Cryptic sexual populations account for genetic diversity and ecological success in a widely distributed, asexual fungus-growing ant

Christian Rabeling[a,b,1], Omar Gonzales[a], Ted R. Schultz[b], Maurício Bacci, Jr.[c], Marcos V. B. Garcia[d], Manfred Verhaagh[e], Heather D. Ishak[a], and Ulrich G. Mueller[a]

[a]Section of Integrative Biology, University of Texas, Austin, TX 78712; [b]Department of Entomology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012; [c]Center for the Study of Social Insects, São Paulo State University, Rio Claro, SP 13506-900, Brazil; [d]Embrapa Amazônia Ocidental, Manaus, AM 69010-970, Brazil; and [e]Staatliches Museum für Naturkunde Karlsruhe, 76133 Karlsruhe, Germany

Edited by Bert Hölldobler, Arizona State University, Tempe, AZ, and approved June 14, 2011 (received for review April 6, 2011)

Sex and recombination are central processes in life generating genetic diversity. Organisms that rely on asexual propagation risk extinction due to the loss of genetic diversity and the inability to adapt to changing environmental conditions. The fungus-growing ant species *Mycocepurus smithii* was thought to be obligately asexual because only parthenogenetic populations have been collected from widely separated geographic localities. Nonetheless, *M. smithii* is ecologically successful, with the most extensive distribution and the highest population densities of any fungus-growing ant. Here we report that *M. smithii* actually consists of a mosaic of asexual and sexual populations that are nonrandomly distributed geographically. The sexual populations cluster along the Rio Amazonas and the Rio Negro and appear to be the source of independently evolved and widely distributed asexual lineages, or clones. Either apomixis or automixis with central fusion and low recombination rates is inferred to be the cytogenetic mechanism underlying parthenogenesis in *M. smithii*. Males appear to be entirely absent from asexual populations, but their existence in sexual populations is indicated by the presence of sperm in the reproductive tracts of queens. A phylogenetic analysis of the genus suggests that *M. smithii* is monophyletic, rendering a hybrid origin of asexuality unlikely. Instead, a mitochondrial phylogeny of sexual and asexual populations suggests multiple independent origins of asexual reproduction, and a divergence-dating analysis indicates that *M. smithii* evolved 0.5–1.65 million years ago. Understanding the evolutionary origin and maintenance of asexual reproduction in this species contributes to a general understanding of the adaptive significance of sex.

Attini | clonality | Formicidae | thelytoky | mutualism

The vast majority of metazoans reproduces sexually, enjoying the benefits of genetic recombination (1–3) such as rapid adaptability to novel ecological conditions (4, 5) and the purging of deleterious mutations from their genomes (6, 7). However, relative to sexually reproducing organisms, an asexual female doubles its fitness by transmitting its entire genetic material to the next generation (8). Despite such obvious short-term fitness advantages, asexual organisms occur only sporadically throughout the tree of life and are predicted to be evolutionarily short-lived and doomed to early extinction (9–11). In contrast to the short-term advantages of asexuality, the adaptive value of sexuality, that is, genetic recombination, is expected to be of long-term benefit (2, 12–14). There remain in evolutionary biology significant unexplored questions about whether sexual reproduction is favored by natural selection over short evolutionary time spans and, if not, why sexual reproduction persists as the prevalent mode of reproduction, given that the selective benefits are deferred. Studying the origin and evolution of parthenogenetic lineages, and understanding how genetic diversity is generated and preserved in such lineages, is essential to answering these questions.

Asexual reproduction by females, or thelytokous parthenogenesis, has recently been reported in queens of the fungus-growing ant *Mycocepurus smithii* in three geographically distant populations in Latin America: Puerto Rico (15), Panama (16), and Brazil (17). The widespread geographic distribution of asexuality and the complete absence of males from field collections and laboratory colonies suggested that *M. smithii* might be obligately asexual (16, 17), and one study proposed that asexuality in this species might be ancient (16). Among bees, wasps, and ants, thelytokous parthenogenesis has so far been observed in the Cape honey bee (18, 19) and in 12 distantly related species of ants (17, 20–23). Population-genetic studies of some species revealed a diversity of highly complex genetic systems, including different cytogenetic mechanisms used to produce workers and queens, facultative sexual reproduction, and clonal male lineages (23–27). Asexual eusocial Hymenoptera produce diploid offspring via meiotic parthenogenesis, or automixis, in which a limited amount of genetic variability is generated through fusion of sister nuclei (28–31). In contrast, mitotic parthenogenesis, or apomixis, in which offspring are genetic clones of their mothers, has not been demonstrated unambiguously in social insects.

Although many theoretical studies predict the costs and benefits of sex, little is known about the evolution of asexuality at the organism level (2). To study the origin and maintenance of parthenogenesis and to elucidate the mechanisms generating genetic diversity in parthenogenetic lineages, we investigated the evolutionary history of the asexual fungus-growing ant *M. smithii*. To test for obligate asexuality in *M. smithii*, we developed highly variable short tandem repeat (or microsatellite) markers and analyzed colonies from multiple populations across the species's broad range, extending from Mexico to Argentina and including some Caribbean islands (32, 33). To identify the genetic structure within and between populations of *M. smithii* and to infer the cytogenetic mechanism underlying parthenogenetic reproduction, we genotyped sterile workers and reproductive queens from 234 colonies. Clonality was inferred by genetic identity between nest mates. Controlled laboratory breeding experiments complemented our field observations. To test for a potential hybrid origin of parthenogenesis in *M. smithii*, we reconstructed a molecular phylogeny of the genus *Mycocepurus*. An additional fine-scaled mitochondrial phylogeny of asexual and sexual *M. smithii* populations was used to investigate whether asexuality arose

Author contributions: C.R. designed research; C.R. and O.G. performed research; C.R., T.R.S., M.B., M.V.B.G., M.V., H.D.I., and U.G.M. contributed new reagents/analytic tools; C.R. and T.R.S. analyzed data; C.R., O.G., T.R.S., M.B., M.V.B.G., M.V., H.D.I., and U.G.M. revised the manuscript; and C.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: DNA sequences reported in this paper have been deposited in the GenBank database (accession nos. JN054745–JN055353).

[1]To whom correspondence should be addressed. E-mail: crabeling@gmail.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1105467108/-/DCSupplemental.

once or multiple times independently from sexually reproducing ancestors. Lastly, we performed a divergence-dating analysis to estimate the time span over which parthenogenesis has persisted in *M. smithii*, because asexuality was previously proposed to be of ancient origin.

Results

Population-Genetic Analyses. A total of 1,930 *M. smithii* individuals from 234 colonies collected at 39 different localities in Latin America (Fig. 1 and Table S1) was genotyped at 12 variable microsatellite loci yielding 106 alleles (range: 2–15 alleles per locus). The number of alleles per locus per individual never exceeded two, indicating diploidy of females. Of the genotyped populations, 89.7% (n = 35) showed population-genetic signatures of clonality, whereas 10.3% (n = 4) showed an increase of unique multilocus genotypes, indicative of genetic recombination caused by sexual reproduction.

Asexual populations. A total of 1,647 individuals from 218 colonies in 35 populations exhibited genetic signatures of clonal reproduction. Asexual reproduction was characterized by sharing of repeated multilocus genotypes among individuals (Table S1), maximum deviation from random mating (F_{IS} = −1; Table S2), and a low genotype-to-individual ratio (i.e., G:N approaching 0, whereas a G:N of 1 indicates that each individual is genetically distinct from another) (Table S1). To determine the number of independently evolved asexual lineages that arose at different localities from the sexual population, we estimated the probability that slightly different multilocus genotypes originated from separate sexual events (p_{sex} > 0.01) instead of arising from accumulated mutations or scoring errors (p_{sex} < 0.01). In addition, clonal diversity (R) was calculated.

Among all *M. smithii* populations, 66 asexual genotypes were identified, 57 of them representing unique multilocus genotypes (R = 0.86; Tables S2 and S3). Five repeated multilocus genotypes were shared between 10 geographically proximate populations (~10–40 km distance), and three unique genotypes were identified in seven geographically distant populations (~700–2,600 km distance; Tables S2 and S3). Calculating the probability that repeated multilocus genotypes from different populations originated from distinct sexual events revealed that identical multilocus genotypes belong to the same clonal lineage (p_{sex} < 0.01), indicating long-distance dispersal events of individuals from the same asexual lineage. No genetic variation was present within repeated multilocus genotypes (F_{IS} = −1), but significant genetic variance was structured among them [analysis of molecular variance (AMOVA); F_{ST} = 0.624, P = 0.01].

A comparison of the 57 unique multilocus genotypes revealed high frequencies of low genetic distances between genotypes, resulting in a bimodal frequency distribution of genetic distances and indicating the potential existence of mutations or scoring errors in clones (34). Eleven multilocus genotype pairs differed from one other genotype only by a single allele, reducing the number of asexual lineages that potentially originated from distinct sexual events to 46 (p_{sex} < 0.01, R = 0.69). Further lowering the threshold and allowing two to six alleles to be shared among multilocus genotypes within an independently evolved clonal lineage, we identified 43 (R = 0.65) to minimally 38 (R = 0.57) independently evolved clonal lineages.

In 20 clonal populations, only a single multilocus genotype was encountered across different colonies. In 15 populations, two to maximally six multilocus genotypes coexisted at a single site (Table S1). In five populations, all or a subset of multilocus genotypes differed by one to six alleles, suggesting a single colonization event followed by diversification within clonal lineages due to the accumulation of mutations or scoring errors (Table S1). In contrast, 12 populations harbored multilocus genotypes differing by 7–15 alleles, indicating independent colonization events of these sites by distantly related clonal foundress queens. The highest diversity of clonal lineages (n = 5) was discovered at a Peruvian lowland rainforest site (Los Amigos).

Genetic uniformity across all loci within colonies suggests either mitotic parthenogenesis (apomixis) as the cytogenetic mechanism underlying thelytokous parthenogenesis in *M. smithii* or, alternatively, automixis with central fusion and low recombination rates. To trace the genotypes of reproductive individuals over multiple generations, we propagated *M. smithii* colonies in the laboratory for six consecutive generations and genotyped all 93 queens at the end of the experiment. All queens were genetically identical across generations, and transitions from a heterozygous locus in the mother to a homozygous locus in the offspring was not observed, as would be expected under automixis with central fusion. Interestingly, in field-collected populations in which 7 of the 11 multilocus genotype pairs differ by only a single allele and are identical at all other loci, we observed that one genotype was heterozygous at a given locus whereas the other was homozygous at the same locus. These transitions could indicate a switch from heterozygosity to homozygosity, as expected under automixis. Without knowing which one of these two is the maternal or the offspring genotype, however, it is not possible to distinguish between a transition from a heterozygous to a homozygous state caused by infrequent recombination or an accumulation of "somatic" mutations.

Recombining populations. Four Amazonian populations, distributed along the Rio Amazonas and the Rio Negro (Fig. 1), exhibited population-genetic signatures of genetic recombination, indicative of sexual reproduction (Tables S1 and S2). Among 283 genotyped individuals, 210 multilocus genotypes were identified, resulting in high genotype-to-individual (G:N) ratios, ranging from 0.71 to 1 (Table S1). Recombinant populations were characterized by inbreeding indices diverging from genetic fixation (F_{IS} = −1), ranging from 0.03 to −0.77, and observed and expected heterozygosities were similar, as expected for populations under Hardy–Weinberg conditions (Table S2).

Because multiple colonies were collected from the Caldeirão population in Amazonas, Brazil (Fig. 1), we investigated this

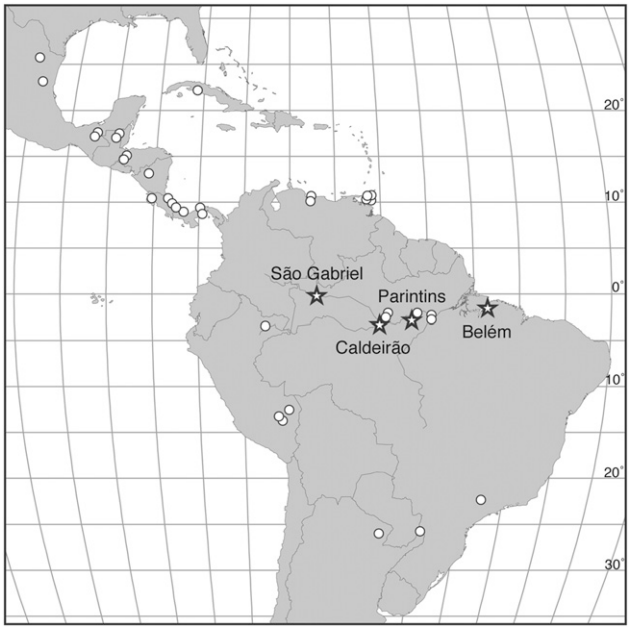

Fig. 1. Geographic distribution of sexual (stars) and asexual (circles) *M. smithii* populations. Localities refer to the sexual populations, distributed along the Rio Amazonas and the Rio Negro. Asexual populations are widely distributed in Latin America, ranging from northern Mexico to northern Argentina. Lines of longitude and latitude are separated by units of 5°.

population in detail to test for sexual reproduction. Genotyping of 243 individuals (234 workers, 5 queens, 4 spermatheca contents) revealed the existence of 173 unique multilocus genotypes, of which 132 multilocus genotypes were represented by single individuals whereas the remaining 41 multilocus genotypes were shared by 111 individuals. Among the shared multilocus genotypes, two or at most six nestmates carried identical genotypes. After removing identical genotypes from the dataset, we tested whether genotypes that differ by only a single allele are derived from distinct sexual events or from somatic mutations or scoring errors. Among those unique genotypes (n = 173), 55 multilocus genotypes likely belonged to the same clonal lineage ($p_{sex} < 0.01$), whereas 118 multilocus genotypes probably originated from distinct sexual events ($p_{sex} > 0.01$). This result indicates that 48.6% (118 multilocus genotypes out of 243 individuals) of the genotyped individuals result from sexual reproduction. Such a mixture of recombinant and clonal offspring within a single population suggests that sexual *M. smithii* queens either occasionally reproduce parthenogenetically or, alternatively, that a larger number of clonally reproducing queens coexists with sexual queens in the same colony. Facultative asexual reproduction by otherwise sexual queens seems more likely, however, given the high number of shared genotypes in the Caldeirão population (n = 41), contrasting with the low number of individuals sharing a multilocus genotype (n = 2–6). After excluding repeated genotypes, observed and expected heterozygosities were almost identical (H_o = 0.372, H_e = 0.369) and the inbreeding index was indicative of random mating (F_{IS} = −0.009) (Table S2).

To directly test whether queens were fertilized, the abdomens of four (out of five) queens were dissected, revealing sperm-filled spermathecae and reproductively active ovaries. The spermatheca contents (n = 4) were identified as sperm under 200× magnification and subsequently genotyped. The sperm from each spermatheca were haploid at all loci, as expected from hymenopteran males developing from unfertilized, haploid eggs. In addition, haploidy at all loci indicates that the queens were singly mated. Furthermore, a subset of paternal alleles matched alleles found in workers which were not present in queens (Table S4). Hence, workers exhibited recombinant genotypes representing both maternal and paternal alleles. The combined evidence demonstrates that the *M. smithii* population from Caldeirão reproduces sexually and, although males have as far as we know never been collected, sperm content clearly reveals their existence.

The genetically recombinant population from São Gabriel da Cachoeira showed that all nestmates (n = 8) were genetically distinct (G:N = 1, H_o = 0.365, H_e = 0.315, F_{IS} = −0.172), consistent with strict sexual reproduction (Tables S1 and S2). However, in the Belém colony (G:N = 0.96, H_o = 0.451, H_e = 0.466, F_{IS} = 0.034) and the Parintins colony (G:N = 0.71, H_o = 0.650, H_e = 0.398, F_{IS} = −0.773), few individuals shared a multilocus genotype, suggesting mixed sexual and parthenogenetic reproduction in these populations.

