Report

Current Biology

Joint Evolution of Asexuality and Queen Number in an Ant

Highlights

- Multi-queen colonies of a fire ant produce queens asexually but workers sexually
- Single-queen colonies produce both queens and workers sexually
- Queens in multi-queen colonies require sperm from single-queen colony males to produce workers
- Distinct asexual/multi-queen lineages may stem from a sexual/single-queen population

Authors

Kip D. Lacy, DeWayne Shoemaker, Kenneth G. Ross

Correspondence

kipdlacy@gmail.com

In Brief

Lacy et al. describe a socially polymorphic population of an ant in which multi-queen colonies produce queens asexually but produce workers sexually via matings with males from the sexually-reproducing single-queen social form. Two distinct asexual lineages from multi-queen colonies likely originated from the same sexual single-queen population.

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Report

Joint Evolution of Asexuality and Queen Number in an Ant

Kip D. Lacy,[1,3,4,*] DeWayne Shoemaker,[2] and Kenneth G. Ross[1]
[1]University of Georgia, Department of Entomology, 413 Biological Sciences Building, 120 Cedar Street, Athens, GA 30602, USA
[2]University of Tennessee Knoxville, Department of Entomology and Plant Pathology, 371 Plant Biotechnology Building, 2505 EJ Chapman Drive, Knoxville, TN 37996-4560, USA
[3]Present address: Laboratory of Social Evolution and Behavior, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA
[4]Lead Contact
*Correspondence: kipdlacy@gmail.com
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SUMMARY

Ants exhibit a striking diversity of reproductive systems, varying in traits such as the number of reproductives per colony [1], the mode of daughter production (sexual or asexual) [2], and the mode of caste determination (genetic or environmental) [3]. Species employing mixed reproductive systems present a unique opportunity to explore the causes and consequences of alternative breeding strategies. Mixed reproductive systems in ants include social polymorphism in colony queen number, whereby single-queen (monogyne) and multiple-queen (polygyne) colonies co-occur within species [4–7], and facultative asexuality, in which female offspring may be produced sexually or asexually within colonies [8–13]. Here, we document a remarkable confluence of multiple mixed reproductive systems in the tropical fire ant, *Solenopsis geminata*, in a population with three important features: (1) polygyne colonies produce workers sexually but queens asexually, whereas monogyne colonies produce both castes sexually; (2) polygyne queens mate with monogyne males to produce workers, but monogyne queens do not mate with polygyne males; and (3) different asexual/polygyne lineages evidently were founded separately by genetically distinct founder queens, which appear to have originated from the same neighboring monogyne population. Multiple asexual/polygyne genomes are transmitted undiluted in this system, but sterile workers produced with sperm from a sexually-reproducing/monogyne population are necessary for the persistence of these lineages. The intersection of social polymorphism, facultative asexuality, and genetic caste determination marks this population of *S. geminata* as an embodiment of the diversity of ant reproductive systems and suggests previously unknown connections between these phenomena.

RESULTS

We collected 73 monogyne and 42 polygyne nests of *S. geminata* [14] in Gainesville, Florida, sampling a mean of 11.5 workers per nest and collecting reproductive queens, daughter (winged virgin) queens, and males opportunistically (Data S1). All individuals were genotyped at nine microsatellite loci (Data S1). The observed genotype distributions revealed striking differences between the social forms in patterns of within-nest genetic variation and inferred breeding strategies. Distributions of multilocus genotypes (MLGs) among monogyne nestmates reflected the standard reproductive mode of social Hymenoptera—one reproductive queen, singly mated to a haploid male, producing daughters (queens and workers) from fertilized diploid eggs and sons from unfertilized haploid eggs (Figure 1A; Table S1) (see also [15]).

We expected queens in polygyne *S. geminata* nests to reproduce similarly, as reported for polygyne *S. invicta* [15–19], but our genetic data indicate otherwise. Daughter queen MLGs typically were homozygous at all loci and were identical to those of nestmate reproductive queens (Figure 1B; Table S1). In contrast, at any given locus, nestmate workers shared one allele found also in nestmate queens while bearing a second allele typically absent from queens and differing among the workers. These unusual genotypic patterns were confirmed in a subset of individuals genotyped at 26 additional microsatellite loci (Data S1 and Table S2). No workers were found with queen-like MLGs, and only a small proportion of daughter queens (0.04–0.06) possessed worker-like rather than queen-like MLGs. No reproductive queen had a worker-like genotype, although one (GPR16-3-Q1) possessed a somewhat worker-like MLG (identical to other queen MLGs at 30 loci but similar to worker MLGs at the remaining five; Table S2; Figure S1).

These patterns may be explained by asexual production of queens and sexual production of workers, as observed occasionally in other ants [8–13]. To test this, we isolated individual reproductive queens in fragments of their polygyne parent colony and genotyped their progeny. All but one of the resulting daughter queens had MLGs (mean = 9.8, nine fragments) identical to those of their mothers, whereas worker MLGs (mean = 11.8, 25 fragments) invariably contained non-maternal alleles (Figure 1C; Table S1). Sperm extracts from the sperm-storage organs of 23 isolated queens featured a single allele at each

