne larvae or adult ovaries for screens with *Cardinium*-specific and universal bacterial primers (Jeyaprakash & Hoy 2000; Zchori-Fein & Perlman 2004). One to three workers, gynes or queens per colony were sampled from 15 colonies with a total of 31 samples; in only two colonies was a single ant tested. To verify PCR amplification, *Formica truncorum* ants and *Drosophila simulans* infected with *Wolbachia* served as positive controls and uninfected *Formica sanguinae* as the negative control. Twelve *M. smithii* individuals were tested using primers Ch-F/Ch-R that amplify *Cardinium* and other related *Bacteroidetes* symbionts (Zchori-Fein & Perlman 2004), with the wasp *Encarsia pergandiella* as a positive control. Two universal bacterial primer pairs, U519F/U1406R and 338-356F/16S-8R, were used to detect bacterial symbionts other than *Wolbachia* or *Cardinium*, with *Escherichia coli* as a positive control and sterile water as a negative control (Jeyaprakash & Hoy 2000; Baker *et al.* 2004). DNA quality of all samples was verified by successful amplification of the mitochondrial cytochrome oxidase I gene (Simon *et al.* 1994).

(e) Antibiotic experiment

Unmated virgin female *M. smithii* gynes were treated with antibiotics to test whether curing infection by potentially parthenogenizing microbes would permit male production. Thirty-five subcolonies were split from three source colonies. Subcolony replicates from the same source colony were blocked in groups of five for a total of seven groups. Each replicate subcolony contained a 20 mm³ garden fragment,

20 winged queens and approximately 75 workers, except one quintuplet set had six queens per subcolony. Within each group, replicates were randomly assigned to one out of four antibiotic treatments: 10 per cent streptomycin, 5 per cent penicillin, 5 per cent tetracycline, and 0.5 per cent rifampicin or a sucrose solution control. Particular antibiotic concentrations were chosen because, in pilot experiments, females readily imbibed antibiotic sugar solutions at maximal concentrations without causing significant mortality (less than 10%) during a seven-day treatment, compared to control females kept without sugar solution that showed near 100 per cent mortality. This difference in mortality demonstrated indirectly that the treated females were ingesting the antibiotic solutions. Replicate subcolonies were habituated for eight weeks in a two-chamber system before antibiotic treatment (Schultz 1993). During this time gardens doubled in size, and virgin females eclosed from pupae hidden in the transferred fungus garden, increasing the number of females per subcolony to an average of 36.5 (± 7.6) that were all then treated with the assigned antibiotic. For treatment, all gynes were removed from each subcolony, and supplied with a drop of either 10 per cent (weight/volume) sucrose solution (control) or a 10 per cent sucrose solution laced with one of the four antibiotics. Fresh sucrose solutions were provided daily for seven days, and treated gynes were then returned to their subcolony. Subcolonies were maintained for 16 months until after the next round of annual gyne production was complete. Number of new queens produced after treatment was calculated per subcolony as number of live queens plus dead queens collected during the experiment, minus queens present at start of experiment.

(f) Fungus switch experiment

Seventy-five laboratory-reared, unmated gynes were randomly chosen from six *M. smithii* nests and placed individually on a brood-free fungus garden fragment (20 mm³) in a 6 cm Petri dish with a moistened plaster of Paris base. Thirty gynes each were randomly assigned to one of three garden types ($n=90$ queens total): native cultivar (from *M. smithii*), closely related cultivar (from *Cyphomyrmex costatus*), or distantly related cultivar (from *Cyphomyrmex longiscapus*). Replicates were maintained for 15 months over three generations and were fed weekly with a sterilized mix of polenta and ground oats as substrate for the fungus. Owing to non-normality, we examined the effect of fungal type on the number of workers and gynes produced using a Kruskal–Wallis test, and the effect of fungal type on queen survival using a Pearson's Chi-square test. All tests were conducted at the significance level of 0.05.

3. RESULTS

(a) Colony collections

One hundred colonies of *M. smithii* collected from five populations in Panama produced over 10 000 new queens (gynes) during five years in the laboratory, an estimated 10–20 times that number of workers, but no males. By contrast, approximately 30 other species of attine ants maintained in the same laboratory produced males. Unmated *M. smithii* queens born in the laboratory produced both worker and gyne offspring (but never males) when separated from their natal colony, starting clonal female lineages that could be propagated without mating over an indefinite number of generations.