Only a single clonal lineage (from Trinidad) shared a multilocus genotype with the sexual population from São Gabriel da Cachoeira, suggesting that sexual lineages may continuously spawn asexual lineages. To further explore whether sexual populations give rise to asexual lineages, we analyzed the genetic structure of unique multilocus genotypes of asexual and sexual populations. Genotypes of sexual populations group as distinct genetic clusters in the 3D plot generated by a nonmetric multidimensional scaling (NMDS) analysis (Fig. 2). In the discriminate analysis of principal components (DAPC) analysis, the asexual genotypes as a whole and the four clusters of sexual genotypes are significantly different from each other [Wilks's lambda = 0.098, approximate F ratio = 80.247, df (12, 685), $P < 0.0001$]. Only a few asexual genotypes grouped inside clusters of sexual genotypes, indicating genetic proximity. Greater genetic distances between clones and sexual clusters most likely indicate that the clones originated from sexual source populations other than the four that were sampled, or perhaps that they are of older evolutionary origin and thus highly diverged. Limited overlap between sexual clusters further indicates that the genetic variability of sexual populations was not exhaustively sampled for *M. smithii* as a species.

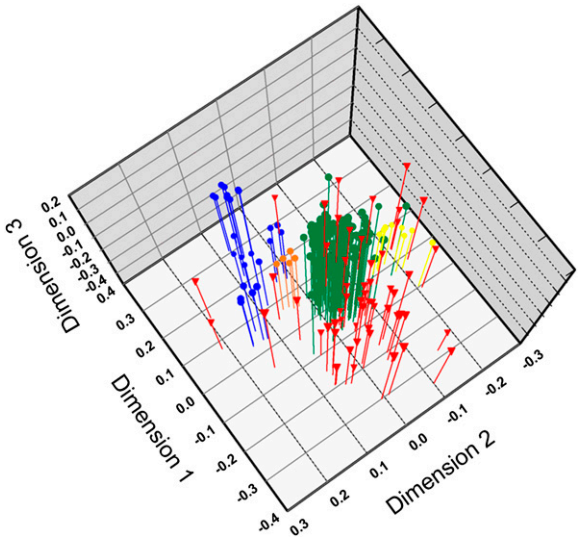

Fig. 2. Plot of 3D object coordinates resulting from an NMDS analysis derived from individual genetic distances. Colored circles represent genotypes of sexual *M. smithii* populations (blue, Belém; orange, Parintins; green, Caldeirão; yellow, São Gabriel da Cachoeira) and red triangles represent genotypes of asexual lineages.

Phylogenetic Analyses. To test the monophyly of *M. smithii* and reconstruct whether asexuality evolved once or multiple times from a sexually reproducing ancestor, we conducted a global phylogenetic analysis of the genus *Mycocepurus* and a local analysis of only *M. smithii* taxa representing a sample from each of the genotyped populations (Table S5).

In the global analyses, the monophyly of the genus *Mycocepurus* was unequivocally supported [Fig. S1; Bayesian posterior probability (BPP) = 1; maximum likelihood bootstrap proportion (MLBP) = 100], which is consistent with a previous analysis (35). Within the genus *Mycocepurus*, nine reciprocally monophyletic, highly supported groups were recognized [Fig. S1; BPP = 1, MLBP ≥ 92], supporting the existence of five new species (Fig. S1). The monophyly of *M. smithii* was well-supported [Fig. S1; BPP = 1, MLBP = 92], suggesting that extant *M. smithii* populations derive from a single most recent common ancestor (MRCA). An undescribed species from the Colombian Amazon was found to be the sister lineage of *M. smithii*, but with only weak statistical support (Fig. S1; BPP = 0.72, MLBP = 56).

For the mitochondrial gene tree of genotyped *M. smithii* populations, the statistical support for relationships between sampled individuals is generally low, as expected from the relatively weak phylogenetic informativeness of the mtDNA markers (Table S6; parsimony-informative characters = 169; 11% of mtDNA dataset). Despite this general problem, the monophyly of *M. smithii* as a species was supported by both the mitochondrial and nuclear data, suggesting that a hybrid origin of asexual reproduction is unlikely in *M. smithii*. The mitochondrial phylogeny further indicates that the sexual populations are separated into at least two distantly related groups (Fig. 3) and that relationships among asexual populations are in some cases correlated with geography. Three sexual populations form a reasonably well supported clade (BPP = 0.96, MLBP = 59) that also includes two clonal pop-

ulations (Fig. 3). The sexual population from Belém forms the sister lineage to a clade consisting of asexual populations from the Amazon and Trinidad. This relationship, however, is only weakly supported (BPP = 0.51). Neither the asexual nor the sexual populations are reconstructed as monophyletic under any possible rooting (Fig. 3), consistent with the hypothesis of independent evolutionary origins of asexuality. Based on Bayes factors (BF), the likelihoods of phylogenies resulting from analyses in which the asexual populations are constrained to be monophyletic are significantly worse fitting to the data than those resulting from unconstrained analyses [ML: 2ln(BF) = 137.82; Bayesian: 2ln (BF) = 124.1], further indicating multiple independent origins of asexuality.

Divergence-dating analysis. The stem-group age (i.e., earliest possible origin) of the fungus-gardening ants was estimated to be 52 million years (Ma) [confidence interval (CI) = 44,60] and the crown-group age was 50 Ma (CI = 43,58), consistent with estimates in Schultz and Brady (35). The estimated crown-group age of the genus *Mycocepurus* is ~10 Ma (CI = 6,14), whereas the stem-group age is considerably older with 37 Ma (CI = 27,46), which is also indicated by a long branch leading to the MRCA shared with the sister lineage *Myrmicocrypta* (Fig. S1). The stem-group age of *Mycocepurus smithii* is ~1.65 Ma (CI = 0.57,2.84), whereas the crown-group origin was estimated to be considerably more recent at 0.5 Ma ago (CI = 0.01,1.19). This relatively recent estimate for the evolutionary origin of *M. smithii* is consistent with the almost complete absence of genetic variability observed in the nuclear DNA sequences.

Discussion

M. smithii consists of a mosaic of sexual and parthenogenetic populations. Although separated by as much as 2,000 km, the sexual populations are located along the Rio Amazonas and the Rio Negro, suggesting the existence of a central widespread sexual (or facultatively sexual/asexual) population that has repeatedly generated asexual, clonally reproducing lineages. These asexual lineages have rapidly dispersed throughout much of Latin America, leading to the current widespread geographic distribution of the species (32, 33). The high clonal diversity in some populations indicates that independently evolved clonal lineages have colonized these habitats separately and repeatedly through time. Once an *M. smithii* lineage has lost the ability to reproduce sexually, the condition seems irreversible, resulting in our finding of genetically identical individuals in each of the 218 parthenogenetic colonies studied. The mitochondrial phylogeny of *M. smithii* (Fig. 3) identifies a statistically well-supported group that includes individuals from both asexual and sexual populations, and places the sexual populations in at least two distantly related clades. These patterns, coupled with the results of phylogenetic constraint analyses, are consistent with independent and repeated losses of sexual reproduction. Given the limitations of our sampling, it is nearly certain that additional sexual source populations, from which such closely related groups of asexual clones originated, were not sampled. The divergence-dating analysis provides a recent estimate (crown-group age: 0.5 Ma; CI = 0.01,1.19) for the origin of the presumably sexual most recent common ancestor of extant *M. smithii* populations, indicating that secondary transitions from sexual to asexual reproduction have occurred recently and possibly continue to occur in the present.

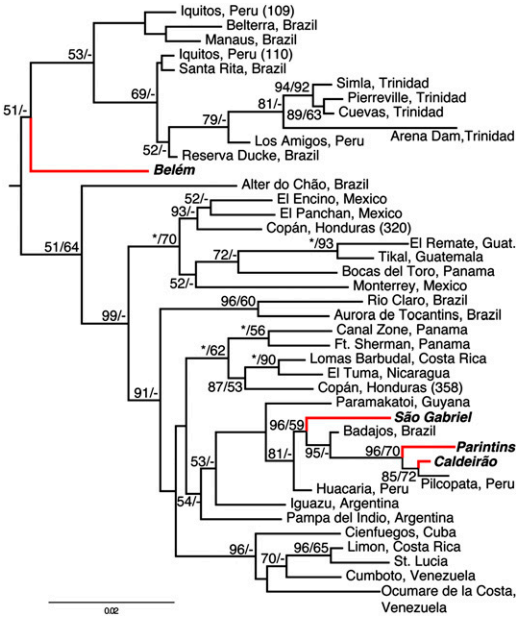

Fig. 3. Midpoint-rooted Bayesian phylogram of *M. smithii* individuals representing each of the genotyped populations based on analyses of three mitochondrial gene fragments. Bayesian posterior probabilities (×100) and ML bootstrap proportions are indicated as BPP/MLBP. Red branches and bold font indicate taxa from sexually reproducing populations. All other taxa represent asexual populations. (Scale bar, number of substitutions per site.)

The combined phylogenetic and population-genetic evidence is consistent with the hypothesis that sexual reproduction was lost in ancestors of parthenogenetic *M. smithii* populations. The spontaneous loss of sexual reproduction has been proposed for the little fire ant *Wasmannia auropunctata*, in which sexual populations in the native range of this invasive species are likely the source of asexual invasive populations (36). The proximate genetic mechanisms causing the loss of sexuality are not well-understood. However, studies of Cape honey bees (37) and of parthenogenetic lineages of *Drosophila melanogaster* (38) show that a single recessive allele can cause thelytoky. These examples suggest that the high propensity for switching from sexual to asexual reproduction in *M. smithii* may be controlled by a small number of genes. Breeding experiments could test whether thelytoky is a qualitative or a quantitative trait in *M. smithii* by introgressing sexual genes into an asexual genetic background and observing the segregation pattern of the offspring.

Cyclical parthenogenesis, the alteration of asexual and sexual life stages (39, 40), is unlikely to occur in *M. smithii*. In each of the 218 parthenogenetic colonies collected in different seasons over an 8-y period (2003–2010), nestmates belonged only to one or very few clonal lineages. The nonrandom geographic distribution of sexual and asexual populations likewise suggests that the switch from sexuality to asexuality is unlikely triggered by season.

In arthropods, the evolution of asexuality is often associated with hybridization (30, 41), a mechanism so far unknown in social Hymenoptera (36). Given the monophyly of *M. smithii* and the phylogenetic congruence between nuclear and mitochondrial markers, hybridization is also unlikely to explain the origin of asexuality in *M. smithii*.

Alternatively, microorganisms such as *Wolbachia*, *Cardinium*, and *Rickettsia* have been shown to induce parthenogenesis in parasitoid wasps (42–44). Even though *Wolbachia* infections have not been detected in social Hymenoptera (45), including *M. smithii* (16), other parthenogenesis-inducing symbionts cannot be ruled out in *M. smithii*.

Although we have so far only examined a scenario in which asexual populations of *M. smithii* have repeatedly arisen from sexual populations, the nonmonophyly of the sexual and asexual populations in the mitochondrial phylogeny equally supports an alternative hypothesis: that sexual populations have repeatedly evolved from widespread asexual populations. Although evolutionary reversals from less complex to more complex ancestral traits have long been deemed unlikely (46, 47), reversals from asexual to sexual reproduction have been suggested for mites and

hawkweed (48, 49). The absence of males (17) and the lack of genetic recombination in asexual populations of *M. smithii* are consistent with the hypothesis that meiosis is dysfunctional in parthenogenetic queens. In species with haplodiploid sex determination, restoring functional meiosis would simultaneously result in recombination and the production of haploid eggs, from which males could develop (41, 50). Therefore, haplodiploid species might theoretically require only a single mutation to reevolve sexuality. However, given (*i*) that all *Mycocepurus* species for which we have biological information reproduce sexually, (*ii*) the high genetic diversity observed in the sexually reproducing *M. smithii* populations, and (*iii*) the genetic variability observed between separate clonal lineages, it seems highly unlikely that extant sexual *M. smithii* individuals descended from asexual ancestors.

Despite the large number of clonal lineages found across the broad geographic distribution of *M. smithii*, mothers and offspring from field and laboratory colonies were genetically identical across multiple generations and males were completely absent from asexual populations, suggesting apomixis as the cytogenetic mechanism underlying thelytoky. Alternatively, it is possible that *M. smithii* queens reproduce via meiotic parthenogenesis (automixis) with central fusion, a cytogenetic mechanism characterized by potentially very low recombination rates, depending on the locus's distance to the centromere, as indicated by genotype pairs that differ only at a single locus. Automixis with central fusion has been documented in social Hymenoptera (18, 19, 26, 28, 29, 51, 52), and a recent study of *W. auropunctata* reported recombination rates as low as 0–2.8% (31). Our current data, however, are insufficient to clearly distinguish between automixis with a low recombination rate and apomixis with rare gene conversion.

Conclusion

M. smithii is a recently evolved, monophyletic species consisting of a mosaic of asexual and sexually reproducing populations. Sex has been lost repeatedly in multiple lineages. Once females have lost the ability to reproduce sexually, the condition seems to be irreversible. The lack of genetic recombination and the complete absence of males in asexual populations and laboratory breeding experiments indicate that meiosis may be dysfunctional in asexual females, and thus that mitotic parthenogenesis (apomixis) is the cytogenetic mechanism underlying parthenogenesis in *M. smithii*. However, automixis with central fusion and low recombination rates cannot be ruled out as a possible alternative mechanism. Sexually reproducing populations were discovered in the center of *M. smithii*'s geographic distribution along the Rio Amazonas and the Rio Negro. *M. smithii* has high local population densities and the most extensive geographic distribution of any fungus-growing ant species, indicating its ecological success. The sympatric existence of sexual and asexual populations in the Amazon suggests that sexual populations continue to enjoy high fitness in the center of the species distribution and are not outcompeted by asexual colonies. The fitness advantage of asexual populations seems to be realized outside the range of sexual populations, where parthenogenetic queens apparently colonize vacant niches and disperse rapidly in the absence of males. Given that kin selection theory predicts that conflict over reproduction should be absent in groups of genetically identical individuals, it would be intriguing to investigate the maintenance of cooperative behavior and social conflict in *M. smithii*. Finally, given the absence of genetic variation within colonies and the presence of phenotypically distinct queen and worker castes, *M. smithii* appears to be a study organism that is well-suited for investigating the proximate mechanisms of environmentally based caste determination and for exploring the genetic basis of phenotypic plasticity.

Materials and Methods

Population-Genetic Analyses. As test statistics for asexuality, we used the existence of repeated multilocus genotypes and maximum deviation from random mating (F_{IS}) (53–55). The genotype-to-individual ratio (G:N ratio) was applied to identify multilocus genotypes (55) (Table S1). Independently evolved asexual lineages (clones) originating from separate sexual events were distinguished from slightly different multilocus genotypes that diversified through accumulation of mutations or scoring errors by calculating the probability, p_{sex}, following the methodology outlined in ref. 34 and implemented in GENCLONE 2.0 (56). The observed and expected heterozygosity for each clonal lineage (57), the proportion of clonal genotypes in a population, *F* statistics, and AMOVA were calculated in GENALEX 6 (58) and Genetic Data Analysis (59). To reveal the underlying population-genetic structure of sexual and asexual populations, we used the multivariate statistical methods (60–62) NMDS, principal component analysis, and DAPC, as implemented in PERMAP (63), GENALEX (58), and SYSTAT (Systat Software).

Phylogenetic Analyses. We conducted analyses of two distinct datasets: first, a global dataset that included 84 *M.* ingroup taxa, 32 of them *M. smithii*, and 87 outgroup taxa. The recently described social parasite *M. castrator* (64) was not included. The alignment consisted of 2,319 bp of protein-coding (exon) sequences of three single-copy nuclear genes and one mitochondrial gene and was divided into 10 partitions. Second, we conducted a local analysis of 41 *M. smithii* taxa representing one individual from each of the genotyped populations (Table S5). We obtained 1,515 bp of three mitochondrial genes and divided the alignment into two partitions (Table S6). Constrained topologies were estimated using Bayesian and ML analyses, and differences in the likelihoods of constrained versus unconstrained topologies were evaluated using Bayes factors (65–67). All ingroup sequence data were generated for this study (Table S5). Best-fit models of sequence evolution were selected for each partition under the Akaike information criterion (68) and hierarchical likelihood ratio tests as calculated in MODELTEST v3.7 (69) (Table S6). We conducted partitioned Bayesian analyses using MrBayes v3.1.2 (70). Burn-in and convergence were assessed using Tracer v1.5 (71). Partitioned ML analyses were carried out in GARLI 0.97.r737 (72).

Divergence-Dating Analysis. We used a Bayesian relaxed clock uncorrelated lognormal approach implemented in the program BEAST v1.4.8 with a Yule process as the tree prior (73–75). The root node was given a normal age prior distribution (mean = 73.5, SD = 4.5), following methodology described in ref. 76. Based on fossil data, lognormal age prior distributions were assigned to three internal nodes, as outlined in ref. 35. For more details on analyses and results, see *SI Materials and Methods* and Tables S1–S8.

