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Mating frequency, colony size, polyethism and sex ratio in fungus-growing ants (Attini)

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Abstract The mating frequency of queens was estimated for eight attine ant species, *Myrmicocrypta ednaella*, *Apterostigma mayri*, *Cyphomyrmex costatus*, *C. rimosus* (four lower attines), *Trachymyrmex isthmicus*, *Sericomyrmex amabilis*, *Acromyrmex octospinosus* and *Atta colombica* (four higher attines), and correlated to colony size, worker polyethism, and sex ratio. Mating frequency was calculated from within-colony relatedness estimated by CAP-PCR DNA fingerprinting. Most queens of lower attines and *T. isthmicus* mated with only one male, while those of the three higher attines mated with multiple males. Mating frequency was positively correlated with colony size. Polyethism among workers was dependent on worker age in lower attines but on body size in higher attines, suggesting some correlation between mating frequency (i.e., within-colony gene diversity) and caste complexity. The sex ratio was biased toward females in species where the mating frequency equaled one, but toward males in species where the mating frequency was greater than two. Changing in nest site from ground surface to deep underground may have facilitated the evolution of large colony size in Attini, and this may have resulted in the evolution of polyandry (a queen mates with multiple males). With the evolution of polyandry in higher attines, *Atta* and *Acromyrmex* in particular have generated high genetic diversity within their colonies and complex social structures.

Key words Attini · CAP-PCR DNA fingerprinting · Polyandry · Polyethism

Introduction

Kin relatedness has been a major issue in animal ecology and sociobiology ever since Hamilton (1964a, 1964b, 1967) stressed the importance of kin selection for the evolution of altruism in animals. In social insects, kin relatedness within a colony is largely dependent on the number of reproductive females and their mating frequency. If high relatedness is needed for the evolution and maintenance of eusociality, monogyny and monandry would be expected to occur widely in social insects. In fact, many species of social insects are polygynous, and the repeated evolution of polygyny within the social insects has attracted considerable attention (e.g., Keller 1993).

Polyandry has been considered common in social insects, and to explain it, Woyciechowski and Lomnicki (1987) proposed a worker-policing hypothesis whereby polyandry decreased kin relatedness among workers and thus enabled queens to reduce the laying of male eggs by workers. However, recent genetic studies have revealed that polyandrous species are not as numerous as previously thought (Ratnieks and Boomsma 1995; Boomsma and Ratnieks 1996). To date, polyandry has been confirmed only in the honeybee *Apis mellifera* (Michener 1974; Adams et al. 1977), the yellowjacket wasps *Vespula maculifrons* and *V. squamosa* (Ross 1986; Ross and Carpenter 1991), the army ant *Eciton burchelli* (Rettenmeyer 1963; Franks and Hölldobler 1987), and the leaf-cutting ants *Atta sexdens* (Kerr 1961), *Atta texana* (Moser 1967), *Atta colombica* (Fjerdingstad and Boomsma 1998; Fjerdingstad et al. 1998), *Acromyrmex octospinosus* (Kerr 1961), and *Acromyrmex versicolor* (Reichardt and Wheeler 1996).

Boomsma and Ratnieks (1996) reviewed studies on paternity in eusocial Hymenoptera and concluded that the “sperm supply hypothesis” (Cole 1983) and “genetic variation hypothesis” (Crozier and Page 1985) have mer-

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it as general explanations for the evolution of multiple mating with different males (polyandry) in the social Hymenoptera. The “sperm supply hypothesis” suggests that polyandry is an adaptation for queens of species with large colonies to ensure high fertility by storing sufficient sperm from multiple males for the production of a large number of worker. Whereas the “sperm supply hypothesis” cannot explain why there has not been selection for large male ejaculate size sufficient to completely fill the queen’s spermatheca (Crozier and Page 1985; Keller and Reeve 1994), this hypothesis is consistent with the significant positive correlation between mating frequency and colony size reported for monogynous ant species (Boomsma and Ratnieks 1996).

