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 descendents 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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buffer (Promega); 3.75 mM MgCl₂ (Promega); 0.25 mM of
for 15 min. Each 10
40 s; and 72
m
M. tardus
sclerotized functional mussel organ of
GENOTYPER v. 3.6. Microsatellite primer Cypho 15–16
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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 longisicapus). 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.

(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)\(^5\times 5.55 \times 10^{-17}\), 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

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.
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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.)

<table>
<thead>
<tr>
<th>antibiotic</th>
<th>percentage of antibiotic solution</th>
<th>no. of subcolonies</th>
<th>no. of queens treated per subcolony</th>
<th>total no. of queens treated</th>
<th>queens produced</th>
<th>males produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>streptomycin</td>
<td>10</td>
<td>7</td>
<td>37 ± 17</td>
<td>264</td>
<td>931</td>
<td>0</td>
</tr>
<tr>
<td>rifampicin</td>
<td>0.5</td>
<td>7</td>
<td>38 ± 16</td>
<td>263</td>
<td>2560</td>
<td>0</td>
</tr>
<tr>
<td>penicillin</td>
<td>5</td>
<td>7</td>
<td>34 ± 16</td>
<td>236</td>
<td>1666</td>
<td>0</td>
</tr>
<tr>
<td>tetracycline</td>
<td>5</td>
<td>7</td>
<td>36 ± 12</td>
<td>249</td>
<td>1558</td>
<td>0</td>
</tr>
<tr>
<td>control (sucrose)</td>
<td>10</td>
<td>7</td>
<td>47 ± 16</td>
<td>332</td>
<td>1129</td>
<td>0</td>
</tr>
<tr>
<td>total</td>
<td></td>
<td></td>
<td></td>
<td>1344</td>
<td>7844</td>
<td>0</td>
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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 $\chi^2 = 3.99, p = 0.1363$.)

<table>
<thead>
<tr>
<th>fungus garden source</th>
<th>M. smithii fungus (control fungus)</th>
<th>C. costatus fungus (closely related fungus)</th>
<th>C. longiscapus fungus (distantly related fungus)</th>
<th>total</th>
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<tr>
<td></td>
<td>no. of workers produced</td>
<td>no. of gynes produced</td>
<td>percentage queens reproducing</td>
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<tr>
<td>77</td>
<td>8</td>
<td>6/30 = 20%</td>
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<tr>
<td>7</td>
<td>1</td>
<td>3/30 = 10%</td>
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<tr>
<td>15</td>
<td>7</td>
<td>6/30 = 20%</td>
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<td>15/90 = 17%</td>
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(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; Peary 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; Peary 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,
Plant cytotypes may represent a strategy where certain cultivars are propagated within a diversity of fungi between different nests. This unpar-allel diversity of cultivars typically specializes on a narrow clade of fungi, parent–offspring conflict and reproductive policing. Societies (Ratnieks 2004; Rabeling 2007). 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 (Ratnicks 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 reassocation) 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 (Ratnicks 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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