(b) Microsatellite fingerprinting and analysis

Microsatellite DNA fingerprinting revealed that workers and gynes had an identical genotype to their mother in 12 *M. smithii* colonies for which both queens and offspring were available. We genotyped nine heterozygous and three homozygous colonies. Under the assumption of sexual reproduction, the probability of heterozygous queens producing only heterozygous offspring in all nine colonies was $(1/2)^{54} = 5.55 \times 10^{-17}$, therefore ruling out sexual reproduction (see electronic supplementary material, table 1). Another potential explanation for the observed genotype distributions (e.g. absence of homozygous offspring in nests with heterozygous queens) is strong selection against homozygotes at some pre-adult stage, but calculation shows that the strength of selection required is so extreme (selection coefficients greater than 0.94) that homozygotes should be very rare in the population (less than 2%). However, 25 per cent (3/12 colonies) of the genotyped colonies were homozygous. Therefore, the alternative explanation that homozygotes produced by a heterozygous queen are lethal can be ruled out. Offspring genotypes were always identical to their mothers' in both heterozygous and homozygous colonies, consistent with clonal reproduction.

(c) Female reproductive tract

Dissections of the female reproductive tracts of *M. smithii* queens collected from mature nests confirmed that they were never inseminated (empty spermathecae), although they had fully developed ovaries containing mature eggs and yellow bodies, indicating that they were active egg layers (figure 1). By contrast, *M. tardus* queens, a closely related species, had sperm-filled spermathecae. *M. smithii* queens also lack the 'mussel organ', a female reproductive structure found in other attine species into which the male's sclerotized genitalia lock during mating (figure 1; Baer & Boomsma 2006).

(d) Molecular screens for endosymbiotic bacteria

PCR screens for endosymbiotic *Wolbachia*, *Cardinium* and other bacteria in *M. smithii* were negative, ruling out infection with these bacteria as the cause of asexuality in *M. smithii*.

(e) Antibiotic experiment

As an additional test that bacteria might cause parthenogenesis in *M. smithii*, we treated 1320 gynes from 28 experimental colonies with four different classes of antibiotics. Antibiotic purging of parthenogenizing bacterial symbionts reinstates male production in some asexual arthropods (Weeks *et al.* 2001; Stouthamer & Mak 2002). However, the antibiotic-treated *M. smithii* queens produced 7488 daughter queens but no males during 16 months post treatment (table 1). The combined molecular and antibiotic evidence therefore indicates absence of a male-eliminating bacterial endosymbiont in *M. smithii*.

(f) Fungus switch experiment

To test the hypothesis that asexuality is induced by the fungal cultivar we conducted a fungal-switch experiment in which *M. smithii*'s normal fungus garden was replaced with a different fungus. Seventy-five newly emerged, unmated *M. smithii* queens were isolated either on

Table 1. Antibiotic treatment of unmated queens. (Total number of queens produced (7844) over 16 months after antibiotic treatment. No males were produced. Concentrations were derived from similar experiments with wasps, and further tested in pilot experiments to maximize dose administered without causing significant mortality (up to 10%). Number of queens treated per subcolony represents the average ± 1 s.d.)

antibiotic	percentage of antibiotic solution	no. of subcolonies	no. of queens treated per subcolony	total no. of queens treated	queens produced	males produced
streptomycin	10	7	37 \pm 17	264	931	0
rifampicin	0.5	7	38 \pm 16	263	2560	0
penicillin	5	7	34 \pm 16	236	1666	0
tetracycline	5	7	36 \pm 12	249	1558	0
control (sucrose)	10	7	47 \pm 16	332	1129	0
total				1344	7844	0

Table 2. Fungus switch experiment. (Seventy-five unmated *M. smithii* queens were placed either on their own fungus (control), a closely related or a distantly related fungus. No males were produced on any fungus type while female workers and new queens (gynes) were produced over three generations. There was no effect of fungus type on number of workers produced (Kruskal–Wallis test $\chi^2=1.69$, $p=0.4289$), on the number of gynes produced (Kruskal–Wallis test $\chi^2=0.0728$, $p=0.9643$) or on queen survival (Pearson's Chi-square test $\chi^2=3.99$, $p=0.1363$.)