ACKNOWLEDGMENTS. We thank the following scientists for generously contributing specimens and institutions for providing permission and access to study sites: G. Alpert, C. Brandão, S. Cappellari, J. Carpenter, S. Cover, R. Feitosa, F. Fernández, J. Fontenla, A. Harada, A. Henriques, A. Himler, J. Lattke, J. Longino, W. Mackay, J. Maes, J. Martins, B. Merz, N. Pitman, R. Poggi, V. Raineri, C. Samper, S. Sánchez-Peña, J. Santisteban, R. Silva, the late R. Snelling, J. Sosa-Calvo, H. Vasconcelos, P. Ward, and E. Wilson; the Autoridad Nacional del Ambiente and Smithsonian Tropical Research Institute, Panama; Conselho Nacional de Desenvolvimento Científico e Tecnológico and the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis; the Forestry Division and Wildlife Section of Trinidad; the Minesterio del Ambiente y Energia, Costa Rica; and the Office of the President of Guyana. P. Armstrong and E. Okonski kindly provided assistance in the laboratory. L. Hayek and S. Arnaud-Haond provided valuable advice on statistical analyses. D. Bolnik, S. Brady, D. Gotzek, D. Hillis, M. Singer, P. Ward, and two anonymous reviewers improved the manuscript with helpful comments. C.R. gratefully acknowledges financial support from Ernst Mayr grants (Museum of Comparative Zoology) and the Green Fund (Harvard University), a National Science Foundation (NSF) Doctoral Dissertation Improvement Grant (DEB-0808164), the Explorer's Club Exploration Fund, a Lewis and Clark Field Scholarship, and research grants from the Section of Integrative Biology and a Miller Endowed University Continuing Fellowship from the University of Texas at Austin; T.R.S. was supported by the NSF (DEB-0949689 and DEB-0431330), the Smithsonian Scholarly Studies Program, and the Smithsonian Restricted Endowments Fund; M.B. received support from the Fundação de Apoio à Pesquisa do Estado de São Paulo (2008/54386-9) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (476250/2008-0 and 304661/2009-0); M.V.B.G. and M.V. acknowledge the Bundesministerium für Bildung und Forschung (BMBF) and the Conselho National de Pesquisa e Tecnologia (BMBF01LT0014/CNPq 690018/00-2) for kindly providing funding for SHIFT Project ENV52-2; U.G.M. was funded by grants from the NSF (DEB-0639879, DEB-0110073, and DEB-998379) and the Wheeler Lost Pines Endowment. This study is a chapter of C.R.'s doctoral dissertation.

1. Barton N (1998) Evolutionary biology. The geometry of adaptation. *Nature* 395: 751–752.
2. Otto SP, Lenormand T (2002) Resolving the paradox of sex and recombination. *Nat Rev Genet* 3:252–261.
3. Avise JC (2008) *Clonality: The Genetics, Ecology, and Evolution of Sexual Abstinence in Vertebrate Animals* (Oxford Univ Press, New York).
4. Hamilton WD (1980) Sex versus non-sex versus parasite. *Oikos* 35:282–290.
5. Lively CM (2010) A review of Red Queen models for the persistence of obligate sexual reproduction. *J Hered* 101(Suppl 1):S13–S20.
6. Kondrashov AS (1982) Selection against harmful mutations in large sexual and asexual populations. *Genet Res* 40:325–332.
7. Muller H (1964) The relation of recombination to mutational advance. *Mutat Res* 1: 2–9.
8. Smith JM (1971) What use is sex? *J Theor Biol* 30:319–335.
9. Bell G (1982) *The Masterpiece of Nature: The Evolution and Genetics of Sexuality* (Croom Helm, London).
10. Maynard Smith J (1978) *The Evolution of Sex* (Cambridge Univ Press, Cambridge, UK).
11. Williams GC (1975) *Sex and Evolution* (Princeton Univ Press, Princeton, NJ).
12. Barton NH, Charlesworth B (1998) Why sex and recombination? *Science* 281: 1986–1990.
13. Burt A (2000) Perspective: Sex, recombination, and the efficacy of selection—Was Weismann right? *Evolution* 54:337–351.
14. Becks L, Agrawal AF (2010) Higher rates of sex evolve in spatially heterogeneous environments. *Nature* 468:89–92.
15. Fernández-Marín H, Zimmerman J, Wcislo W, Rehner S (2005) Colony foundation, nest architecture and demography of a basal fungus-growing ant, *Mycocepurus smithii* (Hymenoptera, Formicidae). *J Nat Hist* 39:1735–1743.
16. Himler AG, Caldera EJ, Baer BC, Fernández-Marín H, Mueller UG (2009) No sex in fungus-farming ants or their crops. *Proc Biol Sci* 276:2611–2616.
17. Rabeling C, et al. (2009) Thelytokous parthenogenesis in the fungus-gardening ant *Mycocepurus smithii* (Hymenoptera: Formicidae). *PLoS One* 4:e6781.
18. Verma S, Ruttner F (1983) Cytological analysis of the thelytokous parthenogenesis in the Cape honeybee (*Apis mellifera capensis* Escholtz). *Apidologie (Celle)* 14:41–57.
19. Baudry E, et al. (2004) Whole-genome scan in thelytokous-laying workers of the Cape honeybee (*Apis mellifera capensis*): Central fusion, reduced recombination rates and centromere mapping using half-tetrad analysis. *Genetics* 167:243–252.
20. Ito F, Touyama Y, Gotoh A, Kitahiro S, Billen J (2010) Thelytokous parthenogenesis by queens in the dacetine ant *Pyramica membranifera* (Hymenoptera: Formicidae). *Naturwissenschaften* 97:725–728.
21. Gotoh A, Billen J, Tsuji K, Sasaki T, Ito F (February 18, 2011) Histological study of the spermatheca in three thelytokous parthenogenetic ant species, *Pristomyrmex punctatus*, *Pyramica membranifera* and *Monomorium triviale* (Hymenoptera: Formicidae). *Acta Zool*, 10.1111/j.1463-6395.2010.00498.x.
22. Timmermans I, Hefetz A, Fournier D, Aron S (2008) Population genetic structure, worker reproduction and thelytokous parthenogenesis in the desert ant *Cataglyphis sabulosa*. *Heredity* 101:490–498.
23. Pearcy M, Goodisman MAD, Keller L (February 2, 2011) Sib mating without inbreeding in the longhorn crazy ant. *Proc Biol Sci*, 10.1098/rspb.2010.2562.
24. Keller L (2007) Uncovering the biodiversity of genetic and reproductive systems: Time for a more open approach. American Society of Naturalists E. O. Wilson Award winner address. *Am Nat* 169:1–8.
25. Heinze J (2008) The demise of the standard ant (Hymenoptera: Formicidae). *Myrmecol News* 11:9–20.
26. Fournier D, et al. (2005) Clonal reproduction by males and females in the little fire ant. *Nature* 435:1230–1234.
27. Pearcy M, Aron S, Doums C, Keller L (2004) Conditional use of sex and parthenogenesis for worker and queen production in ants. *Science* 306:1780–1783.
28. Pearcy M, Hardy O, Aron S (2006) Thelytokous parthenogenesis and its consequences on inbreeding in an ant. *Heredity* 96:377–382.
29. Kellner K, Heinze J (2011) Mechanism of facultative parthenogenesis in the ant *Platythyrea punctata*. *Evol Ecol* 25:77–89.
30. Leach IM, et al. (2009) Thelytoky in Hymenoptera with *Venturia canescens* and *Leptopilina clavipes* as case studies. *Lost Sex: The Evolutionary Biology of Parthenogenesis*, eds Schön I, Martens K, van Dijk P (Springer, Dordrecht, The Netherlands), pp 347–375.
31. Rey O, et al. (March 31, 2011) Meiotic recombination dramatically decreased in thelytokous queens of the little fire ant and their sexually produced workers. *Mol Biol Evol*, 10.1093/molbev/msr082.
32. Mackay WP, Maes JM, Rojas Fernández P, Luna G (2004) The ants of North and Central America: The genus *Mycocepurus* (Hymenoptera: Formicidae). *J Insect Sci* 4:27.
33. Kempf W (1963) A review of the ant genus *Mycocepurus* Forel, 1893 (Hymenoptera: Formicidae). *Stud Entomol* 6:417–432.
34. Arnaud-Haond S, Duarte CM, Alberto F, Serrão EA (2007) Standardizing methods to address clonality in population studies. *Mol Ecol* 16:5115–5139.
35. Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proc Natl Acad Sci USA* 105:5435–5440.
36. Foucaud J, et al. (2007) Sex and clonality in the little fire ant. *Mol Biol Evol* 24: 2465–2473.
37. Lattorff HMG, Moritz RFA, Fuchs S (2005) A single locus determines thelytokous parthenogenesis of laying honeybee workers (*Apis mellifera capensis*). *Heredity* 94: 533–537.
38. Fuyama Y (1986) Genetics of parthenogenesis in *Drosophila melanogaster*. II. Characterization of a gynogenetically reproducing strain. *Genetics* 114:495–509.
39. Normark BB (2003) The evolution of alternative genetic systems in insects. *Annu Rev Entomol* 48:397–423.
40. Decaestecker E, De Meester L, Mergeay J (2009) Cyclical parthenogenesis in *Daphnia*: Sexual versus asexual reproduction. *Lost Sex: The Evolutionary Biology of Parthenogenesis,* eds Schön I, Martens K, van Dijk P (Springer, Dordrecht, The Netherlands).
41. Suomalainen E, Saura A, Lokki J (1987) *Cytology and Evolution in Parthenogenesis* (CRC, Boca Raton, FL).
42. Zchori-Fein E, et al. (2001) A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proc Natl Acad Sci USA* 98:12555–12560.
43. Perlman SJ, Hunter MS, Zchori-Fein E (2006) The emerging diversity of *Rickettsia*. *Proc Biol Sci* 273:2097–2106.
44. Werren JH (1997) Biology of *Wolbachia*. *Annu Rev Entomol* 42:587–609.
45. Wenseleers T, Billen J (2000) No evidence for *Wolbachia*-induced parthenogenesis in the social Hymenoptera. *J Evol Biol* 13:277–280.
46. Dollo L (1893) Les lois de l'évolution. *Bulletin de la Société Belge de Géologie de Paléontologie et d'Hydrologie* 7:164–166.
47. Bull J, Charnov E (1985) On irreversible evolution. *Evolution* 39:1149–1155.
48. Chapman H, Houliston GJ, Robson B, Iline I (2003) A case of reversal: The evolution and maintenance of sexuals from parthenogenetic clones in *Hieracium pilosella*. *Int J Plant Sci* 164:719–728.
49. Domes K, Norton RA, Maraun M, Scheu S (2007) Reevolution of sexuality breaks Dollo's law. *Proc Natl Acad Sci USA* 104:7139–7144.
50. Cook JM (1993) Sex determination in the Hymenoptera: A review of models and evidence. *Heredity* 71:421–435.
51. Foucaud J, et al. (2006) Rare sexual reproduction events in the clonal reproduction system of introduced populations of the little fire ant. *Evolution* 60:1646–1657.
52. Foucaud J, Estoup A, Loiseau A, Rey O, Orivel J (2010) Thelytokous parthenogenesis, male clonality and genetic caste determination in the little fire ant: New evidence and insights from the lab. *Heredity* 105:205–212.
53. Balloux F, Lehmann L, de Meeûs T (2003) The population genetics of clonal and partially clonal diploids. *Genetics* 164:1635–1644.
54. De Meeûs T, Balloux F (2005) *F*-statistics of clonal diploids structured in numerous demes. *Mol Ecol* 14:2695–2702.
55. Halkett F, Simon JC, Balloux F (2005) Tackling the population genetics of clonal and partially clonal organisms. *Trends Ecol Evol* 20:194–201.
56. Arnaud-Haond S, Belkhir K (2007) GENCLONE: A computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Mol Ecol Notes* 7:15–17.
57. Conner JK, Hartl DL (2004) *A Primer of Ecological Genetics* (Sinauer, Sunderland, MA).
58. Peakall R, Smouse PE (2006) GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6:288–295.
59. Lewis P, Zaykin D (2002) GDA (Genetic Data Analysis). http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php.
60. Jombart T, Pontier D, Dufour AB (2009) Genetic markers in the playground of multivariate analysis. *Heredity* 102:330–341.
61. Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. *BMC Genet* 11:94.
62. Lessa EP (1990) Multidimensional analysis of geographic genetic structure. *Syst Biol* 39:242–252.
63. Heady R, Lucas J (2007) PERMAP: Perceptual MAPping Software (Univ of Louisiana at Lafayette). http://www.ucs.louisiana.edu/~rbh8900.
64. Rabeling C, Bacci M (2010) A new workerless inquiline in the Lower Attini (Hymenoptera: Formicidae), with a discussion of social parasitism in fungus-growing ants. *Syst Entomol* 35:379–392.
65. Kass RE, Raftery AE (1995) Bayes factors. *J Am Stat Assoc* 90:773–795.
66. Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldrey JL (2004) Bayesian phylogenetic analysis of combined data. *Syst Biol* 53:47–67.
67. Brown JM, Lemmon AR (2007) The importance of data partitioning and the utility of Bayes factors in Bayesian phylogenetics. *Syst Biol* 56:643–655.
68. Posada D, Crandall KA (2001) Selecting the best-fit model of nucleotide substitution. *Syst Biol* 50:580–601.
69. Posada D, Crandall KA (1998) MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14:817–818.
70. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
71. Rambaut A, Drummond A (2007) Tracer v1.5. http://tree.bio.ed.ac.uk/software/tracer.
72. Zwickl D (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation (Univ of Texas at Austin). http://garli.googlecode.com.
73. Drummond AJ, Nicholls GK, Rodrigo AG, Solomon W (2002) Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data. *Genetics* 161:1307–1320.
74. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with confidence. *PLoS Biol* 4:e88.
75. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7:214.
76. Brady SG, Schultz TR, Fisher BL, Ward PS (2006) Evaluating alternative hypotheses for the early evolution and diversification of ants. *Proc Natl Acad Sci USA* 103:18172–18177.

Supporting Information

Rabeling et al. 10.1073/pnas.1105467108

SI Materials and Methods

Population-Genetic Analyses. ***Population sampling.*** The known distribution range of *Mycocepurus smithii* includes Latin America from northern Mexico to northern Argentina and many Caribbean islands (1–6). To test for asexuality in *M. smithii*, we sampled populations throughout the geographic range between 2003 and 2010 (Table S1). Previous studies demonstrated that *M. smithii* colonies could be either mono- or polygynous, meaning that a single colony could include either a single or multiple reproductively active queens (7–9). Preliminary genotyping of sampled individuals revealed that in some populations more than a single multilocus genotype was present; however, queen and offspring genotypes were genetically identical. Hence, our working hypothesis was that *M. smithii* queens produce workers and queens clonally, either via apomixis or automixis. We attempted to sample entire colonies of *M. smithii* through careful nest excavations, including whenever possible workers, brood, and a reproductively active queen(s). In addition to nest excavations, workers were collected from nest entrances. Scooping up nest entrances with a knife proved to be an efficient way to collect workers because foragers accumulate in a tiny circular chamber below the entrance (7, 10).

Microsatellite development. To characterize *M. smithii* colonies and populations genetically, we developed 12 highly variable short tandem repeat markers (microsatellites). For microsatellite development, genomic DNA was extracted from ∼100 *M. smithii* workers collected from a single population in Rio Claro, Brazil, with a QIAamp DNA Micro Kit (QIAGEN) to obtain ∼100 μg DNA. Genetic Identification Services enriched microsatellite libraries for four different motifs in parallel: CA, GA, AAC, and ATG. Pooled genomic DNA was partially restricted with the enzymes RsaI, HaeIII, BsrB1, PvuII, StuI, ScaI, and EcoRV. Size-selected fragments (300–750 bp) were linked to adapters containing a HindIII restriction site and then captured with magnetic beads. Fragments were ligated into the HindIII site of the plasmid pUC19. Plasmids were propagated in *Escherichia coli* DH5α and stored in 20% glycerol at −80 °C. Cells from the glycerol stock were spread on X-gal/isopropyl-β-D-thiogalactoside/ampicillin plates, picked after incubation, and heated to 100 °C for 10 min in 10 μL PCR Master Mix (1× PCR buffer, 30 nmol $MgCl_2$, 3 nmol each dNTP, 15 pmol M-13 cloning-site primers). Five microliters of polymerase solution (0.075 μL, 5 U Taq DNA polymerase, 0.5 μL 10× PCR buffer, 4.425 μL ddsH_2O) were added to amplify the insert using a PTC-200 Cycler (MJ Research) (94 °C for 3 min; 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 30 s; 72 °C for 4 min). Overall, 100 PCR products (25 for each of the CA, GA, AAC, and ATG libraries) were sequenced on an Applied Biosystems 3100 Genetic Analyzer using BigDye Terminator chemistry.