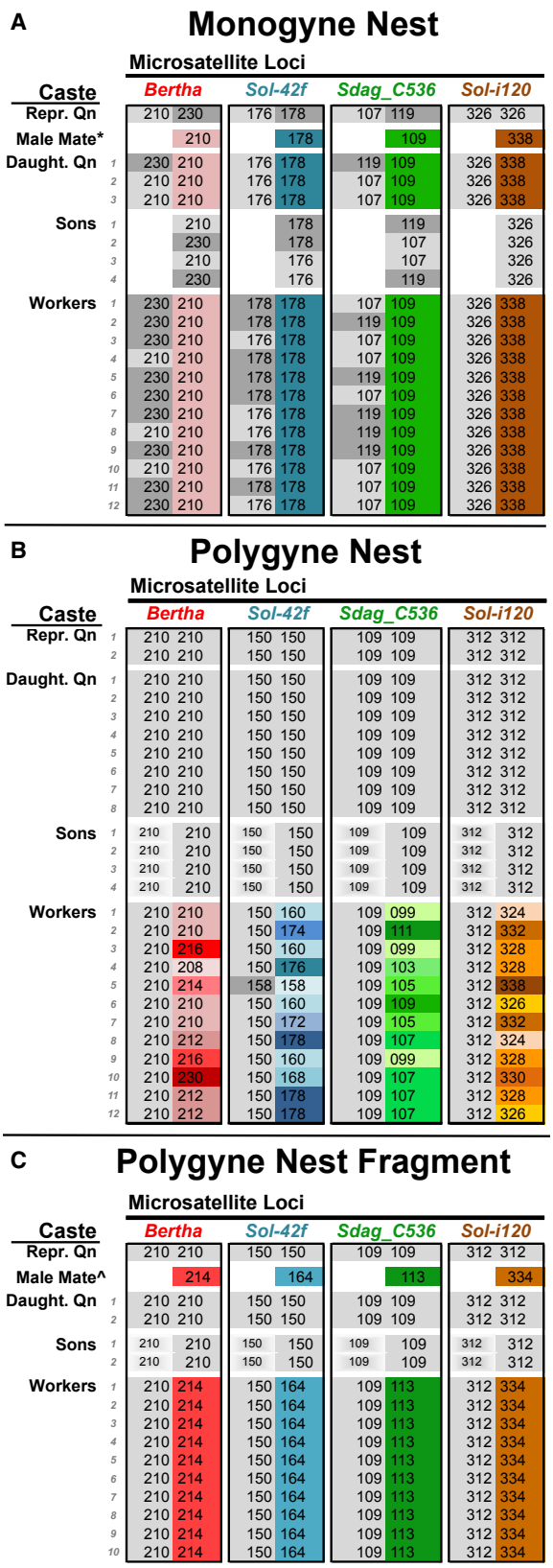

Figure 1. Genotype Distributions at Four Microsatellite Loci Illustrating Reproductive Modes in Monogyne and Polygyne *Solenopsis geminata*

Tables of representative multilocus genotypes are shown for individuals from (A) a monogyne nest, (B) a polygyne nest, and (C) a nest fragment headed by a single polygyne reproductive queen. Diploid genotypes at each locus comprise pairs of three-digit alleles separated by a space. For each panel, unique colors represent different maternal or paternal alleles. Maternal alleles are shaded in gray, while paternal alleles are shaded in other colors. The lighter shade of gray and smaller font size for sons in (B) and (C) reflect the fact that polygyne males may be either haploid or diploid. See also Data S1 and Tables S1, S2, and S4. *; male mate multilocus haplotype inferred from mother and daughter genotypes. ^; male mate multilocus haplotype determined from extracted DNA of pooled sperm from queen's sperm-storage organ.

locus, indicating single matings to haploid males, with the paternal alleles absent from daughter queens but present in offspring workers (Figure 1C; Table S1). These data confirm that polygyne queens typically produce daughter queens asexually and workers sexually (we use polygyne and monogyne to refer to both colony structure and individuals produced in such colonies). The single exceptional daughter queen (GPR16-1-Q1-fp20) had a worker-like genotype (unlike those of 34 of her sisters), indicating that their mother produced a low frequency (0.03) of queen offspring sexually.

Polygyne (asexual) queens exhibit a striking lack of genotypic diversity. We calculated the genotype-to-individual ratio (G:N, number of distinct MLGs divided by number of individuals) and observed heterozygosity (H_o) by randomly resampling single individuals per nest. Median G:N values for each monogyne caste and for polygyne workers were 1.0 (Figure 2A), indicating that non-nestmates typically had distinct MLGs. Values for polygyne reproductive and daughter queens were lower (median = 0.33 and 0.37, respectively), reflecting the fact that queens from different nests at each site often shared MLGs (Table S1). Paralleling these results, monogyne females and polygyne workers yielded matching high mean H_o values (0.62–0.67), while polygyne queens yielded low mean H_o (0.01–0.05; Figure 2B), a result confirmed using the additional microsatellite loci (mean H_o = 0.03; Figure S1; Table S2). Notably, H_O values for rare sexually produced polygyne daughter queens were comparable to those for workers.

The substantial genetic diversity observed in polygyne workers must derive from their fathers which, given the low diversity among polygyne sexuals, we propose to be of monogyne origin. Supporting this scenario, among the 693 genotyped workers from 40 polygyne nests, almost all non-maternal alleles across loci were represented in the monogyne gene pool, whereas non-maternal alleles for at least one, and usually several, loci per worker were absent from the polygyne (asexual) gene pool. Moreover, with one exception, stored-sperm haplotypes obtained for 56 queens from 17 polygyne nests contained at least two alleles found in the monogyne but not the asexual gene pool (Table S3). To further test the monogyne paternity hypothesis, we calculated population assignment probabilities for sperm haplotypes and polygyne worker MLGs using the program *Structure*. Three populations were specified in these analyses—the monogyne and two differentiated asexual gene pools (below). Sperm multilocus haplotypes generally had high assignment probabilities to the monogyne form (mean = 0.87;