The “genetic variation hypothesis” includes the following four sub-hypotheses. (1) Caste system hypothesis: polyandry increases genetic variation within a colony, enhancing the expression of a genetically based caste system. (2) Bet-hedging hypothesis: polyandry increases genetic variation within colonies and decreases the variance of fitness among females by expanding the range of environmental conditions that their offspring can tolerate. (3) Diploid-male hypothesis: polyandry reduces the variance among colonies in the proportion of diploid males that are abandoned as larvae, and the reduced variance is favored when queen fitness is a sigmoidal function of the proportion of a colony’s reproductive diploid (female) brood. (4) Disease/pathogen hypothesis: polyandry increases genetic variation within colonies, thereby reducing the likelihood that parasites or pathogens will diminish the worker/defense force to the point of jeopardizing the colony’s survival and reproduction (Hamilton 1987).

Moreover, mating frequency is expected to influence the sex ratio, if the sex ratio is controlled by workers striving to raise their inclusive fitness under kin selection. While the population-level investment ratio of males to females is expected to be 1:3 in the monogynous and monandrous species and assuming no worker reproduction (Trivers and Hare 1976), polyandry is expected to enhance the relative production of males and shift the sex ratio close to the optimum preferred by the queen (Macevicz 1979; Craig 1980; Page and Metcalf 1984; Page et al. 1993; Tsuji and Yamauchi 1994; Hasegawa and Yamaguchi 1995). In this process, workers are assumed to assess the genetic diversity of each colony and facultatively adjust the sex ratio (Grafen 1986; Boomsma and Grafen 1990, 1991; Boomsma 1993, 1996).

A full evaluation of these hypotheses regarding the relationship between mating frequency and social evolution is still lacking. Existing data on mating frequency of queens are fragmentary, coming from different phyletic lines within the Hymenoptera. The attine ants appear useful in this context because the tribe is composed of 12 genera exhibiting a variety of social structures. Furthermore, the polyandrous nature of some socially derived (*sensu* North et al. 1997) *Atta* and *Acromyrmex* species has already been confirmed (Fjerdingstad and Boomsma 1998; Fjerdingstad et al. 1998). In this study, we report on the mating frequency of attine species at differing

levels of social structure, using results obtained by CAP-PCR DNA fingerprinting.

Methods

A phylogeny of 11 attine genera has been proposed by Schultz and Meier (1995) based on the morphology of larvae. Of these 11 genera, the phyletic position of seven attine genera has been confirmed by Wetterer et al. (1998) based on larval mtDNA sequences. In the present study, we observed colonies of the following eight species of seven genera in Barro Colorado Island (BCI) and Gamboa, in the Canal Zone of the Republic of Panama, in 1993–1997: *Myrmicocrypta ednaella*, *Apterostigma mayri*, *Cyphomyrmex costatus*, *C. rimosus*, *Trachymyrmex isthmicus*, *Sericomyrmex amabalis*, *Acromyrmex octospinosus*, and *Atta colombica*. According to North et al. (1997) who followed Weber’s (1972) idea of dividing the 11 attine genera into lower attines and higher attines on the basis of phylogenetic information (Schultz and Meier 1995) and social behaviors (Weber 1954, 1955, 1956, 1958, 1966, 1972; Hölldobler and Wilson 1990), *M. ednaella*, *Ap. mayri*, *C. costatus*, and *C. rimosus* belong to the lower attines, and *T. isthmicus*, *S. amabalis*, *Ac. octospinosus*, and *At. colombica* to the higher attines. In this paper, therefore, the former four species are called “lower attines” and the latter four “higher attines.”