Twelve loci were chosen to represent a variety of variable repeat motifs, variable product sizes, and similar annealing temperatures, and were combined in four multiplex polymerase chain reactions (Table S7). Specific primers were designed with an optimal annealing temperature (T_m) of 56–58 °C, a GC content of ∼50%, and at least one GC clamp using the Primer3 web site (11).

Genotyping. DNA of single workers, queens, and spermatheca contents was extracted using a 10% Chelex solution (Sigma-Aldrich). Spermatheca contents were extracted following the methodology outlined in Rabeling et al. (8). One microliter of DNA extract was used per 10 μL PCR and amplified using the following conditions: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 90 s, 72 °C for 60 s; 72 °C for 10 min. Using the multiplex PCR, we examined allelic variation within each locus by genotyping 1,930 *M. smithii* samples, yielding a total of 106 alleles across the 12 loci (range = 2–15 alleles per locus; Table S7). The number of alleles per locus per individual never exceeded two, indicating that *M. smithii* females are diploid. Representatives of each multilocus genotype were genotyped twice, using the same DNA extract, and scored blindly to minimize the possibility of erroneously assigning incorrect genotypes to the individuals.

For fragment analysis, 1 μL of PCR product was mixed with 8 μL of HiDi (Applied Biosystems) and 1.5 μL of cheaply amplified size standards using the following primer/ladder sizes: ROX F1, ROX 104, ROX 150, ROX 200, ROX 253, ROX 305, and ROX 424 (12). PCR products were analyzed on an Applied Biosystems 3100 Genetic Analyzer and alleles were scored using GeneScan v3.5 (Applied Biosystems) and GeneMarker v1.5 (SoftGenetics).

Statistical analyses. The goals of the population-genetic analyses were to determine (*i*) whether *M. smithii* is obligately or facultatively asexual, (*ii*) the cytogenetic mechanism underlying parthenogenesis in reproductive females, and (*iii*) the genetic structure and diversity within and among asexual and sexual colonies and populations. According to preliminary analyses performed on laboratory and field colonies, our hypothesis was that workers and queens of a single colony exhibited repeated multilocus genotypes (MLGs). The genotype-to-individual ratio (G:N ratio) is a simple measure for identifying clonality, with ratios ranging from 0 to 1 (13). A value close to 0 is characteristic of a strictly clonal colony/population in which all individuals share the same genotype, whereas a value of 1 is characteristic of a population in which all individuals have distinct genotypes, as expected under sexual reproduction and genetic recombination (Table S1). Because ants are social insects and live in colonies, we devised a second, colony-level measure of asexuality: the genotype-to-colony (G:C) ratio (the number of genotypes observed divided by the number of colonies screened). A value of 1 indicates that a single multilocus genotype was identified in each colony and all colonies were different from each other, as expected under clonal reproduction by a single queen; values between 0 and 1 indicate some sharing of genotypes between different colonies; and values greater than 1 indicate increased genetic diversity within colonies, suggesting either the presence of multiple genetically distinct reproductives in a colony or genetic recombination (Table S1).

Scoring repeated multilocus genotypes of multiple colonies per population revealed that MLGs could differ by only a single allele. These minor differences could either be due to "somatic" mutations or to scoring errors or, alternatively, slightly different MLGs could represent independent asexual lineages that originated separately from a sexually reproducing ancestral population (14) (Table S3). We therefore distinguished between slightly different MLGs belonging to the same asexual lineage, or clone, and slightly different MLGs that belong to the same clone and arose via mutations or scoring errors (13, 14). First, as recommended in Arnaud-Haond et al. (14), we identified MLG pairs in asexual populations with very low genetic distances, as indicated by a small peak in the frequency distribution of genetic distances. Then we calculated p_{sex} (equation 3 in ref. 14) using the software GENCLONE 2.0 (15) to estimate the probability that identical multilocus genotypes arose from independent sexual events or that they belonged to the same clone. If the probability was lower than the implemented threshold value ($\alpha = 0.01$), then identical MLGs were regarded as belonging to the same asexual lineage or clone. In our analysis, we first excluded all identical MLGs, re-

sulting in a total of 57 unique MLGs. Of those 57 MLGs, 11 MLG pairs differed by only a single allele, reducing the number of independently derived asexual lineages to 46. Increasing the allele difference between MLG pairs to 2, 3, 4, 5, and 6 alleles further reduced the number of independently originated clones to 43, 41, 40, 39, and 38 clones, respectively.

Interestingly, seven MLG pairs, all of which came from colonies collected in the same population, differed only at a single locus in which one lineage was homozygous for a given locus and the other lineage was heterozygous (Table S3; Panchan B and C, Copan A and B, Remate A and B and Tikal A, Ocumare B and D, Ocumare B and C, Amigos A and C, Cuevas C and Simla B and C). Currently, we cannot distinguish whether this difference represents a transition from heterozygosity to homozygosity, which would be expected under automixis with central fusion and low recombination rates (16, 17), or whether it represents a case of gene conversion in an apomictic lineage.

We also measured the inbreeding coefficient of *M. smithii* colonies/populations, describing the maximum deviation from random mating and calculated as $F_{IS} = H[bar]_e - H[bar]_o/H[bar]_e$ (13, 14, 18, 19), using the software package Genetic Data Analysis (GDA) (20). Observed heterozygosity (H_o = number of heterozygosities/N) and expected heterozygosity [$H_e = 1 - \Sigma p_i^2$] were calculated using the software GENALEX 6 (21). F statistics and heterozygosities were calculated for each MLG and for each recombinant population separately. To avoid resampling of identical MLGs in recombinant populations, we included only a single representative of each genotype. The analysis of molecular variance was calculated with GENALEX 6 (21). Clonal diversity was calculated as $R = (G - 1)/(N - 1)$, with G representing the number of asexual lineages, or clones, and N representing the number of sampled multilocus genotypes (14).

To reveal the underlying population-genetic structure of sexual and asexual populations, we used a number of multivariate statistical methods (22, 23). Nonmetric multidimensional scaling (NMDS) analyses were used to identify the presence of genetic clusters. In GDA (20), we transformed the genetic variability described by the microsatellite data into a matrix of pairwise Nei's 1972 standard genetic distances (20, 24). The distance matrix was then used to identify clusters that best describe the observed genetic variability in a few dimensions (22, 25–27) using the software PERMAP 11.6 (28). To find a global minimum mapping solution, we used nonmetric ratio and error bounds with a 5% error bound, set the convergence rate control to small step size, and set the convergence limit control to high precision. The analysis was carried out for three dimensions. The 3D distribution of object coordinates was visualized with the software SYSTAT (Systat Software). To determine whether visually identified genetic clusters were significantly different from one another, we used a discriminate analysis of principal components (DAPC) (23) using SYSTAT. In addition, a principal component analysis (PCA) was used to cluster genotypes by genetic similarity, which was 77.55% for the first three principal components (first PC: 45.57%; second PC: 19.48%; third PC: 12.5%).

Breeding experiment. To provide experimental evidence for the cytogenetic mechanism underlying parthenogenetic reproduction, we conducted a laboratory breeding experiment. Six generations of reproductively active queens (n = 93) collected in 2001 in Gamboa, Panama, were raised in laboratory nests over a period of ~1 y (see ref. 29 for a description of the nest setup). Initially, we selected 30 alate virgin queens (five individuals from six colonies) for the breeding experiment. The queens' wings were removed, a procedure known to stimulate reproductive behavior. Each queen was provided with a piece of fungus garden, which was carefully screened to exclude existing eggs and larvae, and 10 sterile workers were added to each colony. As soon as the experimental colonies started producing sexual offspring (i.e., the next generation of virgin queens), those new gynes were separated to initiate the next generation of experimental colonies. After raising six generations of reproductive females from multiple maternal lineages, we genotyped all reproductive and alate queens using the microsatellites described above. All 93 individuals were genetically identical, representing the multilocus genotype Gamboa A (Tables S1, S2, and S3). Transitions from hetero- to homozygosity were not identified at any locus. Even though workers in laboratory and field colonies were never found to have functional ovaries (8), we dissected a subset of workers from the experimental colonies to determine whether workers contribute to colony reproduction. No worker reproduction was detected.

Phylogenetic Analyses. ***Taxon sampling.*** To test the monophyly of *M. smithii* and to infer intraspecific relationships between asexual and sexual populations, we conducted phylogenetic analyses of two distinct datasets. First, we analyzed a global dataset that included 84 *Mycocepurus* ingroup taxa, 32 of them *M. smithii* (Table S5), and 61 non-*Mycocepurus* attines, plus 26 nonattine myrmicine outgroups. The recently described social parasite *M. castrator* (30) was not included in this analysis. Second, we conducted a local ingroup-only analysis including 41 *M. smithii* taxa representing one individual from each of the genotyped populations (Table S5).

DNA sequencing. Given the small size of *Mycocepurus* workers, DNA was extracted from entire single specimens. For queens, only the mesosomas were extracted, using a QIAamp DNA Micro Kit (QIAGEN), diluting the extracted DNA in 40 μL ddH_2O. For the global dataset, we analyzed an alignment including a total of 2,319 bp, consisting of fragments from three single-copy nuclear genes—Elongation Factor 1-α F1 copy (EF1-α; 1,071 bp), Wingless (Wg; 405 bp), and Long Wavelength Rhodopsin (LW Rh; 456 bp)—and one mitochondrial gene—Cytochrome Oxidase I (COI; 387 bp). All data represent protein-coding (exon) sequences; introns of EF1-α, Wg, and LW Rh were excluded from the phylogenetic analysis because they could not be aligned confidently across ingroup and outgroup taxa. All ingroup sequence data were generated for this study; they do not contain missing fragments, except for the LW Rh sequence of *M. goeldii* 278, and were deposited in GenBank (Table S5). The outgroup sequences were acquired from published information (31) and lacked DNA sequence information for COI. The global alignment (including all in- and outgroup taxa) included 909 variable nucleotide positions of which 860 were parsimony-informative (Table S6).

For the local, *M. smithii*-only alignment, we obtained 1,515 bp of the 3′ section of the mitochondrial COI gene (1,173 bp), the t-RNA leucine region (t-RNA Leu; 72 bp), and the 5′ section of the Cytochrome Oxidase II gene (COII; 270 bp). The nontranscribed intergenic spacer, present in some other Attini (32), consists of the triplet TTA in *M. smithii*. All sequence data were translated into amino acid sequences to test for the presence of mitochondrial pseudogenes ("numts"), which have been reported in some Attini (33). The alignment contained 248 informative sites of which 169 were parsimony-informative (Table S6). Primers were modified from several sources and specifically designed for this study (Table S8). DNA sequences were aligned manually in MacClade v4.08 (34). The mitochondrial phylogram was studied both as an unrooted network and as a midpoint-rooted tree because a long branch separates the ingroup from the sister species of *M. smithii*, rendering the correct rooting of the *M. smithii* mitochondrial tree a difficult problem.

Data partitioning. Based on genes and on the variability of codon-position sites within each gene, following the recommended methodology outlined in Ward et al. (35), we partitioned the global dataset into 10 partitions: (*i*) first and second codon position of EF1-α, (*ii*) third position of EF1-α; (*iii–v*) first, second, and third positions of Wg; (*vi–viii*) first, second, and third positions of LW Rh; (*ix*) first and second position of COI, and (*x*) third

position of COI (Table S6). Best-fit models of sequence evolution were selected for each partition under the Akaike information criterion (AIC) (36) and hierarchical likelihood ratio tests (hLRTs) as calculated in MODELTEST v3.7 (37) (Table S6). When different models of sequence evolution were chosen by AIC and hLRT, the more complex model was implemented.

The local, *M. smithii*-only alignment was divided into two partitions. The first partition included the first and second positions of COI and COII and the tRNA leucine region; the second partition included the third positions of COI and COII (Table S6).

Bayesian phylogenetic inference. We conducted partitioned Bayesian analyses using MrBayes v3.1.2 (38) with nucmodel = 4by4 and samplefreq = 500. All parameters, including branch-length rate multipliers, were unlinked across partitions except branch lengths and topology. All analyses were carried out using parallel processing (one chain per central processing unit) with eight chains per run and two runs per analysis (nruns = 2).

To address known problems with branch-length estimation in MrBayes (for example, 35, 39–42), we reduced the branch-length priors. In the global analyses, we used brlenspr = unconstrained: Exp(133.6081222) based on the procedure suggested in Brown et al. (39); in the local analyses, we set brlenspr = unconstrained:Exp (100). For the global analyses, moderately informative Dirichlet priors were specified for branch-length rate multipliers to reflect differences in evolutionary rates between first and second codon positions versus third codon position and between nuclear and mitochondrial genes. In local analyses, which used only two partitions, we set *prset ratepr = variable*. In both sets of analyses, we used the *props* command to increase the proposal rate from 1,000 to 10,000 and to decrease the Dirichlet alpha parameter from 500 to 250 for the rate multipliers (proposal mechanism 26 in MrBayes).

Burn-in and convergence were assessed using Tracer v1.5 (43) by examining potential scale reduction factor values in the MrBayes.stat output files, and by using Bayes factor comparisons of marginal likelihoods of pairs of runs in Tracer, which employs the weighted likelihood bootstrap estimator of Newton and Raftery (44) as modified by Suchard et al. (45), with SE estimated using 1,000 bootstrap pseudoreplicates.

Maximum likelihood analyses. Partitioned maximum likelihood (ML) analyses were carried out in GARLI 0.97.r737 (46) using parallel processing.

ML bootstrap analyses: For the global dataset, ML bootstrap analyses consisted of 1,000 pseudoreplicates; for the local dataset 1,500 pseudoreplicates, both deviating from default settings as follows: genthreshfortopoterm = 5000; scorethreshforterm = 0.10; startoptprec = 0.5; minoptprec = 0.01; numberofprecreductions = 1; treerejectionthreshold = 20.0; topoweight = 0.01; brlenweight = 0.002.

ML "best-tree" analyses: For both the global and local datasets, ML best-tree analyses consisted of 100 searches, deviating from the default settings as follows: topoweight = 0.01; brlenweight = 0.002. The best tree for the global analysis had a score of lnL = −22,005.643; for the local analysis, lnL = −5,141.168.

In all analyses, the value for modweight was calculated as 0.0005× (number of subsets + 1) (46).

Constraint analyses. To test for single versus multiple independent origins of asexuality, sexual and asexual populations were topologically constrained to occupy the opposite sides of a single branch in constrained ML and Bayesian analyses of the ingroup-only mitochondrial data. The marginal likelihoods of the resulting phylogenies were compared with those obtained in unconstrained analyses using Bayes factors (47–50). Bayes factors (BF) were calculated as the ratio of marginal likelihoods from constrained versus unconstrained analyses (i.e., the differences in −lnL) to produce the test statistic 2ln(BF). In the case of the ML analysis comparison, the marginal likelihoods used were point estimates from best-tree analyses as described above; for Bayesian analyses, they were post-burn-in harmonic means of the sampled likelihoods (48, 49, 51) estimated in Tracer v1.5 (43), which employs the weighted likelihood bootstrap estimator of Newton and Raftery (44) as modified by Suchard et al. (45), with SE estimated using 1,000 bootstrap pseudoreplicates. Within the Bayesian statistical framework (47), the resulting test statistics, 137.82 (ML) and 124.1 (Bayesian), indicate that the constrained topologies are significantly worse fitting to the data than the unconstrained topologies, thus providing additional support to a hypothesis of multiple origins of asexuality in *M. smithii*.