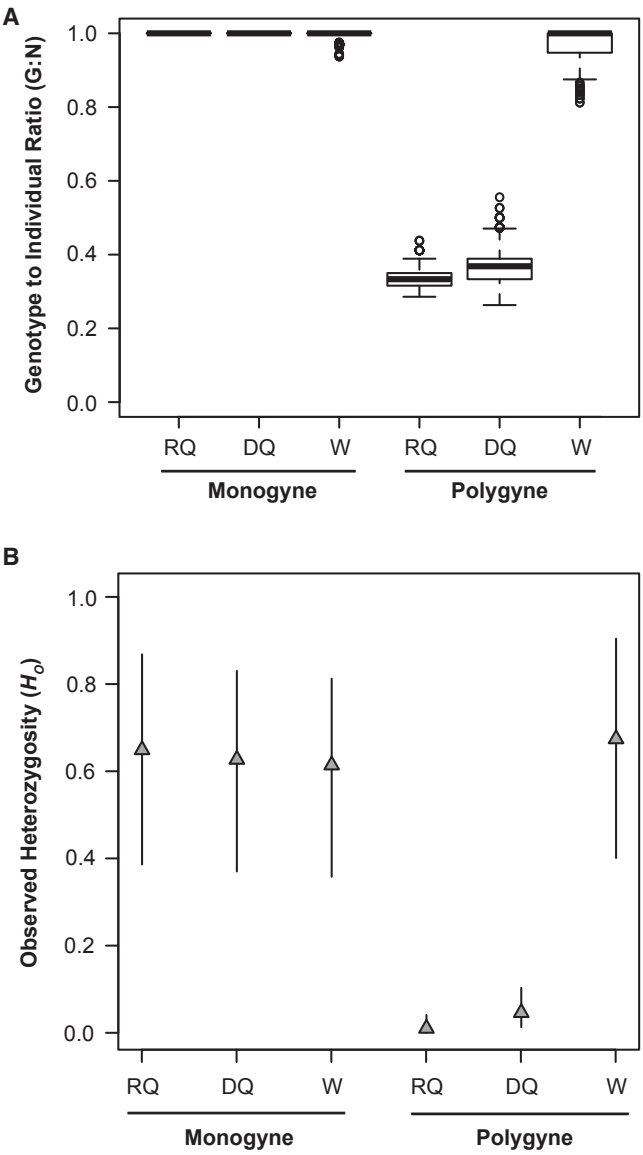

Figure 2. Patterns of Genetic Variation for Different Categories of Females in Monogyne and Polygyne *S. geminata*

Statistics are shown for reproductive queens (RQ), daughter queens (DQ), and workers (W).

(A) Genotype-to-individual ratio (G:N) values calculated from the nine primary study loci using 10,000 random subsamples of a single individual per nest (thus reflecting among-nest diversity). Box-and-whisker plots comprise the median (horizontal line), interquartile range (IQR, box), ± 1.5 × IQR (whiskers), and outliers (circles).

(B) Mean and 99% confidence intervals of overall H_O values obtained by bootstrapping (1,000 iterations) across the single-locus estimates (calculated using 10,000 random subsamples). See also Figure S1, Data S1, and Tables S1 and S4.

Figure 3), with only a single exceptional sperm haplotype predominantly assigned to the same asexual lineage as the mated queen. Individual polygyne workers had roughly equal assignment probabilities to both the monogyne form (mean = 0.5) and their asexual population of origin (mean = 0.45; Figure 3). Interform gene flow evidently is unidirectional—all monogyne daughters possessed alleles absent from the asexual gene pool at one or more loci, indicating they were not sired by polygyne males.

We investigated whether this unexpected mating pattern may be tied to reproductive impairment of polygyne males by dissecting adult males of both forms and comparing reproductive tissue development. Polygyne males exhibited relatively reduced development (Mann-Whitney test, median U = 21, p = 0.004) (Figure S1), consistent with common subfertility or sterility. Sterility is associated with male diploidy in *S. invicta* [20] and other Hymenoptera [21], and we confirmed almost a third of polygyne males as diploids based on heterozygosity at one or more loci (Table S4).

Polygyne queens from our two study sites located 19 km apart, while displaying identical reproductive modes, appear genetically distinct—mean G''_{ST} (a measure of population differentiation varying between zero and one) equals 0.63. All asexual MLGs were site-specific (Table S1), and queens from the two sites had nonoverlapping sets of alleles at 20 of the 35 loci studied and only partial overlap at another five (Figure S2). Virtually all alleles in polygyne queens were found also in the monogyne form (Figure S2), suggesting some genetic affinity between the neighboring populations of the alternate forms; exceptions typically involved highly polymorphic loci for which rarer alleles in the monogyne form may not have been sampled.

Nuclear population genetic differentiation was explored in greater detail using discriminant analysis of principal components (DAPC). An analysis based on 32 microsatellite loci, including polygyne queens from both lineages, monogyne females, and specimens from Mexico [22], identified four clusters with membership corresponding perfectly to these categories (Figure 4A). Each polygyne cluster was linked directly to the monogyne cluster in multidimensional space, suggesting that the two asexual lineages have greater genetic affinity to the monogyne ants than to each other. The polygyne clusters also appear more similar to the Florida monogyne population than to other geographic populations of *S. geminata* [23] or other closely related species [22] (Figure S2).

These analyses raise the possibility that the asexual/polygyne lineages arose from the neighboring monogyne form. The considerable genetic distance between the two asexual lineages could result from differential reductions of a common asexual ancestral genome to homozygosity (“one-monogyne-founder” scenario). Alternatively, the two lineages may have been separately founded by genetically distinct monogyne queens (“two-monogyne-founders” scenario). Finally, a second asexual lineage may have been founded by a queen produced by a backcross mating between a monogyne sexual and an individual from an initial asexual lineage (itself founded by a monogyne queen). To discriminate among scenarios, we conducted simulations in which we repeatedly randomly sampled founder MLGs from the monogyne gene pool and then randomly reduced constituent loci to homozygosity (see STAR Methods). Because backcrossing could occur at any point during the gradual transition of asexual genomes to homozygosity (a transition documented in other asexual Hymenoptera [2]), we conducted two backcross simulations that bracketed this continuum (backcross before any reduction of heterozygosity [“early-backcross” scenario] and after transition to complete homozygosity [“late-backcross” scenario]).

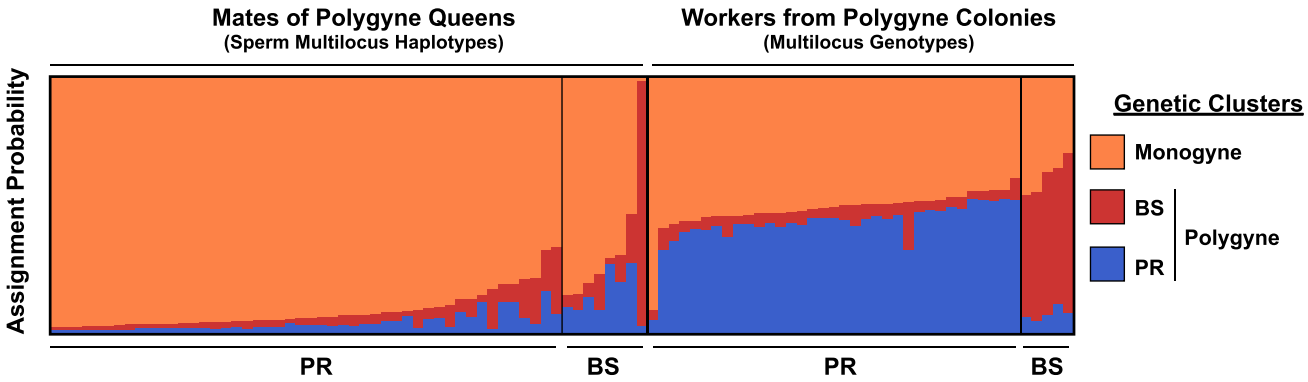

Figure 3. Population Assignment Probabilities for Sperm of Mated Queens and for Workers in Polygyne *S. geminata*