Colony sampling, dissection, and alate sampling

Colonies were collected on the floor of rainforests on BCI and in Gamboa. When a colony was discovered and its nest site had been described, the colony was collected to determine its size in the laboratory. All queens and ten workers per colony were dissected under the microscope to inspect their ovaries and spermathecae. The number of colonies used for dissection was: 18 *M. ednaella*, 25 *Ap. mayri*, 53 *C. costatus*, 69 *C. rimosus*, 43 *T. isthmicus*, 12 *S. amabalis*, 5 *Ac. octospinosus*, and 5 *At. colombica*.

During the nuptial season, November for *C. costatus* and March to May for the other species, the following number of alates were collected per species: 64 *Ap. mayri*, 317 *C. costatus*, 253 *C. rimosus*, 303 *T. isthmicus*, 397 *S. amabalis*, 132 *Ac. octospinosus*, and 1,540 *At. colombica*. *M. ednaella*, *C. costatus*, *C. rimosus*, and *At. colombica* were collected at almost the same site on BCI, and *Ap. mayri*, *T. isthmicus*, *S. amabalis*, and *Ac. octospinosus* were collected at two sites, BCI and Gamboa. *At. colombica* has huge colonies of several million workers, and it is therefore impossible to excavate whole colonies to obtain exact counts on sexual alates. To obtain an approximate estimate, we excavated ten randomly selected fungus gardens in each mature colony. Sexual alates of each species were fixed with Kahle solution (70% formaldehyde and 30% acetic acid) in the field, desiccated in the laboratory at 80°C for 48 h, and weighed to obtain the investment sex ratio of each colony.

Observation of behavior

Five entire colonies were sampled from each of the eight species, with the exception of *Ac. octospinosus* and *At. colombica* which form huge colonies and from which only partial colony samples could be removed. From each of the colony samples, 30–45 workers, 3–4 pupae, 5 larvae, 1–5 eggs, all queens (no queen could be collected in *S. amabalis* and *At. colombica*), and a fungus garden were chosen and placed into an artificial nest. These workers and queens were individually marked with colored paints. Observation of behavior was made for 10 h per colony, by scanning the activities of each ant at intervals of 15 min. During observation periods, water, food, and fungal substrates were always supplied to the foraging arena of the artificial nest. At the end of observations, head widths of all workers were measured under the microscope to categorize them into three body size classes: workers within a range between ± 1 SE of the mean were categorized as “middle-sized”; ants larger and smaller than this intermediate cate-

gory were classified as “large” and “small,” respectively. The workers were further categorized into three age classes according to their body color: yellow=young callows; brown=middle-aged; black=old. Discriminant analysis was conducted on all the variables to assess the degree of division of labor. This statistical method can analyze the correlation between age or body size categories and several labor types. The statistical analyses of three age/body size categories and six labor types in eight attine species were conducted in SYSTAT version 5.2.1.

CAP-PCR DNA fingerprinting analysis

For five to ten colonies of each species, DNA extractions were made from 15 randomly selected adults in 250 µl STE buffer after crushing with a homogenizer. Total genomic DNA was isolated from individual ants using the procedure of Murakami et al. (1997).

Prior to the analysis of the eight attine species, the variation in band patterns was tested preliminarily using four adults of each species per primer. The following eight CA-repeat primers were tested in the PCR: Pr. 1: (CA)7-(A/G/T)-G; Pr. 2: (CA)7-(A/G/T)-A; Pr. 3: (CA)7-(A/G/T)-T; Pr. 4: (CA)7-(A/G/T)-C; Pr. 5: (AC)7-(G/T/C)-G; Pr. 6: (AC)7-(G/T/C)-A; Pr. 7: (AC)7-(G/T/C)-T; Pr. 8: (AC)7-(G/T/C)-C (Ishibashi et al. 1995). PCR conditions followed the procedure reported in Murakami et al. (1997).