Divergence-dating analysis. We used a Bayesian relaxed clock uncorrelated lognormal approach implemented in the program BEAST v1.4.8 with a Yule process as the tree prior (52–54). As the model of sequence evolution, we used GTR+I+Γ with three partitions (codons 1, 2, and 3). To provide identical gene sampling for in- and outgroup taxa, the mitochondrial DNA sequence data were excluded from the divergence-dating analysis and only the nuclear DNA data were retained. Substitution model, rate heterogeneity, and base frequencies were unlinked across codon positions. The root node was assigned to the so-called core myrmicines, a well-supported clade identified in Brady et al. (55), and three taxa, one *Hylomyrma* (note: *Hylomyrma* was erroneously named *Pogonomymex* in ref. 31) and two *Myrmica* species, were used to root the tree. According to the estimates obtained by Brady et al. (55), the root node was given a normal age prior distribution (mean = 73.5, SD = 4.5). Lognormal age prior distributions were assigned to three internal nodes, the *Apterostigma pilosum*-complex stem group (mean = 2.7, SD = 0.3, zero offset 15.0), the *Cyphomyrmex rimosus* stem group (mean = 2.2, SD = 0.5, zero offset 15.0), and the *Trachymyrmex* stem group (mean = 1.5, SD = 0.5, zero offset 15.0), taking into account fungus-growing ant fossils and following the methodology outlined in Schultz and Brady (31). Two fossils, *Trachymyrmex primaevus* and a putative leafcutter ant fossil depicted in Grimaldi and Engel (56), were not included in our analysis because the placement of these fossils within the tribe Attini is uncertain (31). Markov chain Monte Carlo runs were run for 10 million generations, and the first 1 million generations were discarded as burn-in. Searches achieved sufficient mixing, as indicated by high effective sample size values for all parameters, by plateaus in divergence time estimates over generations after burn-in, and by repeatability of results over 10 independent runs. The results from all independent runs were combined in Tracer v1.5 and reported as mean values ± 95% upper and lower boundaries (43).

To use consistent in- and outgroup taxon sampling and to prevent estimating disproportionally old root nodes for the ingroup clades, only a single representative of each *Mycocepurus* species was used during the divergence-dating analysis, except for *M. smithii*, for which two genetically divergent individuals were included to estimate the crown-group age (i.e., most recent possible origin) for the species. In addition, to test whether the mitochondrial sequence data (present for the *Mycocepurus* ingroup but not for the outgroup taxa) had an effect on the outcome of the divergence-dating analyses, 10 parallel runs were executed, including and excluding COI sequences. The divergence estimates of the root node and internal nodes were significantly older for the dataset including mitochondrial sequence data. Hence, the mitochondrial data were discarded for our final divergence-dating analysis, and only the sequence information for single-copy nuclear genes was retained, providing identical gene sampling for in- and outgroup taxa.

1. Wheeler WM (1907) The fungus-growing ants of North America. *Bull Am Mus Nat Hist* 23:669–807.
2. Kempf W (1963) A review of the ant genus *Mycocepurus* Forel, 1893 (Hymenoptera: Formicidae). *Stud Entomol* 6:417–432.
3. Kempf WW (1972) Catálogo abreviado das formigas da região Neotropical. *Stud Entomol* 15:3–344.
4. Weber NA (1972) *Gardening Ants. The Attines* (Memoirs Am Philos Soc, Philadelphia).
5. Brandão CRF (1991) Adendos ao catálogo abreviado das formigas da região Neotropical (Hymenoptera: Formicidae). *Rev Bras Entomol* 35:319–412.
6. Mackay WP, Maes JM, Rojas Fernández P, Luna G (2004) The ants of North and Central America: The genus *Mycocepurus* (Hymenoptera: Formicidae). *J Insect Sci* 4:27.
7. Rabeling C, Verhaagh M, Engels W (2007) Comparative study of nest architecture and colony structure of the fungus-growing ants, *Mycocepurus goeldii* and *M. smithii*. *J Insect Sci* 7:40.
8. Rabeling C, et al. (2009) Thelytokous parthenogenesis in the fungus-gardening ant *Mycocepurus smithii* (Hymenoptera: Formicidae). *PLoS One* 4:e6781.
9. Eidmann H (1936) Ökologisch-faunistische Studien an südbrasilianischen Ameisen. *Arb Physiol Angew Entomol Berlin-Dahlem* 3:81–114.
10. Fernández-Marín H, Zimmerman J, Wcislo W, Rehner S (2005) Colony foundation, nest architecture and demography of a basal fungus-growing ant, *Mycocepurus smithii* (Hymenoptera, Formicidae). *J Nat Hist* 39:1735–1743.
11. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132:365–386.
12. DeWoody JA, et al. (2004) Universal method for producing ROX-labeled size standards suitable for automated genotyping. *Biotechniques* 37:348–352.
13. Halkett F, Simon JC, Balloux F (2005) Tackling the population genetics of clonal and partially clonal organisms. *Trends Ecol Evol* 20:194–201.
14. Arnaud-Haond S, Duarte CM, Alberto F, Serrão EA (2007) Standardizing methods to address clonality in population studies. *Mol Ecol* 16:5115–5139.
15. Arnaud-Haond S, Belkhir K (2007) GENCLONE: A computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Mol Ecol Notes* 7:15–17.
16. Pearcy M, Hardy O, Aron S (2006) Thelytokous parthenogenesis and its consequences on inbreeding in an ant. *Heredity* 96:377–382.
17. Rey O, et al. (March 31, 2011) Meiotic recombination dramatically decreased in thelytokous queens of the little fire ant and their sexually produced workers. *Mol Biol Evol*, 10.1093/molbev/msr082.
18. Balloux F, Lehmann L, de Meeûs T (2003) The population genetics of clonal and partially clonal diploids. *Genetics* 164:1635–1644.
19. De Meeûs T, Balloux F (2005) *F*-statistics of clonal diploids structured in numerous demes. *Mol Ecol* 14:2695–2702.
20. Lewis P, Zaykin D (2002) GDA (Genetic Data Analysis). http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php.
21. Peakall R, Smouse PE (2006) GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6:288–295.
22. Jombart T, Pontier D, Dufour AB (2009) Genetic markers in the playground of multivariate analysis. *Heredity* 102:330–341.
23. Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. *BMC Genet* 11: 94.
24. Nei M (1972) Genetic distance between populations. *Am Nat* 106:283–292.
25. Lessa EP (1990) Multidimensional analysis of geographic genetic structure. *Syst Biol* 39:242–252.
26. Novembre J, Stephens M (2008) Interpreting principal component analyses of spatial population genetic variation. *Nat Genet* 40:646–649.
27. Ross KG, Gotzek D, Ascunce MS, Shoemaker DDW (2010) Species delimitation: A case study in a problematic ant taxon. *Syst Biol* 59:162–184.
28. Heady R, Lucas J (2007) PERMAP: Perceptual MAPping Software (Univ of Louisiana at Lafayette). http://www.ucs.louisiana.edu/~rbh8900.
29. Schultz T (1993) Stalking the wild attine. *Notes Undergr* 8:7–10.
30. Rabeling C, Bacci M (2010) A new workerless inquiline in the Lower Attini (Hymenoptera: Formicidae), with a discussion of social parasitism in fungus-growing ants. *Syst Entomol* 35:379–392.
31. Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proc Natl Acad Sci USA* 105:5435–5440.
32. Wetterer JK, Schultz TR, Meier R (1998) Phylogeny of fungus-growing ants (Tribe Attini) based on mtDNA sequence and morphology. *Mol Phylogenet Evol* 9:42–47.
33. Martins J, Jr, et al. (2007) Nuclear mitochondrial-like sequences in ants: Evidence from *Atta cephalotes* (Formicidae: Attini). *Insect Mol Biol* 16:777–784.
34. Maddison DR, Maddison WP (2000) *MacClade 4* (Sinauer, Sunderland, MA).
35. Ward PS, Brady SG, Fisher BL, Schultz TR (2010) Phylogeny and biogeography of dolichoderine ants: Effects of data partitioning and relict taxa on historical inference. *Syst Biol* 59:342–362.
36. Posada D, Crandall KA (2001) Selecting the best-fit model of nucleotide substitution. *Syst Biol* 50:580–601.
37. Posada D, Crandall KA (1998) MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14:817–818.
38. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
39. Brown JM, Hedtke SM, Lemmon AR, Lemmon EM (2010) When trees grow too long: Investigating the causes of highly inaccurate Bayesian branch-length estimates. *Syst Biol* 59:145–161.
40. Marshall DC, Simon C, Buckley TR (2006) Accurate branch length estimation in partitioned Bayesian analyses requires accommodation of among-partition rate variation and attention to branch length priors. *Syst Biol* 55:993–1003.
41. Marshall DC (2010) Cryptic failure of partitioned Bayesian phylogenetic analyses: Lost in the land of long trees. *Syst Biol* 59:108–117.
42. Spinks PQ, Shaffer HB (2009) Conflicting mitochondrial and nuclear phylogenies for the widely disjunct Emys (Testudines: Emydidae) species complex, and what they tell us about biogeography and hybridization. *Syst Biol* 58:1–20.
43. Rambaut A, Drummond A (2007) Tracer v1.5. http://tree.bio.ed.ac.uk/software/tracer.
44. Newton MA, Raftery AE (1994) Approximate Bayesian inference with the weighted likelihood bootstrap. *J R Stat Soc, B* 56:3–48.
45. Suchard MA, Weiss RE, Sinsheimer JS (2001) Bayesian selection of continuous-time Markov chain evolutionary models. *Mol Biol Evol* 18:1001–1013.
46. Zwickl D (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. PhD dissertation (Univ of Texas at Austin). http://garli.googlecode.com.
47. Kass RE, Raftery AE (1995) Bayes factors. *J Am Stat Assoc* 90:773–795.
48. Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldrey JL (2004) Bayesian phylogenetic analysis of combined data. *Syst Biol* 53:47–67.
49. Brown JM, Lemmon AR (2007) The importance of data partitioning and the utility of Bayes factors in Bayesian phylogenetics. *Syst Biol* 56:643–655.
50. Rabeling C, Brown JM, Verhaagh M (2008) Newly discovered sister lineage sheds light on early ant evolution. *Proc Natl Acad Sci USA* 105:14913–14917.
51. Brandley MC, Schmitz A, Reeder TW (2005) Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid lizards. *Syst Biol* 54:373–390.
52. Drummond AJ, Nicholls GK, Rodrigo AG, Solomon W (2002) Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data. *Genetics* 161:1307–1320.
53. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with confidence. *PLoS Biol* 4:e88.
54. Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7:214.
55. Brady SG, Schultz TR, Fisher BL, Ward PS (2006) Evaluating alternative hypotheses for the early evolution and diversification of ants. *Proc Natl Acad Sci USA* 103:18172–18177.
56. Grimaldi DA, Engel MS (2005) *Evolution of the Insects* (Cambridge Univ Press, New York).
57. Simon C, et al. (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann Entomol Soc Am* 87:651–701.
58. Ward PS, Downie DA (2005) The ant subfamily Pseudomyrmecinae (Hymenoptera: Formicidae): Phylogeny and evolution of big-eyed arboreal ants. *Syst Entomol* 30: 310–335.
59. Abouheif E, Wray GA (2002) Evolution of the gene network underlying wing polyphenism in ants. *Science* 297:249–252.
60. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3:294–299.
61. Hasegawa E, Tinaut A, Ruano F (2002) Molecular phylogeny of two slave-making ants: *Rossomyrmex* and *Polyergus* (Hymenoptera: Formicidae). *Ann Zool Fenn* 39:267–271.
62. Artiss T, Schultz TR, Polhemus DA, Simon C (2001) Molecular phylogenetic analysis of the dragonfly genera *Libellula*, *Ladona*, and *Plathemis* (Odonata: Libellulidae) based on mitochondrial cytochrome oxidase I and 16S rRNA sequence data. *Mol Phylogenet Evol* 18:348–361.
63. Wetterer JK, Schultz TR, Meier R (1998) Phylogeny of fungus-growing ants (Tribe Attini) based on mtDNA sequence and morphology. *Mol Phylogenet Evol* 9:42–47.

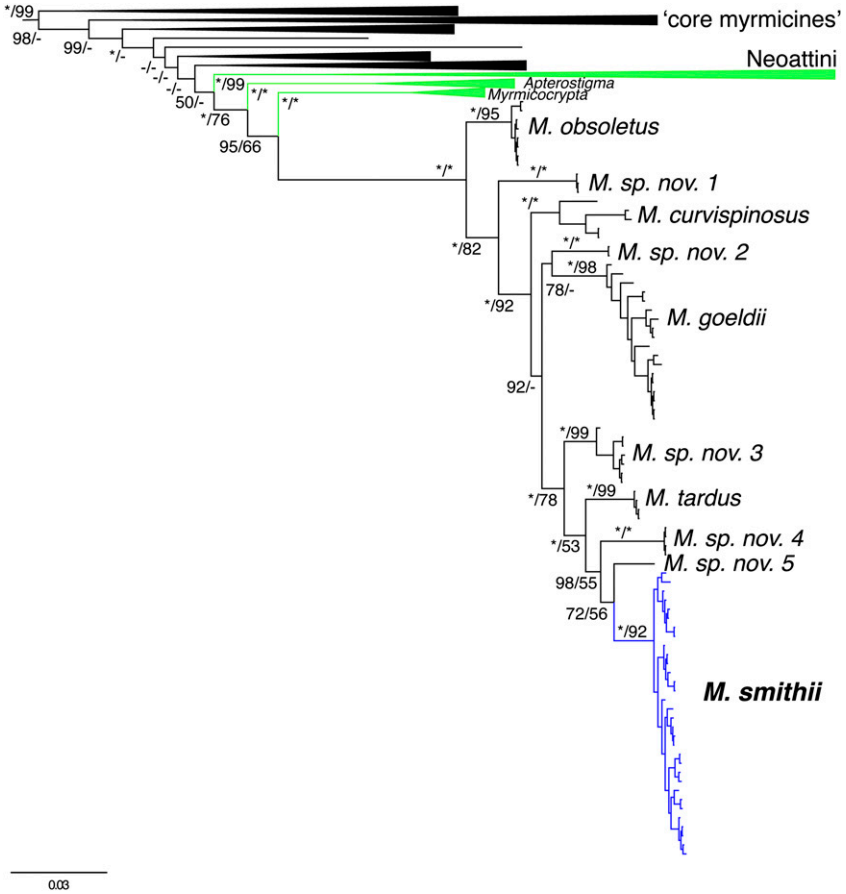

Fig. S1. Phylogram of the fungus-growing ant genus *Mycocepurus* generated by a Bayesian analysis of three nuclear protein-coding genes and one mitochondrial gene. Bayesian posterior probabilities (×100) (BPP) and ML bootstrap proportions (MLBP) are indicated as BPP/MLBP; values of BPP = 100 or MLBP = 100 are indicated by an asterisk. Relationships between 87 outgroup taxa are collapsed to better depict relationships among *Mycocepurus* species. (Scale bar, number of substitutions per site.)