Colored vertical bars display the mean assignment probabilities from *Structure* to the monogyne gene pool and the gene pools of the two asexual lineages (BS and PR). Mean probabilities were taken across 100 resampling iterations in which individuals comprising the stipulated source populations, as well as polygyne workers, were resampled. Each vertical bar represents one multilocus haplotype for the mates of polygyne queens, whereas each bar represents a single colony for polygyne workers. Black lines separate samples from different sites. See also Figure S1, Data S1, and Tables S1, S3, and S4.

Comparison of simulation and empirical results (Figure 4B) revealed that the empirical between-lineage G''_{ST} estimate does not differ significantly from simulation values for the two-monogyne-founders or early-backcross scenarios ($p = 0.196$ and 0.068, respectively) but differs from the late-backcross and one-monogyne-founder simulation values ($p < 0.001$ for both).

To complement the nuclear genetic results, we analyzed diversity at the mitochondrial gene *COI*. No haplotypes were shared between polygyne sites or between social forms. Site-exclusive polygyne haplotypes are distinguished by at least seven substitutions and separated by two monogyne-exclusive haplotypes in a minimum-spanning network (Figure S3). Maximum parsimony (MP) phylogenies of the haplotypes revealed similar patterns (Figure 4C)—all minimum-length trees, which featured polyphyletic site-exclusive polygyne haplotype groups, had shorter lengths (30 steps) than did trees in which polygyne monophyly was enforced (minimum length = 33). An estimated maximum likelihood (ML) phylogeny mirrored the MP topologies (Figure S3). Additional likelihood trees were constructed with enforced monophyly of polygyny haplotypes—these were rejected in favor of the unconstrained ML phylogeny (Shimodaira-Hasegawa test, p=0.047). Together, nuclear and mitochondrial analyses indicate that the two asexual/polygyne lineages were founded separately by genetically distinct queens, both of which may have originated from the neighboring monogyne form.

DISCUSSION

We document co-occurrence of polygyny with facultative asexuality in a socially polymorphic population of *S. geminata*. Most polygyne daughter queens were produced asexually (~95%); in contrast, all polygyne workers were produced sexually, as were a small minority of queens. This difference in modes of queen and worker production yields an association between genotype and caste, with queens generally having low and workers having high heterozygosity. Similar genotype-caste associations occur in other ant species, and the phenomenon is generally referred to as "genetic caste determination" [3, 8–13, 24–27]. While it is tempting to conclude that caste is deterministically encoded by genotype in such cases, under controlled conditions, individuals of all genotypes can develop as either caste, at least at some rate [28, 29]. Thus, from a developmental standpoint, genotype strongly biases (but does not deterministically encode) caste identity in ants with genetic caste determination. In polygyne *S. geminata*, asexually produced, highly homozygous offspring are biased toward queen development, whereas sexually produced, highly heterozygous offspring are biased toward worker development.

Caste biases may be mediated by both genotypic and environmental effects on larval size. In ants, caste is associated with body size at the time of pupation [30], with queens being larger than workers in *S. geminata* [31] and other ants [30]. Thus, bias toward queen development in asexually produced *S. geminata* likely results from a predisposition of such individuals toward large larval body size. It is possible that the highly homozygous asexual genomes induce genetic queen bias due to homozygosity for recessive alleles at loci important in influencing larval size. In turn, larvae biased toward queen development likely command access to an inordinately large portion of the colony's nutritional resources, thus limiting food available to the sexually produced brood and thereby relegating them to smaller size and worker-biased development (genetic caste bias generates a conditional environmental bias, similar to [32]). Thus, while queen bias and worker bias appear to be distinct phenomena, both may result from relatively straightforward cause-and-effect relationships based on the superimposition of genetic queen bias in asexual offspring on existing nutrient flow dynamics in complex societies with dependent young.

Remarkably, polygyne *S. geminata* workers in our study apparently were produced exclusively via matings to monogyne males. Two phenomena may contribute to this mating pattern. First, many polygyne males are subfertile—they exhibit reduced reproductive development compared to monogyne males, possibly resulting from diploidy (below). Second, matings between sexuals of the polygyne form, while rare (1.8% of observed matings), may yield few or no workers. Half of offspring produced by matings between members of the same asexual

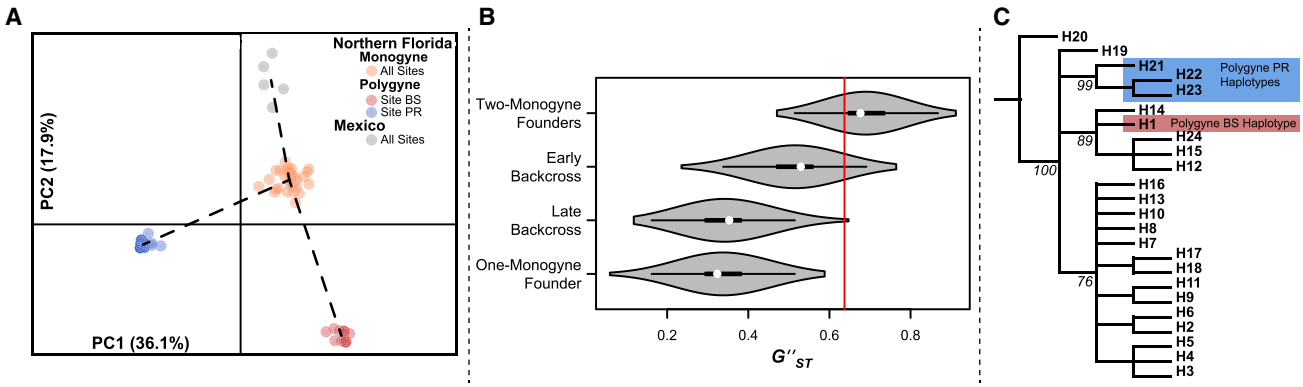

Figure 4. Nuclear and Mitochondrial Population Genetic Analyses of *S. geminata*

(A) Scatterplot comprising projections on the first two principal components (PCs) from DAPC analyses based on genotypes at 32 microsatellite loci. Percentages of the total variance in the original data explained by the two PCs are shown on the relevant axes. Cluster centroids calculated using all principal components are connected by a minimum-spanning tree (dashed lines).