The preliminary analysis showed that the lowest band-sharing proportions were obtained for Pr. 1 in *C. costatus* and *Ac. octospinosus*, Pr. 2 in *C. rimosus* and *At. colombica*, Pr. 4 in *T. isthmicus*, Pr. 5 in *M. ednaella* and *Ap. mayri*, and Pr. 7 in *S. amabilis*. Therefore, these primers were used for analyzing mean relatedness. For *Apterostigma*, *Sericomyrmex*, and *Acromyrmex* only three, five, and two colonies, respectively, had queens, and calculation of parent-offspring relatedness was therefore limited to those colonies with queens. For *Atta*, no queen had been collected, therefore, parent-offspring relatedness could not be estimated. The estimated relatedness (r) was calculated from the within- (w) and between- (b) colony band-sharing proportion by the equation $r=(w-b)/(1-b)$ (Reeve et al. 1992). To calculate b -values, randomly selected colonies were run on the same electrophoresis gel. Assuming equal sperm usage of queens and no worker reproduction, the mating frequency of the queen (K) was estimated as $K=2/(4r-1)$ (Pamilo 1982).

Results

Nest size, colony size, and colony composition

Ap. mayri and *C. rimosus* usually nested in or beneath rotting wood, *C. costatus* was found under stones, and

M. ednaella and *T. isthmicus* below ground at a depth of 10 cm, while the other attine species nested at greater depths (Table 1). The three deep-nesting species, *S. amabilis*, *Ac. octospinosus*, and *At. colombica*, had thousands of workers and many chambers, while nests of other species had only one chamber where the queen, workers, and brood lived together on a fungus garden.

In *C. rimosus*, 64 of 69 collected colonies were monogynous. The remaining 5 colonies had two queens; both of queens were inseminated and had yellow bodies, indicating past oviposition for both. However, in all 5 cases, one of the two queens possessed five or six mature oocytes, while the other queen had only one or two mature oocytes in her ovaries, suggesting that these two-queen colonies were functionally monogynous.

Ten of 25 *Ap. mayri* colonies were monogynous, while the other 15 were queenless, perhaps indicating that this species, like other species in the genus (Weber 1972), form polydomous colonies. All colonies of the other six species had only one inseminated queen. While queens of *M. ednaella* and *C. costatus* had 4 ovarioles and *Ap. mayri*, *C. rimosus*, and *T. isthmicus* had 6 ovarioles, *S. amabilis* had over 10 ovarioles, and *Ac. octospinosus* and *At. colombica* had more than 50 ovarioles. Consequently, the number of mature oocytes per queen was more than 100 in *At. colombica*, but less than 10 in all lower-attine species and in *T. isthmicus* (Fig. 1). Queens of *Ac. octospinosus* had an average of 28 ± 3.94 mature oocytes per queen. Ovaries of workers were undeveloped with no mature oocytes in all species. In particular, ovaries of *At. colombica* workers were almost completely degenerated, indicating that workers of this species are incapable of oviposition.

Relatedness and mating frequency

The CA-repeat primers amplified highly polymorphic bands. The mean number of amplified bands was 11.3 ± 2.8 (SD) in *M. ednaella*, 9.9 ± 4.0 in *Ap. mayri*, 10.8 ± 4.0 in *C. costatus*, 11.1 ± 4.2 in *C. rimosus*, 10.9 ± 4.5 in *T. isthmicus*, 16.7 ± 2.03 in *S. amabilis*,

Table 1 Nest site, nest structure, and colony size for mature nests of eight attine species

Lower attines

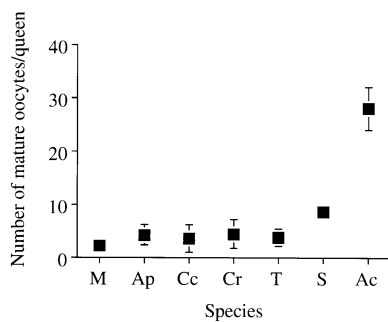
	<i>Myrmicocrypta ednaella</i>	<i>Apterostigma mayri</i>	<i>Cyphomyrmex costatus</i>	<i>Cyphomyrmex rimosus</i>
Nest site	Below ground	In rotting wood	Under rocks	In rotting wood
Depth (cm)	10	—	0	—
Number of chambers	1	1	1	1
Mean colony size (range)	60 (10–190)	20 (1–45)	40 (2–144)	60 (3–334)