Table S1. *Mycocepurus smithii* populations sampled across Latin America

Country	State	Locality/population	Number of individuals	Number of queens	Number of workers	Number of unique genotypes	Number of colonies	Genotype: colony ratio	Genotype: individual ratio
Argentina	Chaco	Pampa del Indio	5	0	5	1	1	1	0.2
Argentina	Misiones	Iguazú National Park	22	0	22	1	3	0.33	0.05
Brazil	***Amazonas***	***Caldeirão***	***243****	***5***	***234***	***173***	***11***	***15.72***	***0.71***
Brazil	Amazonas	Manaus	263	13	250	3	36	0.08	0.01
Brazil	***Amazonas***	***Parintins***	***7***	***0***	***7***	***5***	***1***	***5***	***0.71***
Brazil	Amazonas	Reserva Ducke	8	0	8	1	1	1	0.13
Brazil	Amazonas	Santa Rita	15	0	15	1	3	0.33	0.07
Brazil	***Amazonas***	***São Gabriel da Cachoeira***	***8***	***0***	***8***	***8***	***1***	***8***	***1***
Brazil	Pará	Alter do Chão	9	0	9	1	1	1	0.1
Brazil	***Pará***	***Belém***	***25***	***0***	***25***	***24***	***3***	***8***	***0.96***
Brazil	Pará	Belterra	22	0	22	2	4	0.5	0.09
Brazil	São Paulo	Rio Claro	390	138[†]	252	2	59	0.03	0.01
Costa Rica	Limón	Cahuita	11	0	11	1	1	1	0.09
Costa Rica	Guanacaste	Lomas Barbudal	25	0	25	2	5	0.4	0.08
Costa Rica	Limón	Limón	28	10[‡]	18	1	2	0.5	0.04
Cuba		Cienfuegos	20	0	20	1	2	0.5	0.05
Guatemala	Peten	El Remate	45	0	45	2	9	0.22	0.04
Guatemala	Peten	Tikal	15	0	15	1	3	0.33	0.07
Guyana	Potaro-Siparuni	Paramakatoi	24	0	24	1	3	0.33	0.04
Honduras	Copán	Copán Archeological Museum	15	0	15	1	3	0.33	0.07
Honduras	Copán	Copán Ruinas	30	0	30	3	6	0.5	0.1
Mexico	Chiapas	El Panchan	30	0	30	4	6	0.67	0.13
Mexico	Chiapas	Palenque	10	0	10	2	2	1	0.2
Mexico	Nuevo León	Monterrey	50	0	50	1	6	0.17	0.02
Mexico	Tamaulipas	El Encino	35	0	35	1	5	0.2	0.03
Nicaragua	Matagalpa	El Tuma	25	0	25	1	5	0.2	0.04
Panama	Bocas del Toro	Bocas del Toro	33	0	33	1	4	0.25	0.03
Panama	Colon	Ft. Sherman	35	0	35	3	4	0.75	0.09
Panama	Colon	Gamboa (breeding experiment)	93	93[§]	0	1	2	0.5	0.01
Panama	Colon	Gamboa	20	0	20	2	1	2	0.1
Peru	Cusco	Huacaria	40	0	40	1	4	0.25	0.03
Peru	Cusco	Pilcopata	5	0	5	1	1	1	0.2
Peru	Loreto	Explorama Lodge, Iquitos	47	2	45	1	4	0.25	0.02
Peru	Madre de Dios	CICRA, Los Amigos	149	10	139	6	15	0.4	0.04
Trinidad		Las Cuevas	20	0	20	3	2	1.5	0.15
Trinidad		Arena Dam	20	0	20	1	2	0.5	0.05
Trinidad		Pierreville	20	0	20	1	1	1	0.05
Trinidad		Simla Research Station	18	1	17	3	2	1.5	0.17
Venezuela	Aragua	Ocumare de la Costa	40	0	40	5	8	0.63	0.13
Venezuela	Aragua	Rio Cumboto	10	0	10	3	2	1.5	0.3
Total		39 localities	1,930	272	1,654	276	234		

Number of individuals describes the sample total including queens and workers. Number of unique genotypes is the number of unique multilocus genotypes. Number of colonies corresponds to either the number of nest entrances or the number of chambers from which individuals were collected. The genotype:colony ratio describes the ratio between the number of genotypes and the number of sampled colonies (*SI Materials and Methods*). The genotype:individual ratio describes the ratio between the number of genotypes and the number of sampled individuals. A value of the genotype:individual ratio approaching 0 describes genetic uniformity within a colony; a value of 1 describes sexual reproduction under random mating. Recombining populations are italicized and highlighted in bold.

*Number of individuals includes the number of male mates estimated from the spermatheca content extracted and genotyped from four queens.

[†]A total of 12 of the 138 queens were reproductively active; the remaining 126 individuals were queen larvae.

[‡]All 10 queens were alates emerging from the maternal colony and were not reproductively active at the time of collection.

[§]All queens were raised in six consecutive generations in a breeding experiment in laboratory colonies and represent offspring from two colonies initially collected in close proximity in Gamboa, Panama.

Table S2. Sample size, observed and expected heterozygosity, and inbreeding coefficient of multilocus genotypes and recombinant populations (indicated by bold and italicized font)

Country	State	Locality/population	Clone	*n*	H_o	H_e	F_{IS}
Argentina	Chaco	Pampa del Indio	PampaA	5	0.667	0.333	−1
Argentina	Misiones	Iguazú National Park	IguazuA [6]	22	0.917	0.458	−1
Brazil	***Amazonas***	***Caldeirão***	***n/a***	***243 (173)***	***0.372***	***0.369***	***−0.009***
Brazil	Amazonas	Manaus	ManausA	5	0.667	0.333	−1
			ManausB [6]	5	0.917	0.458	−1
			ManausC [7]	253	0.417	0.208	−1
Brazil	***Amazonas***	***Parintins***	***n/a***	***7 (5)***	***0.650***	***0.398***	***−0.773***
Brazil	Amazonas	Reserva Ducke	DuckeA [7]	8	0.417	0.208	−1
Brazil	Amazonas	Santa Rita	RitaA	15	0.667	0.333	−1
Brazil	***Amazonas***	***São Gabriel da Cachoeira***	***n/a***	***8 (8)***	***0.365***	***0.315***	***−0.172***
Brazil	Pará	Alter do Chão	AlterA	9	0.75	0.375	−1
Brazil	***Pará***	***Belém***	***n/a***	***25 (24)***	***0.451***	***0.466***	***0.034***
Brazil	Pará	Belterra	BelterraA	17	0.417	0.208	−1
			BelterraB	5	0.667	0.333	−1
Brazil	São Paulo	Rio Claro	RioClaroA	295	0.5	0.25	−1
			RioClaroB	95	0.5	0.25	−1
Costa Rica	Limón	Cahuita	CahuitaA	11	0.333	0.167	−1
Costa Rica	Guanacaste	Lomas Barbudal	LomasA	20	0.833	0.417	−1
			LomasB	5	0.75	0.375	−1
Costa Rica	Limón	Limón	LimonA	28	0.583	0.292	−1
Cuba		Cienfuegos	CubaA [4]	20	0.667	0.333	−1
Guatemala	Peten	El Remate	RemateA [1]	35	0.417	0.208	−1
			RemateB	10	0.5	0.25	−1
Guatemala	Peten	Tikal	TikalA [1]	15	0.417	0.208	−1
Guyana	Potaro-Siparuni	Paramakatoi	ParamakatoiA	24	0.75	0.375	−1
Honduras	Copán	Copán Archeological Museum	MuseumA	15	0.583	0.292	−1
Honduras	Copán	Copán Ruinas	CopanA	9	0.25	0.125	−1
			CopanB	16	0.167	0.083	−1
			CopanC	5	0.5	0.25	−1
Mexico	Chiapas	El Panchan	PanchanA	15	0.5	0.25	−1
			PanchanB	5	0.667	0.333	−1
			PanchanC	5	0.583	0.292	−1
			PanchanD	5	0.5	0.25	−1
Mexico	Chiapas	Palenque	PalenqueA	5	0.5	0.25	−1
			PalenqueB	5	0.417	0.208	−1
Mexico	Nuevo Leon	Monterrey	MonterreyA	50	0.417	0.208	−1
Mexico	Tamaulipas	El Cielo	ElCieloA	35	0.5	0.25	−1
Nicaragua	Matagalpa	El Tuma	ElTumaA	25	0.667	0.333	−1
Panama	Bocas del Toro	Bocas del Toro	BocasA	33	0.333	0.167	−1
Panama	Colon	Ft. Sherman	ShermanA [5]	10	0.583	0.292	−1
			ShermanB	15	0.583	0.292	−1
			ShermanC [2]	10	0.583	0.292	−1
Panama	Colon	Gamboa (breeding experiment)	GamboaA [5]	93	0.583	0.292	−1
Panama	Colon	Gamboa	GamboaB [2]	13	0.583	0.292	−1
			GamboaC	7	0.583	0.292	−1
Peru	Cusco	Huacaria	HuacariaA	40	0.667	0.333	−1
Peru	Cusco	Pilcopata	PilcopataA	5	0.667	0.333	−1
Peru	Loreto	Explorama Lodge, Iquitos	IquitosA	47	0.5	0.375	−1
Peru	Madre de Dios	CICRA, Los Amigos	AmigosA	23	0.833	0.417	−1
			AmigosB	6	0.75	0.375	−1
			AmigosC	22	0.75	0.375	−1
			AmigosD	41	0.75	0.375	−1
			AmigosE	18	0.75	0.375	−1
			AmigosF	39	0.75	0.375	−1
Trinidad		Las Cuevas	CuevasA	10	0.25	0.125	−1
			CuevasB [3]	3	0.417	0.208	−1
			CuevasC [8]	7	0.5	0.25	−1
Trinidad		Arena Dam	ArenaDamA [3]	20	0.417	0.208	−1

Table S2. Cont.

Country	State	Locality/population	Clone	*n*	H_o	H_e	F_{IS}
Trinidad		Pierreville	PierrevilleA	20	0.667	0.333	−1
Trinidad		Simla Research Station	SimlaA [4]	2	0.667	0.333	−1
			SimlaB	8	0.583	0.292	−1
			SimlaC [8]	8	0.5	0.25	−1
Venezuela	Aragua	Ocumare de la Costa	OcumareA [3]	5	0.417	0.208	−1
			OcumareB	5	0.583	0.292	−1
			OcumareC	5	0.5	0.25	−1
			OcumareD	21	0.667	0.333	−1
			OcumareE	4	0.75	0.375	−1
Venezuela	Aragua	Rio Cumboto	CumbotoA	2	0.667	0.333	−1
			CumbotoB	3	0.667	0.333	−1
			CumbotoC	5	0.75	0.375	−1
Total (asexual)				1,647	0.589	0.671	0.123
Total (sexual)				283	0.387	0.458	0.154
Total (sexual and asexual)				1,930	0.430	0.545	0.210

Statistics are presented separately for each multilocus genotype in asexual populations. A total of eight identical multilocus genotypes is shared between colonies from different localities, which are indicated by numbers in square brackets. For recombinant populations, the number of multilocus genotypes is given in parentheses following the sample number; a single representative for each multilocus genotype was included to calculate the observed and expected heterozygosity and the inbreeding coefficient. n/a, not applicable.

Table S3. Genotypes for 66 multilocus genotypes of clonal *M. smithii* colonies/populations at 12 microsatellite loci

	A9		D117		A5		C6		C119		A6		C104		D11		B1		B4		C2		D8	
PampaA	145	161	202	202	246	257	259	265	123	126	117	117	216	219	288	291	098	114	205	205	236	236	165	177
IguazuA	145	147	202	218	238	242	246	259	117	120	111	113	210	213	288	291	098	106	205	207	236	236	174	177
ManausA	145	148	202	239	240	246	250	265	111	120	103	105	204	213	288	288	090	112	207	207	236	236	162	162
ManausB	145	147	202	218	238	242	246	259	117	120	111	113	210	213	288	291	098	106	205	207	236	236	174	177
ManausC	145	148	202	239	240	242	250	265	120	120	103	105	213	213	288	288	112	112	207	207	236	236	162	162
DuckeA	145	148	202	239	240	242	250	265	120	120	103	105	213	213	288	288	112	112	207	207	236	236	162	162
RitaA	135	147	202	205	242	250	253	265	114	117	103	111	207	210	288	288	114	118	207	207	236	236	174	174
AlterA	143	147	202	205	242	254	259	265	111	117	103	105	204	210	288	288	108	116	207	207	236	236	180	183
BelterraA	145	148	205	242	250	252	265	265	117	120	105	105	210	213	288	288	112	112	205	205	236	236	174	174
BelterraB	135	147	202	208	242	250	253	265	114	117	103	111	207	210	288	288	114	118	207	207	236	236	174	174
RioClaroA	141	141	218	236	252	257	265	271	123	123	111	119	216	216	288	288	114	124	205	205	236	236	174	177
RioClaroB	141	147	218	218	246	252	253	259	117	117	109	111	210	210	288	288	098	114	205	205	236	236	165	174
CahuitaA	135	135	196	202	245	245	259	259	117	117	103	115	210	210	288	291	112	130	207	207	230	230	174	174
LomasA	135	143	202	205	245	245	253	265	117	120	115	117	210	213	288	291	112	128	207	207	233	236	174	177
LomasB	135	143	202	205	245	245	253	265	117	120	109	117	210	213	288	291	112	128	207	207	236	236	174	177
LimonA	145	145	202	205	244	257	259	265	114	132	103	117	207	225	288	288	108	108	207	207	236	236	171	174
CubaA	143	143	202	205	250	250	259	265	114	120	111	111	207	213	285	291	108	112	207	207	233	236	171	174
RemateA	135	143	202	205	245	245	259	259	117	117	105	105	210	210	288	291	112	124	205	207	236	236	174	174
RemateB	135	143	202	205	245	245	259	259	117	117	105	107	210	210	288	291	112	124	205	207	236	236	174	174
TikalA	135	143	202	205	245	245	259	259	117	117	105	105	210	210	288	291	112	124	205	207	236	236	174	174
ParamakatoiA	145	150	202	205	250	257	253	265	114	120	101	111	207	213	288	288	090	112	207	207	236	236	168	174
MuseumA	143	148	205	205	245	250	253	268	111	117	115	115	204	210	288	291	122	122	207	207	230	233	174	174
CopanA	135	141	205	205	245	245	265	265	120	120	107	107	213	213	288	288	112	120	207	207	236	239	174	174
CopanB	135	141	205	205	245	245	265	265	120	120	107	107	213	213	288	288	120	120	207	207	236	239	174	174
CopanC	135	141	205	205	245	245	259	265	120	120	115	119	213	213	288	291	118	124	207	207	236	239	174	174
PanchanA	143	148	205	205	238	245	237	268	117	117	103	119	210	210	288	291	112	120	207	207	236	236	174	174
PanchanB	135	141	202	202	245	247	265	268	114	117	103	107	207	210	288	288	098	124	207	207	236	236	159	174
PanchanC	135	141	202	202	245	247	265	268	114	117	103	107	207	210	288	288	098	098	207	207	236	236	159	174
PanchanD	135	135	196	205	245	245	253	259	120	126	103	103	213	219	291	291	098	112	207	207	236	236	174	177
PalenqueA	143	148	205	205	238	245	259	268	117	117	103	119	210	210	288	291	112	120	207	207	236	236	174	174
PalenqueB	141	143	202	205	245	245	259	259	117	117	103	113	210	210	288	291	098	098	205	207	236	236	174	174
MonterreyA	143	143	202	208	245	245	253	259	120	123	103	103	213	216	288	288	112	130	207	207	239	239	174	174
ElCieloA	143	143	205	205	245	247	253	268	117	123	119	119	210	216	288	288	112	124	207	207	236	239	174	174
ElTumaA	137	141	202	205	245	245	250	265	117	123	115	123	210	216	288	291	112	122	207	207	236	236	174	174
BocasA	135	135	196	202	244	244	259	259	117	117	103	115	210	210	288	291	112	132	207	207	230	230	174	174
ShermanA	145	145	202	205	244	257	259	265	114	132	103	115	207	225	288	288	108	108	207	207	236	236	171	174
ShermanB	137	141	202	202	245	245	265	265	114	123	113	123	207	216	288	294	098	100	207	207	236	236	159	174
ShermanC	137	141	202	202	245	245	265	265	114	123	113	125	207	216	288	294	098	102	207	207	236	236	159	174
GamboaA	145	145	202	205	244	257	259	265	114	132	103	115	207	225	288	288	108	108	207	207	236	236	171	174
GamboaB	137	141	202	202	245	245	265	265	114	123	113	125	207	216	288	294	098	102	207	207	236	236	159	174
GamboaC	137	141	202	202	245	245	265	265	114	123	115	125	207	216	288	294	098	102	207	207	236	236	159	174
HuacariaA	145	165	202	202	246	246	250	265	111	120	111	121	204	213	288	291	090	090	205	205	236	242	174	177
PilcopataA	148	148	202	237	246	250	250	250	111	120	111	125	204	213	288	288	090	124	205	207	236	236	177	180
IquitosA	143	147	202	205	242	246	250	265	117	120	105	107	210	213	288	288	090	112	207	207	236	236	171	174
AmigosA	141	147	202	205	246	246	256	265	111	120	111	115	204	213	288	291	090	112	207	207	236	239	168	171
AmigosB	141	161	202	205	250	257	259	259	111	120	109	113	204	213	288	288	090	112	205	207	236	236	171	174

Table S3. Cont.