(B) Violin plots depicting estimates of G''_{ST} between the two polygyne subpopulations derived from four simulations (gray area; kernel density plots of frequencies in 1000 simulation iterations) and boxplots comprising the median (white dot), interquartile range (IQR, box), and ± 1.5 × IQR (whiskers) for the same data. The red line displays the empirical estimate of G''_{ST} from a resampling of single MLGs (mean from 1,000 resampling iterations).

(C) Maximum parsimony phylogeny of a 784 bp segment of the mtDNA *COI* gene (strict consensus of three minimum-length [=30 steps] trees). Symmetric resampling support values greater than 70 (out of 100 replicates) are shown in italics for relevant nodes. Polygyne haplotypes are highlighted with colored boxes; the remaining haplotypes were found exclusively in monogyne individuals. See also Figures S2 and S3 and Table S2.

lineage necessarily are determined to develop as diploid males rather than workers (due to predicted homozygosity at the complementary sex determination [CSD] locus in half of zygotes; homozygosity or hemizygosity at this locus triggers male development, while heterozygosity triggers female development in fire ants and other Hymenoptera with CSD [16, 21]). Furthermore, if queen bias is encoded in asexual genomes (as hypothesized above), daughters produced from same-lineage matings would preferentially develop as queens. Thus, asexual/polygyne *S. geminata* may be dependent on males from the sexually reproducing monogyne form for the production of workers, which are essential for colony growth and survival.

Continued production of polygyne males may appear paradoxical given their evident lack of mating success, but it possibly can be explained entirely by constraints imposed by CSD. Queens presumably are heterozygous at the CSD locus, and asexually-produced diploid males likely result from reductions to homozygosity at this locus (and genome wide), stemming from genetic recombination (as in other asexual Hymenoptera [2]). Some diploid males could also be produced via rare same-lineage matings, as described above. While we could confirm only a third of genotyped polygyne males to be diploid, this is likely a substantial underestimate because offspring ploidy could not be inferred for mother queens homozygous at all marker loci (84% of polygyne queens studied). Thus, most or even all of the polygyne males in our study may be diploids, a circumstance with some precedent in *S. invicta* [16, 33].

The two asexual/polygyne *S. geminata* lineages we studied evidently were founded separately by genetically distinct queens, yet both lineages likely originated from the same sexual/monogyne population. These lineages might share the same functional basis for asexuality (e.g., a mutation that interferes with typical meiosis); such a case could involve a rare recessive asexuality-inducing element circulating in the monogyne population that occasionally appears in homozygous condition in the offspring of two carriers (consistent with the two-monogyne-founders simulation). Such an element could also be propagated via backcrossing between a monogyne queen and a male from a young asexual lineage (consistent with the early-backcross simulation, if the allele is instead dominant). While speculative, these scenarios illustrate how standing variation for simple molecular mechanisms of asexuality could foster repeated origins of asexual/polygyne *S. geminata* populations from the monogyne form.

It is unclear why facultative asexuality and polygyny co-occur in our study population. The connection could be strictly genetic, with asexuality and polygyny resulting from linked mutations in relevant reproductive and social-regulatory machinery (i.e., [6, 34]), similar to the coordinated effects of supergenes on myriad phenotypic traits in other socially polymorphic ants [6, 7]. Linkage may predate the foundation of asexual/polygyne lineages (via development of a supergene conferring both traits), or it may arise as a direct result of asexuality (all loci are linked in asexual genomes). It is also possible that asexual reproduction may facilitate polygyny via pleiotropic effects of reduced genotypic diversity. For instance, nestmate queens are genetically homogeneous, and genetic variation influences the cuticular semiochemical profiles underlying systems of nestmate recognition and regulation of queen number in ants [35, 36]. Thus, queens of the same asexual lineage may have identical profiles, possibly contributing to the stability of multi-queen associations (see [37]). This scenario might help explain the absence of polygyne reproductive queens with worker-like MLGs—such queens presumably would not share the preponderant semiochemical profiles and thus could be discriminated against by workers as they attempt to become supernumerary reproductives.

The unidirectional mating system and genetic caste determination evident in polygyne *S. geminata* are especially striking

when considered in a superorganismal context [38]. Sterile workers—the superorganismal soma—are produced sexually, while queens—the superorganismal germline—are produced asexually. This closely resembles the phenomenon of "hybridogenesis" found occasionally in asexual populations in diverse animal taxa, in which paternal chromosomes are present in somatic tissue but are absent from eggs [39]. Indeed, similar ant breeding systems have been termed "social hybridogenesis" [9–13, 25, 26, 32] and feature asexual lineages (or other types of closed gene pools) with queen-biased development of asexual (or within-lineage) offspring and worker-biased development of eggs fertilized by sperm from closely related species or lineages. Asexual/polygyne *S. geminata* clearly exhibit social hybridogenesis, with distinct lineages propagating the superorganismal germline asexually but parasitizing sperm from a sexual population for somatic growth. These lineages appear to have originated from the same sexual population they parasitize, indicating that the local monogyne form serves dual roles as both source of and host for these lineages. Co-occurrence of the transition from monogyny to polygyny and from standard ant reproduction to facultative asexuality with genetic caste determination suggests proximate connections between these phenomena, functional studies of which will yield new insights into the evolutionary diversification of ant breeding strategies.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - mtDNA Sequence Analyses
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- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.03.018.