offspring (F1 + F2)	fungus garden source			total
	<i>M. smithii</i> fungus (control fungus)	<i>C. costatus</i> fungus (closely related fungus)	<i>C. longiscapus</i> fungus (distantly related fungus)	
no. of workers produced	77	8	128	213
no. of gynes produced	7	1	7	15
percentage queens reproducing	6/30 = 20%	3/30 = 10%	6/30 = 20%	15/90 = 17%

(i) their own fungus, (ii) a closely related fungus, or (iii) on a distantly related fungus. Surviving colonies were raised for two successive generations during which 21 unmated queens produced exclusively female offspring (213 F1 workers and 15 F1 gynes). These 15 gynes later produced 17 F2 workers, but no males (table 2). Asexual reproduction by queens was independent of cultivar type (table 2), and cultivar substitution did not induce male production (electronic supplementary material). Most significantly, queens always produced workers before gynes, suggesting queen control over offspring caste, rather than extrinsic factors such as the cultivated fungus.

4. DISCUSSION

Six lines of evidence support complete and endogenous asexuality in *M. smithii*: absence of males, significant degeneration of the female mating apparatus, DNA fingerprint identity between mothers and daughters, absence of sex-ratio-biasing endosymbiotic bacteria, and the inability to induce male production by antibiotic treatment or fungal substitution. *M. smithii* represents, to our knowledge, the first case of a male-less species of ant, and the first case where females produce both reproductive and worker offspring by asexual means.

Degeneration of the female mating apparatus renders cryptic sex unlikely for *M. smithii* (Judson & Normark 1996; Normark *et al.* 2003) and could suggest that the evolutionary origin of *M. smithii* from a sexual ancestor may not be very recent. A more remote rather than recent origin of asexuality is also consistent with the widespread Neotropical distribution of *M. smithii* from northern Argentina to northern Mexico. However, it is possible

the degeneration of the unused mating apparatus could progress quickly. A coalescent analysis within a larger phylogenetic treatment of the *Mycocepurus* genus is needed to infer the age of asexuality in *M. smithii*.

Asexuality in *M. smithii* could be caused by infection with endosymbionts such as *Wolbachia* bacteria (Stouthamer *et al.* 1999), *Cardinium* bacteria (Zchori-Fein & Perlman 2004), undescribed microbes that can manipulate reproduction (Zchori-Fein & Perlman 2004), or by the fungal cultivar (Mueller 2002). *Wolbachia* has been found in several sexually reproducing ant species (Wenseleers *et al.* 1998) including fungus-growing ants (Van Borm *et al.* 2003), but is absent in seven partially asexual ant species (Wenseleers & Billen 2000; Fournier *et al.* 2005; Percy *et al.* 2005). We document here that endosymbiotic microbes also appear to be absent in the asexual *M. smithii*.

While asexual reproduction of haploid males developed from unfertilized eggs (arrhenotokous parthenogenesis) is a normal part of hymenopteran reproduction, asexual reproduction of diploid females from unfertilized eggs (thelytokous parthenogenesis) is exceedingly rare in ants. Only seven distantly related ant species produce females asexually (Wenseleers & Billen 2000; Fournier *et al.* 2005; Percy *et al.* 2005; Ohkawara *et al.* 2006) primarily by unmated workers, ranging from facultative asexual reproduction after queen death to nearly obligate asexuality in which the queen caste is absent or morphologically reduced (Itow *et al.* 1984; Schilder *et al.* 1999). However, males occur in all seven of these ant species, contrasting with the complete absence of males in *M. smithii*. Three distinct reproductive strategies of asexuality therefore appear to exist in ants: (i) worker reproduction of females with a trend towards queen loss (*Messor capitatus*,

Platytherea punctata, *Cerapachys biroi*, *Pristomyrmex pungens*; Percy *et al.* 2005), (ii) a mixed strategy where mated queens produce workers sexually but new queens asexually (*Cataglyphis cursor*, *Wasmannia auropunctata*, *Vollenhovia emeryi*; Fournier *et al.* 2005; Percy *et al.* 2005; Ohkawara *et al.* 2006), and (iii) strict queen thelytoky with sterile workers (*M. smithii*). *Pristomyrmex pungens* was proposed as an obligate asexual since it reproduces by worker thelytoky; however, males with functional genitalia and normal spermatogenesis occur, indicating the potential for sex (Itow *et al.* 1984; Tsuji 1988). By contrast, *M. smithii* has evolved a unique strategy in which queens produce both workers and new queens asexually, where workers are completely sterile (electronic supplementary material).