	A9		D117		A5		C6		C119		A6		C104		D11		B1		B4		C2		D8	
AmigosC	141	147	202	205	246	246	256	265	111	120	111	115	204	213	288	291	090	090	207	207	236	239	168	171
AmigosD	148	161	202	202	246	250	256	262	111	123	103	111	204	216	288	288	112	122	205	207	236	236	174	183
AmigosE	148	161	202	205	242	246	243	262	111	120	111	111	204	213	288	288	112	122	205	207	236	236	174	177
AmigosF	148	161	202	205	246	254	253	265	114	120	101	103	207	213	288	288	090	112	207	207	236	239	174	174
CuevasA	141	148	208	208	250	250	253	253	120	120	111	111	213	213	285	288	112	114	207	207	236	236	171	171
CuevasB	141	147	205	205	246	254	259	265	120	120	103	103	213	213	288	288	090	112	207	207	236	236	171	177
CuevasC	145	148	205	205	244	248	253	259	120	126	103	103	213	219	288	291	112	112	207	207	236	236	171	171
ArenaDamA	141	147	205	205	246	254	259	265	120	120	103	103	213	213	288	288	090	112	207	207	236	236	171	177
PierrevilleA	141	150	202	205	242	248	259	259	111	117	113	113	204	210	288	288	112	114	207	207	230	236	171	174
SimlaA	143	143	202	205	250	250	259	265	114	120	111	111	207	213	285	291	108	112	207	207	233	236	171	174
SimlaB	145	148	205	205	244	248	253	259	120	126	103	103	213	219	288	291	112	114	207	207	236	236	171	171
SimlaC	145	148	205	205	244	248	253	259	120	126	103	103	213	219	288	291	112	112	207	207	236	236	171	171
OcumareA	141	147	205	205	246	254	259	265	120	120	103	103	213	213	288	288	090	112	207	207	236	236	171	177
OcumareB	141	141	202	205	246	250	259	265	123	132	103	115	216	225	288	288	108	112	207	207	236	236	171	174
OcumareC	141	141	202	205	246	250	259	265	123	132	103	115	216	225	285	288	108	112	207	207	236	236	171	174
OcumareD	141	141	202	205	246	250	259	265	123	123	103	115	216	225	288	288	108	112	207	207	236	236	171	174
OcumareE	145	145	202	205	244	257	259	265	114	135	103	115	207	207	288	288	108	108	207	207	236	236	171	174
CumbotoA	145	145	202	205	244	257	259	265	114	129	103	115	207	222	288	288	108	112	207	207	236	236	171	174
CumbotoB	141	147	202	208	248	250	259	265	123	129	103	111	216	222	288	288	108	112	207	207	236	236	174	174
CumbotoC	161	161	202	208	250	257	246	265	111	120	111	117	204	213	288	288	108	124	207	207	236	242	171	174

The number of unique multilocus genotypes is 57, because 8 genotypes are shared among the following populations: Tikal A and Remate A, Gamboa B and Sherman C, Ocumare A and Arena Dam A and Cuevas B, Simla A and Cuba A, Gamboa A and Sherman A, Manaus B and Iguazu A, Ducke A and Manaus C, and Simla C and Cuevas C. Individuals of the Simla and Cuba, Manaus and Iguazu, Manaus and Ducke, and Ocumare and Arena Dam populations do not seem to be closely related based on their mitochondrial genotypes (Fig. 3). Therefore, we caution that identical multilocus genotypes may not in every case indicate common descent but in some cases result from convergent evolution, that is, fragments identical in size but not in sequence. Alternatively, mitochondrial heteroplasmy, the occurrence of multiple mitochondrial haplotypes within a single individual, is also consistent with the observed pattern. Future studies will have to explore this seemingly paradoxical result.

Table S4. Comparison of genotypes (allele sizes measured as base pairs) at 12 microsatellite loci from one selected sexual and one selected asexual population of *M. smithii*

Individuals	A9	A9	D117	D117	A5	A5	C6	C6	C119	C119	A6	A6	C104	C104	D11	D11	B1	B1	B4	B4	C2	C2	D8	D8
Sexual																								
Queen (1)	141	149	202	205	242	250	246	246	117	117	111	113	210	210	288	288	112	114	207	207	233	236	162	171
Queen (1)	143	143	202	202	242	252	246	256	117	117	103	113	210	210	288	288	112	112	205	205	233	236	171	174
Queen (1)	143	143	202	202	242	252	246	256	117	117	103	113	210	210	288	288	112	112	205	207	233	236	171	174
Queen (1)	143	149	202	202	250	252	246	256	117	117	103	111	210	210	288	288	112	112	205	207	233	236	171	174
Queen (1)	143	149	202	202	250	250	250	259	117	117	103	111	210	210	288	288	112	112	205	207	236	236	171	171
Male (1)	143		205		250		259		***120***		111		***213***		288		112		207		236		171	
Male (1)	143		205		242		259		117		103		210		288		112		205		236		162	
Male (1)	143		202		250		259		117		111		***207***		288		***108***		207		236		171	
Male (1)	***148***		202		242		246		117		111		210		288		112		207		236		171	
Worker (1)	143	143	202	205	250	250	259	259	117	***120***	103	111	210	***213***	288	288	112	112	207	207	236	236	171	171
Worker (1)	149	149	202	205	242	250	246	246	117	***120***	103	113	210	***213***	288	288	112	112	205	207	236	236	174	177
Worker (1)	143	143	202	205	250	250	250	259	117	***120***	111	111	210	***213***	288	288	112	112	207	207	236	236	171	171
Worker (1)	149	149	202	202	250	252	246	256	117	117	103	111	210	210	288	288	112	112	205	207	236	236	171	171
Worker (1)	143	143	202	202	242	252	246	256	117	117	103	113	210	210	288	288	112	112	207	207	233	236	171	171
Asexual																								
Queen (6)	141	141	218	236	253	257	265	271	123	123	111	119	216	216	288	288	114	124	204	204	236	236	174	177
Worker (168)	141	141	218	236	253	257	265	271	123	123	111	119	216	216	288	288	114	124	204	204	236	236	174	177
Brood (121)	141	141	218	236	253	257	265	271	123	123	111	119	216	216	288	288	114	124	204	204	236	236	174	177
Queen (6)	141	147	218	218	246	253	253	259	117	117	109	111	210	210	288	288	97	114	204	204	236	236	165	174
Worker (84)	141	147	218	218	246	253	253	259	117	117	109	111	210	210	288	288	97	114	204	204	236	236	165	174
Brood (5)	141	147	218	218	246	253	253	259	117	117	109	111	210	210	288	288	97	114	204	204	236	236	165	174

In the leftmost column, the number in parentheses following the caste/sex of the individual indicates the number of samples with the depicted genotype. Representative genotypes for a sexual population are from the Caldeirão population. "Male" genotypes were determined from the spermatheca content of inseminated queens. Paternal alleles appear as bold, italicized numbers. Worker genotypes of this sexual population were selected to represent a diversity of maternal and paternal alleles, including recombinant genotypes. Note that not all alleles present in the sperm were recovered in the worker genotypes and, conversely, workers carried a few alleles not detected in either queens or males, indicating that the allelic diversity present in this population was not sampled exhaustively. Samples representing the asexual population were collected in Rio Claro, São Paulo. In this asexual population, only two identical multilocus genotypes were found among 390 genotyped individuals representing queens, workers, and female brood; males are not known to occur in strictly asexual populations (8).

Table S5. *Mycocepurus* **taxa used for the phylogenetic analyses with GenBank accession numbers**

Species	Extraction code	Collector's code	Country	Sample locality	EF1-α F1 exon 1	EF1-α F1 exon 2	Wg exon 1	LW Rh exon 1	LW Rh exon 2	COI	tRNA Leu	COII
M. curvispinosus	M228	UGM0950612	Costa Rica	Parque Nacional Santa Rosa	JN054745	JN054829	JN055079	JN054913	JN054996	JN055163	n/a	n/a
M. curvispinosus	M285	AGH010405-01	Panama	Pipeline Rd. Km 6, Parque Nacional Soberanía	JN054746	JN054830	JN055080	JN054914	JN054997	JN055164	n/a	n/a
M. curvispinosus	M286	AGH010405-01	Panama	Pipeline Rd. Km 6, Parque Nacional Soberanía	JN054747	JN054831	JN055081	JN054915	JN054998	JN055165	n/a	n/a
M. curvispinosus	M296	E.Deulefent M2169	Colombia	Villa Roca	JN054748	JN054832	JN055082	JN054916	JN054999	JN055166	n/a	n/a
M. curvispinosus	M317	CR071221-09	Costa Rica	Lomas Barbudal	JN054749	JN054833	JN055083	JN054917	JN055000	JN055167	n/a	n/a
M. goeldii	M028	CR050121-05	Brazil	Pareci Novo, Rio Grande do Sul	JN054750	JN054834	JN055084	JN054918	JN055001	JN055168	n/a	n/a
M. goeldii	M145	MB050906-07	Brazil	Rio Claro, São Paulo	JN054751	JN054835	JN055085	JN054919	JN055002	JN055169	n/a	n/a
M. goeldii	M241	CR060903-01	Brazil	Manaus, Amazonas	JN054752	JN054836	JN055086	JN054920	JN055003	JN055170	n/a	n/a
M. goeldii	M263	CR060819-05	Brazil	Alter do Chão, Pará	JN054753	JN054837	JN055087	JN054921	JN055004	JN055171	n/a	n/a
M. goeldii	M278	J.Martins061011-05	Brazil	Júlio de Castilhos, Rio Grande do Sul	JN054754	JN054838	JN055088	n/a	n/a	JN055172	n/a	n/a
M. goeldii	M280	R.Feitosa061001	Brazil	Lizarda, Tocantins	JN054755	JN054839	JN055089	JN054922	JN055005	JN055173	n/a	n/a
M. goeldii	M281	Dietz&Silva041006	Brazil	Ponte Alta do Bom Jesus, Tocantins	JN054756	JN054840	JN055090	JN054923	JN055006	JN055174	n/a	n/a
M. goeldii	M299	CR070716-05	Brazil	Brasília, Distrito Federal	JN054757	JN054841	JN055091	JN054924	JN055007	JN055175	n/a	n/a
M. goeldii	M307	CR061002-02	Brazil	Rio Claro, São Paulo	JN054758	JN054842	JN055092	JN054925	JN055008	JN055176	n/a	n/a
M. goeldii	M328	UGM080921-01	Brazil	Estação Ecológica do Panga, Minas Gerais	JN054759	JN054843	JN055093	JN054926	JN055009	JN055177	n/a	n/a
M. goeldii	M329	UGM080928-01	Brazil	Piracanjuba, Goiás	JN054760	JN054844	JN055094	JN054927	JN055010	JN055178	n/a	n/a
M. goeldii	M330	UGM080929-02	Brazil	Jussara, Goiás	JN054761	JN054845	JN055095	JN054928	JN055011	JN055179	n/a	n/a
M. goeldii	M331	UGM081003-01	Brazil	Roadside nr. Cuiabá, Mato Grosso	JN054762	JN054846	JN055096	JN054929	JN055012	JN055180	n/a	n/a
M. goeldii	M333	CR081003-01	Brazil	Rio Claro, São Paulo	JN054763	JN054847	JN055097	JN054930	JN055013	JN055181	n/a	n/a
M. goeldii	M335	CR081003-04	Brazil	Rio Claro, São Paulo	JN054764	JN054848	JN055098	JN054931	JN055014	JN055182	n/a	n/a
M. goeldii	M337	CR081002-02	Brazil	Rio Claro, São Paulo	JN054765	JN054849	JN055099	JN054932	JN055015	JN055183	n/a	n/a
M. goeldii	M339	CR081002-07	Brazil	Rio Claro, São Paulo	JN054766	JN054850	JN055100	JN054933	JN055016	JN055184	n/a	n/a
M. goeldii	M340	CR060831-12	Brazil	Caldeirão, Amazonas	JN054767	JN054851	JN055101	JN054934	JN055017	JN055185	n/a	n/a
M. obsoletus	M243	CR060906-02	Brazil	Parintins, Amazonas	JN054768	JN054852	JN055102	JN054935	JN055018	JN055186	n/a	n/a
M. obsoletus	M249	CR060906-03	Brazil	Parintins, Amazonas	JN054769	JN054853	JN055103	JN054936	JN055019	JN055187	n/a	n/a
M. obsoletus	M255	CR060813-04	Brazil	Alter do Chão, Pará	JN054770	JN054854	JN055104	JN054937	JN055020	JN055188	n/a	n/a
M. obsoletus	M256	CR060813-06	Brazil	Alter do Chão, Pará	JN054771	JN054855	JN055105	JN054938	JN055021	JN055189	n/a	n/a
M. obsoletus	M260	CR060816-06	Brazil	Alter do Chão, Pará	JN054772	JN054856	JN055106	JN054939	JN055022	JN055190	n/a	n/a
M. obsoletus	M266	CR060908-01	Brazil	Maranhão, Amazonas	JN054773	JN054857	JN055107	JN054940	JN055023	JN055191	n/a	n/a
M. obsoletus	M300	CR070717-01	Brazil	Brasília, Distrito Federal	JN054774	JN054858	JN055108	JN054941	JN055024	JN055192	n/a	n/a
M. obsoletus	M314	SCC081112-01	Brazil	Brasília, Distrito Federal	JN054775	JN054859	JN055109	JN054942	JN055025	JN055193	n/a	n/a
M. smithii	M070	CR050318-03	Cuba	Cienfuegos	JN054776	JN054860	JN055110	JN054943	JN055026	JN055231	JN055313	JN055272
M. smithii	M071	CR050318-03	Cuba	Cienfuegos	JN054777	JN054861	JN055111	JN054944	JN055027	JN055194	n/a	n/a

Table S5. Cont.

Species	Extraction code	Collector's code	Country	Sample locality	EF1-α F1 exon 1	EF1-α F1 exon 2	Wg exon 1	LW Rh exon 1	LW Rh exon 2	COI	tRNA Leu	COII
M. smithii	M109	CR040613-01	Peru	Explorama Lodge, Iquitos	JN054778	JN054862	JN055112	JN054945	JN055028	JN055232	JN055314	JN055273
M. smithii	M110	CR040613-02	Peru	Explorama Lodge, Iquitos	JN054779	JN054863	JN055113	JN054946	JN055029	JN055233	JN055315	JN055274
M. smithii	M129	UGM950107-07	Trinidad	Simla Research Station	n/a	n/a	n/a	n/a	n/a	JN055234	JN055316	JN055275
M. smithii	M131	UGM950112-09	Trinidad	Pierreville	n/a	n/a	n/a	n/a	n/a	JN055235	JN055317	JN055276
M. smithii	M132	UGM950114-08	Trinidad	Arena Dam	JN054780	JN054864	JN055114	JN054947	JN055030	JN055236	JN055318	JN055277
M. smithii	M152	UGM960116-01	Panama	Canal Zone	n/a	n/a	n/a	n/a	n/a	JN055237	JN055319	JN055278
M. smithii	M170	UGM950616-01	Costa Rica	Limón	n/a	n/a	n/a	n/a	n/a	JN055238	JN055320	JN055279
M. smithii	M175	TRS960416-12	Guyana	Paramakatoi	JN054781	JN054865	JN055115	JN054948	JN055031	JN055239	JN055321	JN055280
M. smithii	M182	TRS960428-22	Panama	Ft. Sherman	JN054782	JN054866	JN055116	JN054949	JN055032	JN055240	JN055322	JN055281
M. smithii	M186	TRS920821-17	Brazil	São Gabriel da Cachoeira, Amazonas	n/a	n/a	n/a	n/a	n/a	JN055241	JN055323	JN055282
M. smithii	M198	UGM950110-02	Trinidad	Las Cuevas	n/a	n/a	n/a	n/a	n/a	JN055242	JN055324	JN055283
M. smithii	M218	CR060627-07	Mexico	El Encino, Tamaulipas	JN054783	JN054867	JN055117	JN054950	JN055033	JN055243	JN055325	JN055284
M. smithii	M226	UGM030329-02	Argentina	Iguazú National Park	JN054784	JN054868	JN055118	JN054951	JN055034	JN055244	JN055326	JN055285
M. smithii	M230	UGM960116-01	Panama	Gamboa	JN054785	JN054869	JN055119	JN054952	JN055035	JN055195	n/a	n/a
M. smithii	M264	CR060820-03	Brazil	Belterra, Pará	JN054786	JN054870	JN055120	JN054953	JN055036	JN055245	JN055327	JN055286
M. smithii	M267	CR060908-04	Brazil	Badajós, Amazonas	JN054787	JN054871	JN055121	JN054954	JN055037	JN055246	JN055328	JN055287
M. smithii	M268	CR060909-01	Brazil	Parintins, Amazonas	JN054788	JN054872	JN055122	JN054955	JN055038	JN055247	JN055329	JN055288
M. smithii	M275	CR061011-03	Brazil	Rio Claro, São Paulo	JN054789	JN054873	JN055123	JN054956	JN055039	JN055248	JN055330	JN055289
M. smithii	M276	CR061013-10	Brazil	Rio Claro, São Paulo	JN054790	JN054874	JN055124	JN054957	JN055040	JN055196	n/a	n/a
M. smithii	M279	RRSilva041009	Brazil	Aurora do Tocantins, Tocantins	JN054791	JN054875	JN055125	JN054958	JN055041	JN055249	JN055331	JN055290
M. smithii	M305	G.Alpert020221	St. Lucia	Gros Islet, Point du Cap	JN054792	JN054876	JN055126	JN054959	JN055042	JN055250	JN055332	JN055291
M. smithii	M318	CR071221-05	Costa Rica	Lomas Barbudal	JN054793	JN054877	JN055127	JN054960	JN055043	JN055251	JN055333	JN055292
M. smithii	M319	CR071229-05	Nicaragua	El Tuma	JN054794	JN054878	JN055128	JN054961	JN055044	JN055252	JN055334	JN055293
M. smithii	M320	CR080103-01	Honduras	Copán, Archeological Museum	JN054795	JN054879	JN055129	JN054962	JN055045	JN055253	JN055335	JN055294
M. smithii	M321	CR080108-04	Guatemala	El Remate	JN054796	JN054880	JN055130	JN054963	JN055046	JN055254	JN055336	JN055295
M. smithii	M322	CR080109-01	Guatemala	Tikal	JN054797	JN054881	JN055131	JN054964	JN055047	JN055255	JN055337	JN055296
M. smithii	M323	CR080110-04	Mexico	El Panchan, Chiapas	JN054798	JN054882	JN055132	JN054965	JN055048	JN055256	JN055338	JN055297
M. smithii	M324	CR080111-02	Mexico	El Panchan, Chiapas	JN054799	JN054883	JN055133	JN054966	JN055049	JN055197	n/a	n/a
M. smithii	M325	CR080813-06	Venezuela	Ocumare de la Costa	JN054800	JN054884	JN055134	JN054967	JN055050	JN055257	JN055339	JN055298
M. smithii	M326	CR080815-01	Venezuela	Parque Nacional Henri Pittier, Rio Cumboto	JN054801	JN054885	JN055135	JN054968	JN055051	JN055258	JN055340	JN055299
M. smithii	M341	CR060831-10	Brazil	Caldeirão, Amazonas	JN054802	JN054886	JN055136	JN054969	JN055052	JN055259	JN055341	JN055300
M. smithii	M342	CR060925-02	Brazil	Manaus, Amazonas	JN054803	JN054887	JN055137	JN054970	JN055053	JN055260	JN055342	JN055301
M. smithii	M343	CR040528-03	Peru	Pilcopata	JN054804	JN054888	JN055138	JN054971	JN055054	JN055261	JN055343	JN055302
M. smithii	M344	CR040605-04	Peru	CICRA, Los Amigos	JN054805	JN054889	JN055139	JN054972	JN055055	JN055262	JN055344	JN055303
M. smithii	M353	CR060808-03	Brazil	Belém, Pará	JN054806	JN054890	JN055140	JN054973	JN055056	JN055263	JN055345	JN055304
M. smithii	M354	CR060814-03	Brazil	Alter do Chão, Pará	JN054807	JN054891	JN055141	JN054974	JN055057	JN055264	JN055346	JN055305