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AUTHOR CONTRIBUTIONS

K.D.L., D.S., and K.G.R. designed the study; K.D.L. and D.S. collected samples and performed genotyping; K.D.L. performed all analyses and wrote the original draft; D.S. and K.G.R. supervised the project; and all authors contributed to data interpretation and to editing and writing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Gentra PureGene Tissue Kit (4g)	QIAGEN	Cat No. 158667
Deposited Data		
Microsatellite Data from *S. geminata* from Mexico and other North American Fire Ants	[22]	https://datadryad.org/resource/doi:10.5061/dryad.39sr7
Microsatellite data and mitochrondrial sequence data form global populations of *S. geminata*	[23]	https://datadryad.org/resource/doi:10.5061/dryad.256kh
Mitochondrial COI Haplotypes	This paper	Genbank: MK733396-MK733419
Microsatellite data and male reproductive development scores	This paper	https://doi.org/10.5061/dryad.jt0r643
Oligonucleotides		
See Data S1 for full lists of primers.	This paper	https://doi.org/10.5061/dryad.jt0r643
Software and Algorithms		
GeneMarker	SoftGenetics	https://softgenetics.com/GeneMarker.php
Microchecker	[40]	http://www.nrp.ac.uk/nrp-strategic-alliances/elsa/software/microchecker/
Adegenet	[41]	https://github.com/thibautjombart/adegenet
Sequencher	Gene Codes	https://www.genecodes.com/
Geneious	Geneious	https://www.geneious.com/
PopART	[42]	http://popart.otago.ac.nz/index.shtml
TNT	[43]	http://www.lillo.org.ar/phylogeny/tnt/
Mmod	[44]	https://github.com/dwinter/mmod
GenePop on the Web	[45, 46]	http://genepop.curtin.edu.au/
Structure	[47, 48]	https://web.stanford.edu/group/pritchardlab/structure.html
IQTREE	[49–51]	http://www.iqtree.org
PartitionFinder2	[52]	http://www.robertlanfear.com/partitionfinder/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Corresponding Author, Kip D. Lacy (kipdlacy@gmail.com).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study was conducted using field-collected colonies of the tropical fire ant, *Solenopsis geminata*. Colonies were housed in the laboratory in 40 cm x 50 cm x 10 cm plastic trays containing plaster-bottom nests at 32°C on a 14 hour/10 hour light/dark cycle, and were fed a mixed diet of high-sugar food, high-protein food [53], frozen crickets, and millet seeds.

METHOD DETAILS

Collection and Sampling

Monogyne and polygyne *Solenopsis geminata* nests were collected from multiple locations in Gainesville, Alachua County in northern Florida, US in the spring and early summer of 2014-2017. Polygyne nests were collected from two sites approximately 19km apart: Parker Road (PR) [29°34'02.2"N 82°28'28.1"W] and Boulware Springs (BS) [29°37'11.8"N 82°17'22.5"W]. Monogyne nests were collected from PR, BS, and a third location in Alachua Co.: Kanapaha Oaks (KO) [29°33'45.6"N 82°27'23.6"W]. A mean of 11.5 workers (pupae or adults) was sampled from each nest, along with reproductive (wingless, inseminated) queens (from 16 monogyne and 25 polygyne nests), daughter (winged, virgin) queens (from 22 monogyne and 23 polygyne nests), and males (from 14 monogyne and 20 polygyne nests) collected opportunistically as available. Also, sperm was extracted from the sperm-storage organ of 56 reproductive queens (from 17 polygyne nests) (see Data S1 for complete information on sample sizes).

To obtain known offspring of individual queens from polygyne nests, we established colony fragments with single reproductive queens isolated from field-collected nests from both sites; the fragments, consisting of a few thousand workers and brood, were

housed individually in shoebox-size plastic trays. Because some brood were offspring of queens from the polygyne source nest other than the isolated queen, we waited six weeks before sampling pupae so that all such brood had eclosed as adults. We genotyped individuals from 44 fragments, including the reproductive queens heading 36 fragments, daughter queens from nine fragments (88 total; mean = 9.8 per fragment), workers from 25 fragments (294 total; mean = 11.8 per fragment), and males from seven fragments (21 total; mean = 3.0 per fragment). We dissected the sperm-storage organs of 23 isolated queens for DNA extraction (see Data S1 for complete sample sizes).

Microsatellite Genotyping

We genotyped all samples (2277 individuals from 73 monogyne and 42 polygyne nests) at nine previously described polymorphic microsatellite loci (*Bertha, Sdag_C294, Sdag_C536, Sol_i114, Sol_i120, Sol_i126, Sol_i129, Sol-42f, and Sol-49*) [54]. A subset of sampled individuals (one individual from each of 30 monogyne nests; 43 polygyne queens—13 from seven nests at site BS and 30 from 15 nests at site PR) also was genotyped at 26 additional microsatellite loci (*Jackstraw, Jerry_Garcia*, *Sdag_C1*, *Sdag_C121*, *Sdag_C185*, *Sdag_C204*, *Sdag_C216*, *Sdag_C234*, *Sdag_C264*, *Sdag_C278*, *Sdag_C316*, *Sdag_C334*, *Sdag_C368*, *Sdag_C485*, *Sol_i113*, *Sol_i125*, *Sol_i127*, *Sol_i136*, *Sol-6*, *Sol-20*, *Sol-55*, *Sol-J1*, *Sol-M2*, *Sol-M3*, *Sol-M5*, and *Wharf_Rat*) [54]. This subset of samples was chosen for supplemental genotyping with two goals: 1) to confirm initial unexpected findings regarding the mode of reproduction in the polygyne form, and 2) to explore genetic differentiation between, and the phylogenetic origins of, the two asexual/polygyne lineages. With the first goal in mind, we genotyped a reproductive queen, a daughter queen, and a worker from 14 polygyne nests (including three nest fragments). With the second goal in mind, we genotyped 43 total queens from all known asexual multilocus genotypes (MLGs) that had been identified with the nine primary loci. This included four MLGs from site PR and five MLGs from site BS. For the monogyne form, we genotyped a single individual from each of 30 colonies from multiple collection sites in order to capture a meaningful portion of the population genetic variation in this form (because of high nestmate relatedness, the colony, rather than individual, is the relevant unit of genetic interest in the monogyne form).