Higher attines

	<i>Trachymyrmex isthmicus</i>	<i>Sericomyrmex amabilis</i>	<i>Acromyrmex octospinosus</i>	<i>Atta colombica</i>
Nest site	Below ground	Below ground	Below ground	Below ground
Depth (cm)	10–15	10–50	50–100	>100
Number of chambers	1	1	1–10	>100
Mean colony size (range)	60 (6–236)	200 (9–536)	>1,000	>100,000

Table 2 Modified regression estimates of relatedness between queen and worker (Q-W) and between worker and worker (W-W) in eight attine species

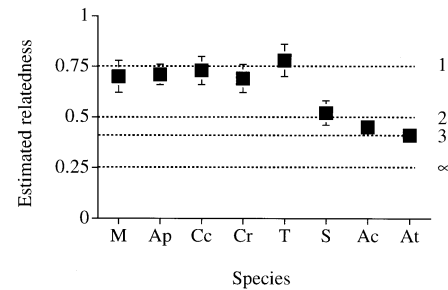
Colony	$n_{\text{colonies}}(n_{\text{individuals}})$	Band-sharing proportion			Modified regression Estimated $r=(w-b)/(1-b)$	
		Within colony (w)		Between colonies (b)	Q-W	W-W
		Q-W	W-W			
Lower attines						
<i>M. ednaella</i>	8 (120)	0.70±0.06	0.80±0.06	0.36±0.09	0.54±0.08	0.70±0.08
<i>Ap. mayri</i>	6 (90)	0.56±0.06	0.74±0.05	0.13±0.03	0.49±0.07	0.71±0.05
<i>C. costatus</i>	10 (150)	0.70±0.08	0.83±0.04	0.37±0.05	0.53±0.14	0.73±0.07
<i>C. rimosus</i>	7 (105)	0.66±0.10	0.77±0.05	0.27±0.06	0.53±0.12	0.69±0.07
Higher attines						
<i>T. isthmicus</i>	7 (105)	0.79±0.07	0.89±0.04	0.50±0.02	0.58±0.15	0.78±0.08
<i>S. amabalis</i>	9 (135)	0.70±0.05	0.75±0.03	0.48±0.04	0.40±0.07	0.52±0.06
<i>Ac. octospinosus</i>	9 (135)	0.69±0.09	0.66±0.03	0.36±0.05	0.54±0.14	0.45±0.04
<i>At. colombica</i>	5 (75)	–	0.68±0.02	0.43±0.01	–	0.41±0.03

**Fig. 1** The mean (\pm SD) number of mature oocytes per ovary in queens of seven attine species (*M* *Myrmicocrypta ednaella*, $n=18$; *Ap* *Apterostigma mayri*, $n=10$; *Cc* *Cyphomyrmex costatus*, $n=48$; *Cr* *C. rimosus*, $n=65$; *T* *Trachymyrmex isthmicus*, $n=40$; *S* *Sericomyrmex amabalis*, $n=12$; *Ac* *Acromyrmex octospinosus*, $n=5$). *Atta colombica* queens had a great number of mature oocytes per ovary (>100), and it was impossible to count them exactly

13.1±4.0 in *Ac. octospinosus*, and 19.24±2.52 in *At. colombica*.