Table S5. Cont.

Species	Extraction code	Collector's code	Country	Sample locality	EF1-α F1 exon 1	EF1-α F1 exon 2	Wg exon 1	LW Rh exon 1	LW Rh exon 2	COI	tRNA Leu	COII
M. smithii	M355	S.Sanchez-01	Mexico	Monterrey, Nuevo León	n/a	n/a	n/a	n/a	n/a	JN055265	JN055347	JN055306
M. smithii	M356	AGH020607-05	Panama	Bocas del Toro	n/a	n/a	n/a	n/a	n/a	JN055266	JN055348	JN055307
M. smithii	M357	UGM030406-03	Argentina	Pampa del Indio	n/a	n/a	n/a	n/a	n/a	JN055267	JN055349	JN055308
M. smithii	M358	CR080104-02	Honduras	Copan Ruinas	n/a	n/a	n/a	n/a	n/a	JN055268	JN055350	JN055309
M. smithii	M360	TRS920816-07	Brazil	Reserva Ducke, Manaus, Amazonas	n/a	n/a	n/a	n/a	n/a	JN055269	JN055351	JN055310
M. smithii	M363	CR060905-01	Brazil	Santa Rita, Amazonas	n/a	n/a	n/a	n/a	n/a	JN055270	JN055352	JN055311
M. smithii	M364	CR040530-04	Peru	Huacaria	n/a	n/a	n/a	n/a	n/a	JN055271	JN055353	JN055312
M. tardus	M162	UGM960125-01	Panama	Pipeline Rd. Km6, Parque Nacional Soberanía	JN054808	JN054892	JN055142	JN054975	JN055058	JN055198	n/a	n/a
M. tardus	M173	UGM950202-03	Panama	Pipeline Rd. Km6, Parque Nacional Soberanía	JN054809	JN054893	JN055143	JN054976	JN055059	JN055199	n/a	n/a
M. tardus	M309	UGM960202-02	Panama	Pipeline Rd. Km6, Parque Nacional Soberanía	JN054810	JN054894	JN055144	JN054977	JN055060	JN055200	n/a	n/a
M. tardus	M310	UGM960202-01	Panama	Pipeline Rd. Km6, Parque Nacional Soberanía	JN054811	JN054895	JN055145	JN054978	JN055061	JN055201	n/a	n/a
M sp. nov. 1	M250	CR060906-05	Brazil	Parintins, Amazonas	JN054812	JN054896	JN055146	JN054979	JN055062	JN055202	n/a	n/a
M sp. nov. 1	M251	CR060906-09	Brazil	Parintins, Amazonas	JN054813	JN054897	JN055147	JN054980	JN055063	JN055203	n/a	n/a
M sp. nov. 1	M252	CR060906-07	Brazil	Parintins, Amazonas	JN054814	JN054898	JN055148	JN054981	JN055064	JN055204	n/a	n/a
M sp. nov. 2	M245	CR060915-01	Brazil	Manaus, Amazonas	JN054815	JN054899	JN055149	JN054982	JN055065	JN055205	n/a	n/a
M sp. nov. 2	M246	CR060919-05	Brazil	Manaus, Amazonas	JN054816	JN054900	JN055150	JN054983	JN055066	JN055206	n/a	n/a
M sp. nov. 3	M095	CR040603-1-4	Peru	CICRA, Los Amigos	JN054817	JN054901	JN055151	JN054984	JN055067	JN055207	n/a	n/a
M sp. nov. 3	M102	CR040608-03	Peru	CICRA, Boca Amigos	JN054818	JN054902	JN055152	JN054985	JN055068	JN055208	n/a	n/a
M sp. nov. 3	M103	CR040608-04	Peru	CICRA, Boca Amigos	JN054819	JN054903	JN055153	JN054986	JN055069	JN055209	n/a	n/a
M sp. nov. 3	M117	CR040615-01/06	Peru	Explorama Lodge, Iquitos	JN054820	JN054904	JN055154	JN054987	JN055070	JN055210	n/a	n/a
M sp. nov. 3	M135	AGH030616-03	Ecuador	Tiputini Biodiversity Station	JN054821	JN054905	JN055155	JN054988	JN055071	JN055211	n/a	n/a
M sp. nov. 3	M136	AGH030613-04	Ecuador	Tiputini Biodiversity Station	JN054822	JN054906	JN055156	JN054989	JN055072	JN055212	n/a	n/a
M sp. nov. 3	M345	CR040529-02	Peru	Pilcopata	JN054823	JN054907	JN055157	JN054990	JN055073	JN055213	n/a	n/a
M sp. nov. 4	M153	TRS960415-16	Guyana	Paramakatoi	JN054824	JN054908	JN055158	JN054991	JN055074	JN055214	n/a	n/a
M sp. nov. 4	M311	TRS960415-17	Guyana	Paramakatoi	JN054825	JN054909	JN055159	JN054992	JN055075	JN055215	n/a	n/a
M sp. nov. 4	M312	TRS960415-12	Guyana	Paramakatoi	JN054826	JN054910	JN055160	JN054993	JN055076	JN055216	n/a	n/a
M sp. nov. 4	M313	TRS960415-13	Guyana	Paramakatoi	JN054827	JN054911	JN055161	JN054994	JN055077	JN055217	n/a	n/a
M sp. nov. 5	M294	A.Parente M713	Colombia	Amacayacu National Park	JN054828	JN054912	JN055162	JN054995	JN055078	JN055218	n/a	n/a

Outgroup taxa are listed in Schultz and Brady (31). Collection information can be requested from the first author.

Table S6. Sequence characteristics and best-fit models of sequence evolution as calculated by hLRTs and the AIC

Gene	Number of sites	All taxa: Variable sites	All taxa: PI sites	Ingroup: Variable sites	Ingroup: PI sites	hLRTs	AIC	Model Bayesian	Model partitioned ML
Global analysis									
Ef1-α Exon1&2	1,071	370	363	43	35				
Ef1-α Pos1&2	714	37	34	1	1	TIM+I+G	TIM+I+G	GTR+I+G	TIM+I+G
Ef1-α Pos3	357	333	329	42	33	GTR+I+G	GTR+I+G	GTR+I+G	GTR+I+G
Wg Exon	405	187	164	20	18				
Wg Pos1	135	36	21	0	0	K80+G	TrNef+G	GTR+G	TrNef+G
Wg Pos2	135	19	15	1	1	K80+G	K80+G	K80+G	K80+G
Wg Pos3	135	132	128	19	17	HKY+G	GTR+G	GTR+G	GTR+G
LWR Exon1&2	456	206	193	25	23				
LWR Pos1	152	56	50	10	10	HKY+I+G	HKY+I+G	HKY+I+G	HKY+I+G
LWR Pos2	152	28	26	0	0	GTR+G	GTR+G	GTR+G	GTR+G
LWR Pos3	152	122	117	15	13	HKY+I+G	HKY+I+G	HKY+I+G	HKY+I+G
COI	387	146	140	146	140				
COI Pos1&2	258	30	28	30	28	TrN+I+G	TIM+I+G	GTR+I+G	TIM+I+G
COI Pos3	129	116	112	116	112	TrN+G	TrN+G	GTR+G	TrN+G
Total	***2,319***	***909***	***860***	***234***	***216***	n/a	n/a	n/a	n/a
Local analysis									
COI-II + tRNA Leu	1,515	n/a	n/a	248	169				
COI-II Pos1&2 + tRNA Leu	1,034	n/a	n/a	54	33	HKY+I+G	TrN+I+G	GTR+I+G	TrN+I+G
COI-II Pos3	481	n/a	n/a	194	136	TrN+G	TIM+I+G	GTR+I+G	TIM+I+G
Total	***1,515***	n/a	n/a	***248***	***169***	n/a	n/a	n/a	n/a

"Model" columns indicate the models of sequence evolution implemented in the Bayesian and likelihood analyses. The global dataset consists of nuclear and mitochondrial DNA sequence data for 84 *Mycocepurus* ingroup taxa and 87 attine and myrmicine outgroup taxa. The local dataset consists exclusively of mitochondrial sequence data for 41 *M. smithii* individuals. PI, parsimony-informative.

Table S7. Microsatellite loci developed for the fungus-gardening ant species *M. smithii*

Locus	Repeat motif	Primer (5′-3′)	T_m (°C)	Multiple ×	Dye	Size range	Number of alleles	GenBank accession number
A5	$(AC)_{14}$	F: GAACTTCGACGTGTAATTCG R: GCCACGGATAATTTCGAT	56–57	B	FAM	238–256	12	JN055219
A6	$(AC)_{15}$	F: CTCCTCCGGCTTTTCTCT R: GATCGCGTACGGGTATATG	56–57	C	FAM	101–123	12	JN055220
A9	$(GT)_{13}$	F: AACCTTCCCTTTGCGAAT R: TATGTTTTGTGCCGTCGTTA	56–57	A	FAM	135–165	10	JN055221
B1	$(TC)_{17}$	F: GTGAGACGTGTTCGACGAG R: GACTCGGAACCGACTTTCT	56–58	D	HEX	90–132	15	JN055222
B4	$(GC)_8$	F: GATTTGCATACGTCTGTCTAGC R: GCCTATTTCGTGTAAGGTAATG	56–57	D	FAM	205–207	2	JN055223
C2	$(TTG)_6$-A-$(TTG)_5$	F: CGCGTGATTCCTAGACAAC R: AACGTGAGTCAGAACAATACG	56–57	D	FAM	230–242	5	JN055224
C6	$(TTG)_6$-TTA-$(TTG)_4$	F: ACCAGGTTACAGGCGTAGAT R: CGATACCATCACCACGACTA	56–57	B	HEX	237–271	11	JN055225
C104	$(CAA)_8$	F: CGTCTACCAGTTCTGATTGC R: ATCTGACATTTTGTCCAACG	56–57	C	FAM	204–225	8	JN055226
C119	$(CAG)_4$-$(CAA)_8$-$(ATC)_3$	F: CGATTCTACATCGATTCTGCR R: ATCTGACATTTTGTCCAACG	56–57	B	FAM	111–135	9	JN055227
D8	$(CAT)_{11}$-$(CGT)_5$	F: CGGACATGTTCTTCGAGAT R: CGCGACCTTTGAAAGTAGAT	56–57	D	HEX	159–189	10	JN055228
D11	$(GAT)_{10}$-GAC-$(GAT)_4$	F: ACTTCGTTCCTCCATCTTCC R: CGCATCATCAGTTTGTTCAC	56–57	C	FAM	285–294	4	JN055229
D117	$(TCA)_{27}$	F: GATGTCATAGCAGGGCATTA R: TGTCGCGTTGTGTGTCTAT	56–57	A	FAM	196–242	8	JN055230

T_m is the optimal annealing temperature. Loci were amplified in four multiplexed PCR reactions (A–D). The number of alleles and the size range were determined from genotyping 1,930 individuals from 39 localities in Latin America. Clone sequences were deposited in GenBank under the accession numbers given. F, forward; R, reverse. HEX, hexachlorofluorescein; FAM, carboxyfluorescein.

Table S8. Primers used for PCR amplification and DNA sequencing

Primer	Sequence (5′-3′)	Position	Source
EF1-α F1 copy			
F1-494F	AAGGAGGCTCAGGAGATGGG	*Apis* 494–513	(31)
F1-1044R	CGTCTTACCATCGGCATTGCC	*Apis* 1044–1019	(31)
F1-792F	TTGGCGTGAAGCAGCTGATCG	*Apis* 792–812	(31)
F1-1189R	ACCTGGTTTYAAGATRCCGGT	*Apis* 1189–1169	(31)
F1-1109F	CCGCTTCAGGATGTCTATAA	*Apis* 1109–1128	(31)
F1-1551R	CCGCGTCTCAGTTCYTTTAC	*Apis* 1551–1532	(31)
F1-1424F	GCGCCKGCGGCTCTCACCACCGAGG	*Apis* 1424–1448	(55)
F1-1829R	GGAAGGCCTCGACGCACATMGG	*Apis* 1829–1808	(55)
Wg			
MycoWg578F	TGCACGGTGAAGACTTGCTGGATGCG	*Pheidole* 578–603	Modified from ref. 58
Wg1032R	ACYTCGCAGCACCARTGGAA	*Pheidole* 1032–1013	(59)
LW Rh			
LR143F	ACAAAGTGCCACCGGAGATGCT	*Apis* 144–165	Modified from ref. 58
MycoLR639ER	CTTACCGGTTTCCATCCGAACA	*Apis* ~639–624	Modified from ref. 58
COI-II			
LCO1490	GGTCAACAAATCATAAAGATATTGG	*D. yakuba* 1490–1515	(60)
HCO2198	TGATTTTTTGGTCACCCTGAAGTTTA	*D. yakuba* 2198–2223	(60)
CI13	ATAATTTTTTTTATAGTTATACC	*Apis* 2002–2025	(61)
CI14	ATTTCTTTTTTTCCTCTTTC	*Apis* 2549–2568	(61)
MycoJerry	CAACAYYTATTTTGATTTTTTGG	*Apis* ~2181–2203	Modified from ref. 57
MycoBen	CAYGAYACHTATTATGTAGTRGC	*Apis* ~2613–2591	Modified from ref. 62
MycoGeorge	ATACCTCGTCGATATTCTGA	*D. yakuba* 2773–2792	Modified from ref. 63
Marilyn	TCATAAGTTCARTATCATTG	*D. yakuba* 3364–3383	(63)
Lewis	TATTATTTGRGARTCCCTCT	*Apis* ~2660–2679	This study

Position numbers correspond to *Apis mellifera* GenBank accession number X52884 (EF1-αF1), *Pheidole morrisi* GenBank accession number AY101369.1 (Wg), *A. mellifera* GenBank accession number U26026 (LW Rh), *Drosophila yakuba* GenBank accession number X03240 (COI and COII), and the *A. mellifera* nucleotide position given in ref. 57 (COI). For all genes, the PCR product was amplified directly from the DNA extract.