Total genomic DNA was extracted from each individual (or sperm-storage organ) using the PureGene Core Kit A (QIAGEN). Microsatellites were amplified using previously described methods [54]. PCR amplicons were diluted (34:1 or 45:1, depending on the locus) and pooled before 1.5 μL was added to a 96-well plate. Liz 600 size standard (0.1 μL) and formamide were added to all dilutions before analysis on an ABI-3730XL-96 capillary sequencer (Applied Biosystems) at the Georgia Genomics and Bioinformatics Core at the University of Georgia. Microsatellite genotypes initially were scored using GeneMarker (SoftGenetics; https://softgenetics.com/GeneMarker.php) then were manually confirmed. We inspected microsatellite data quality both manually and with Microchecker [40] and found no evidence of genotype miscalling due to stutter, large allele dropout, or presence of null alleles.

Nuclear Genotypic Analyses

The genotype to individual ratio (G:N) [55] and observed heterozygosity (H_o) were calculated by randomly resampling a single individual from each nest and calculating these statistics for each of 10,000 iterations; this was done in order to avoid potentially biasing the estimates by using non-independent nestmate genotypes. G:N was calculated as the number of unique genotypes divided by the total number of individuals (nests). For each locus, H_o was calculated by dividing the total number of heterozygotes by the total number of individuals then averaging across the iterations; overall H_o was obtained by bootstrapping (1000 iterations) across the single-locus estimates.

To identify contributions to worker paternity by males of each social form, MLGs of workers and sperm were analyzed to determine whether they contained paternal alleles found within either the "asexual gene pool," comprising the complete set of alleles represented in the multilocus genotypes of polygyne reproductive queens, or the "monogyne gene pool," comprising the complete set of alleles represented in monogyne worker multilocus genotypes. The program *Structure* [47, 48] (https://web.stanford.edu/group/pritchardlab/structure.html) was used to calculate population assignment probabilities in a Bayesian framework for 1) the multilocus haplotypes of sperm from the sperm-storage organs of polygyne queens, and 2) the multilocus genotypes of workers from polygyne colonies. Based on our findings that the monogyne form appears to be a single outbred population and that two genetically differentiated groups occur at the separate polygyne study sites (see Hardy-Weinberg equilibrium and G''_{ST} results below), we specified three predefined genetic clusters (the monogyne gene pool, and reproductive queens from polygyne sites PR and BS) with the USEPOPINFO option. We also implemented the correlated allele frequencies option in calculating the assignment probabilities to each genetic cluster (appropriate given that the monogyne population and two asexual lineages clustered together in DAPC analyses including other nominal conspecific *S. geminata* populations). Because of the non-independence of nestmates, we ran the *Structure* analysis 100 times after resampling a single individual per nest (both for polygyne workers and for the predefined genetic clusters). Because each sperm multilocus haplotype represents an independent, presumably random male from the monogyne population, sperm haplotypes were not subjected to the resampling procedure.

We assessed the proportion of polygyne daughter queens with "worker-like" MLGs using the following criteria: 1) the MLG did not match that of reproductive queens or daughter queens from other nests, 2) the MLG was heterozygous at one or more loci for alleles not found in known asexual MLGs. Daughter queens with worker-like MLGs are noted in Data S1 and labeled in Table S1. Identifying such MLGs was typically straightforward, the sole exception being one at site BS found in three individuals from a single colony. This MLG resembled an asexual MLG because it was highly homozygous and shared many alleles in common with confirmed asexual MLGs. However, it was observed in only one nest, was not found in any reproductive queens, and was heterozygous at one locus for an allele not found in other polygyne queens from that site. Thus, we considered this MLG to be "somewhat worker-like," and

we calculated the proportion of polygyne daughter queens with worker-like MLGs both with and without these three individuals included. Additionally, one reproductive queen contained a somewhat-worker-like MLG—it matched a common site PR asexual MLG at the nine primary loci and 21 of the additional loci, but was heterozygous at five additional loci (Table S2; Figure S1).

Discriminant analysis of principal components (DAPC) using the nuclear genotypic data was conducted with the R package adegenet [41] (https://github.com/thibautjombart/adegenet); the procedure naively identified clusters of genetically similar individuals using the find.cluster() function. We used the dapc() function to conduct preliminary DAPCs retaining all principal components (PCs) and discriminant functions, used these results to generate the optimal number of PCs using the optim.a.score() function, then conducted a final DAPC retaining only this optimum number. We visualized all pairwise relationships for the first three PCs using scatterplots with superimposed minimum spanning networks connecting cluster centroids. To investigate possible local origins of the two polygyne subpopulations studied, we used data from 32 microsatellite loci in 30 polygyne queens from site PR, 13 polygyne queens from site BS, and 30 monogyne females, along with five previously published MLGs of *S. geminata* individuals from Mexico [22] as an outgroup. To investigate possible origins involving geographically widespread nominal *S. geminata* populations, we surveyed 23 microsatellite loci in Florida *S. geminata* and other nominal conspecific populations from around the world [23]. To investigate possible hybrid origins of the asexual/polygyne subpopulations, we included other members of the *S. geminata* species-group [22] along with Florida *S. geminata* in a third set of analyses, again employing 32 loci.

Simulations

We conducted simulations to test whether our empirical data are most consistent with one of three broad scenarios for the origin of our studied polygyne subpopulations from the local monogyne form: 1) a single origin followed by differentiation of two derivative lineages, 2) two genetically distinct monogyne founders, or 3) separate origins involving a primary origin from the monogyne form followed by a secondary origin stemming from a backcross product of the original polygyne lineage and the local monogyne population. As a first step, we randomly sampled two alleles per locus (with replacement—the probability of sampling an allele was conditioned on its frequency in the monogyne population) from the monogyne gene pool to reconstruct single hypothetical founder multilocus genotypes (MLGs). This strategy for reconstructing random founder genotypes is appropriate given that: 1) the monogyne form is in Hardy-Weinberg equilibrium (exact test, $p = 0.10$) (see also [4, 19]), 2) population genetic geographic structure is minimal in this form (jackknife mean values of Meirmans and Hedrick's G''_{ST} statistic = 0.05 between monogyne populations located 200 km apart in Gainesville and Tallahassee, Florida [56]; values calculated using the R package "mmod" [44]), and 3) our study loci are in linkage equilibrium in this form in Gainesville (mean pairwise $p > 0.55$ for all marker pairs). After sampling founder MLGs in distinctive ways (described below), we next reduced the sampled, highly heterozygous founder MLGs to complete homozygosity using one randomly selected allele at each locus. This step is appropriate because most polygyne queens studied were homozygous at all surveyed loci, presumably due to gradual stochastic reductions of heterozygosity following onset of asexuality.