Theory predicts that long-term asexuality increases extinction potential because absence of recombination constrains a species' ability to evolve, by generating novel genotypes and purging deleterious alleles at lower rates, eventually rendering asexual lineages inferior to competing sexual lineages (Barton & Charlesworth 1998). This cost of asexuality may be exacerbated in *M. smithii* through its dependence on asexual cultivar propagation (Mueller *et al.* 1998, 2005). While cultivars of *M. smithii* are not ancient asexuals (Mueller *et al.* 1998; Mueller 2002), they are cultivated clonally within a nest and between nests over many years, making them vulnerable to fungal pathogens evolving within the gardens (Currie *et al.* 2003). Dependence of an asexual host on an asexual symbiont could therefore present a double cost of asexuality. Alternatively, the asexuality of both ant and fungus may permit the ants to fully exploit the evolutionary advantages of asexuality for unknown reasons. In cyclic parthenogens such as *Daphnia* and aphids, asexual forms predominate during resource abundance and switch to sexual reproduction when resources become limiting (Williams 1975; Bell 1982; Lynch 1984). However, *M. smithii* showed no switch to sexuality across seasonal differences, environmental gradients, geographical range (Argentina to Mexico), and diverse laboratory conditions studied to date.

Assuming a double asexuality handicap, it is unclear how *M. smithii* could sustain one of the most widespread distributions and greatest local abundances of all attine ant species (Rabeling 2004; Rabeling *et al.* 2007). Strict clonal reproduction could eliminate kin-selected queen-worker conflicts that are thought to plague sexual insect societies (Ratnieks *et al.* 2006), and *M. smithii* therefore provides a unique experimental system to test theories of parent-offspring conflict and reproductive policing. In addition, unlike all other fungus-growing ant species that typically specialize on a narrow clade of fungi, *M. smithii* is the only attine ant species known to cultivate a diversity of fungi between different nests. This unparalleled diversity of cultivars propagated within *M. smithii* arises because *M. smithii* frequently switches to novel, distantly related fungal crops (Mueller *et al.* 1998; A. G. Himler & U. G. Mueller 2004, unpublished data). Frequent switching between fungal crops (symbiont reassociation) may mitigate the double asexuality handicap because switching generates novel combinations of ant farmer and crop genomes. This potentially creates sufficient variation in synergistic ant-crop phenotypes to outpace coevolution by crop pathogens (Mueller 2002; Van Doninck *et al.* 2002) and cope with environmental

fluctuations. One possibility is that *M. smithii* is a geographic parthenogen (Vandel 1928), i.e. it tends to reproduce asexually in more extreme altitudes, further north, or more extreme environments than their sexual relatives (Bell 1982; Lynch 1984). However, *M. smithii* has one of the most extensive distributions of fungus-growing ants (Argentina to Mexico), and it is always sympatric with other, sexual attine species (except for some Caribbean island populations). Another possibility is that *M. smithii* can colonize a diversity of habitats because it represents a 'general purpose genotype' (GPG) able to tolerate broad environmental conditions (Lynch 1984), an explanation applied to the ancient asexual darwinulid ostracods (Van Doninck *et al.* 2002). If so, *M. smithii* would be the first case of a symbiosis GPG and support the suggestion that clonality leads towards greater ecological generalization rather than specialization.

The widespread distribution and ecological abundance of *M. smithii* challenge traditional views proposing that sexuality enables fungus-growing ants to assume the coevolutionary arms races of their asexual cultivars (i.e. effectively converting crop-pathogen arms races into races between ant farmers and crop pathogens; Herre *et al.* 1999). Asexuality of *M. smithii* precludes such a hypothesized arms race transfer, and the ecological success of the dual asexual symbiosis between clonal ant farmers and their clonal crops therefore defies current theoretical expectations, perhaps adding a novel form of asexual scandal if *M. smithii* is shown to be of more ancient than recent evolutionary origin (Judson & Normark 1996; Normark *et al.* 2003). We predict that *M. smithii* will emerge not only as a new empirical system to test theories of parent-offspring conflict and policing in eusocial insects (Ratnieks *et al.* 2006), but also as a model permitting controlled symbiont-switch experiments in order to understand the evolutionary persistence of asexual lineages within a network of coevolving sexual pathogens and asexual mutualists.

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