To simulate a single origin of polygyny/asexuality with differentiation between polygyne sites due to distinct patterns of reduction in heterozygosity ("one-monogyne-founder" scenario), we twice independently randomly reduced a single sampled founder MLG from the monogyne form to homozygosity, with the two unique derivative homozygous MLGs then representing extant members of the two asexual lineages. Thus, this procedure represents a scenario in which a single founder queen from the monogyne form gave rise to two distinct subpopulations via divergent descent.

To simulate two genetically distinct monogyne founders of polygyne/asexual subpopulations ("two-monogyne-founders" scenario), we randomly and independently reconstructed two hypothetical founder MLGs from the monogyne form then subsequently randomly reduced each to homozygosity. Thus, this approach represents a scenario in which two monogyne founder queens separately established what became independent asexual lineages.

Finally, to simulate one primary origin of polygyny/asexuality combined with a secondary origin involving a "backcross" to the monogyne form, we treated the first heterozygous MLG derived from the monogyne form as representing the founder of a primary polygyne subpopulation and the second sampled monogyne MLG as the source of a gamete haplotype from a monogyne individual that mated to a reproductive from the primary polygyne subpopulation to produce a "backcross" daughter queen. That is, we combined a haploid version of the primary polygyne MLG (representing a gamete from a polygyne individual) with a second reduced (haploid) monogyne MLG (representing a gamete from a monogyne individual) to create a "sexually-produced" MLG, which was then randomly reduced to homozygosity to represent the secondary polygyne subpopulation. Because such a backcross could have occurred at any point during the gradual erosion of heterozygosity in the asexual genome, we bracketed the possibilities by conducting one version of the simulation with the backcross occurring before any heterozygosity was lost ("early-backcross" scenario) and one with the backcross occurring after complete reduction to homozygosity ("late-backcross" scenario). To implement these, we either randomly reduced the primary polygyne MLG to homozygosity independently of the randomly sampled gamete ("early-backcross" scenario), or used a diploid version of the randomly sampled gamete as the primary reduced MLG ("late-backcross" scenario).

We estimated G''_{ST} between the subpopulations over 1000 iterations for each different simulation scenario. The resulting distributions of G''_{ST} values from the simulations were compared to the empirical distribution generated from 1000 resampling iterations (one individual per nest).

mtDNA Sequence Analyses

We sequenced a 784bp portion of the *mitochondrial cytochrome oxidase subunit 1* (*COI*) gene for a subset of monogyne and polygyne individuals. These sequences were aligned manually with Sequencher (https://www.genecodes.com/) and Geneious (https://www.geneious.com/). A minimum spanning network of the haplotypes was constructed in PopART [42] (http://popart.otago.ac.nz/index.shtml), maximum parsimony (MP) phylogenies were constructed using TNT [43] (http://www.lillo.org.ar/phylogeny/tnt/), and maximum likelihood (ML) analyses were conducted in IQTREE [49, 51] (http://www.iqtree.org/). Partitioning of the data for the ML analyses was conducted using PartitionFinder2 [52] (http://www.robertlanfear.com/partitionfinder/), and the optimal partitioning strategy was defined using ModelFinder in IQTREE [50]. The TIM2 and F81 substitution models were selected for partition one (third codon position) and partition two (first and second codon positions), respectively. Using these parameters, we generated ML trees both with polygyne haplotypes constrained as monophyletic and without any such constraints—these trees were subjected to the Shimodaira-Hasegawa test [57].

Dissections of Male Reproductive Tracts

Two to five adult males were sampled randomly from each of eleven nests of each social form (mean = 4.05 males/nest). Males were dissected and their reproductive tract development was scored on a discrete scale from 1 to 4, according to the following scheme: '1' –reproductive tract not easily visible, very small and deflated, translucent; '2′ –reproductive tract more easily visible, small and deflated, typically with a faint white coloration; '3′ –reproductive tract easily visible, moderate size and turgidity, often with creamy white coloration; '4' –reproductive tract easily visible, enlarged and turgid, with creamy white coloration. The males were studied with the investigator blind to social form of origin.

QUANTIFICATION AND STATISTICAL ANALYSIS

To test whether genotypes in the Gainesville monogyne form conformed to Hardy-Weinberg expectations, we conducted exact probability tests using single resampled workers per nest with Genepop on the Web [45, 46] (http://genepop.curtin.edu.au/) and employing the default Markov chain parameter values. Significant differences in G:N values between groups were judged by non-overlap of the 95% confidence intervals derived from resampling analyses. Significant differences in H_o values between groups were judged by non-overlap of the 99% confidence intervals derived from 1000 bootstrap iterations across the single-locus values.

We used non-parametric Mann-Whitney U-Tests to determine whether the distributions of reproductive development scores for males of the two forms differed significantly. Because of the small number of discrete values and presumed non-independence of nestmate males, a randomization test was performed as well—a single male's score was randomly selected from each nest then randomly assigned to one of two 11-nest pools; means were calculated for each pool and statistically significant differences between the forms were inferred by determining if the mean differences between the actual scores for the two forms fell within the 2.5% tails of the score-difference distributions from randomized males obtained over 10,000 replicates.

G''_{ST} values were calculated between two simulated polygyne subpopulations over 1000 iterations. Probabilities of match between the simulation and empirical results and statistical significance were inferred by determining the percentiles in the distributions of simulation values corresponding to the mean value of the empirically derived G''_{ST} estimate.

DATA AND SOFTWARE AVAILABILITY

The microsatellite data and male reproductive development scores are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.jt0r643. The accession numbers for the mitochondrial haplotypes are Genbank: MK733396-MK733419.