Band-sharing proportions and estimated relatednesses of each species are shown in Table 2. The estimated relatedness of each species varied from 0.41 to 0.78 between workers and 0.40 to 0.58 between the queen and worker. The average estimated relatedness between workers in lower attines and *T. isthmicus* was close to 0.75. This indicates that the queens of these species mated with single males (Fig. 2). In *S. amabalis*, the estimated mean relatedness between workers was 0.50. Assuming equal sperm utilization, this suggests an effective mating frequency of two. With an effective mating frequency of three, the relatedness of daughters drops to 0.41. The estimated relatedness of daughters was 0.45 in *Ac. octospinosus* and 0.41 in *At. colombica*, suggesting an effective mating frequency near three in these species.

Thus, all queens were estimated to mate with a single male in *M. ednaella*, *Ap. mayri*, *C. rimosus*, *C. costatus*, and *T. isthmicus* – species with small colony sizes. The

**Fig. 2** Estimated relatednesses within colonies of eight attine species. Broken lines represent the predicted mating frequency per queen (*At Atta colombica*)

mating frequency of most queens in *S. amabalis*, which had a mean colony size of about 200, was inferred to be below but close to two. In *Ac. octospinosus*, which had relatively large spermathecae and often formed large colonies containing several thousand workers, the mating frequency was two to three. In *At. colombica* with huge colonies consisting of one queen and several million workers in mature colonies, mean mating frequency was estimated to be three (Table 2 and Fig. 2).

Polyethism among workers

Scans of worker behavior inside the nest chamber or in foraging arenas of artificial nests were used to examine worker polyethism using discriminant analysis of behavioral differences among the three age groups and three body size groups. Six worker activities were analyzed: repose, fungus care, nest defense, extranidal repose, and walking outside the nest.

Age polyethism

Discriminant analysis in the four lower attines showed that all six activities were significantly correlated with

Table 3 Correlation between six activities and two factors in a discriminant analysis of polyethism (*R* repose, *W* walk, *FC* fungus care, *D* defense, *RO* repose outside the nest, *WO* walk outside the nest; *FA* factor in the analysis of age effect, *FB* factor in the analysis of body size effect)

	Activities					
	R	W	FC	D	RO	WO
<i>M. ednaella</i>						
FA1	0.22*	0.48*	0.81***	0.14*	-0.54*	-0.99***
FA2	0.14*	-0.69*	0.53***	-0.65*	0.11*	-0.018***
FB1	-0.68	-0.37	0.02	-0.31	0.61	0.45
FB2	0.63	-0.24	0.40	-0.41	0.40	-0.14
<i>Ap. mayri</i>						
FA1	-0.62***	0.63***	-0.82***	0.41*	-0.15*	0.90***
FA2	0.19***	-0.51***	0.11***	-0.23*	-0.02*	0.28***
FB1	-0.53	0.06	-0.34	0.13	-0.12	0.79
FB2	0.42	-0.34	0.71	0.37	-0.26	-0.56
<i>C. costatus</i>						
FA1	-0.45*	-0.54*	-0.86***	0.07*	0.50*	0.83***
FA2	0.55*	-0.22*	0.18***	-0.14*	0.13*	-0.51***
FB1	-0.13	0.05	0.49	0.03	-0.21	0.06
FB2	0.04	-0.69	0.14	-0.50	0.27	-0.12
<i>C. rimosus</i>						
FA1	-0.46*	0.29*	-0.62**	0.55**	0.63**	0.83***
FA2	0.60*	0.32*	-0.18**	-0.51**	-0.12**	-0.13***
FB1	0.33	-0.13	0.69	-0.46	0.13	-0.07
FB2	0.20	0.74	0.08	0.60	0.37	-0.03
<i>T. isthmicus</i>						
FA1	-0.44*	0.25	-0.78***	0.31	0.14	0.76***
FA2	-0.50*	0.66	0.34***	-0.11	0.19	-0.31***
FB1	-0.51	0.44	0.76**	-0.23	-0.27	-0.50*
FB2	-0.39	0.30	-0.63**	0.09	0.22	0.74*
<i>S. amabilis</i>						
FA1	0.07	-0.18	-0.84***	0.64*	0.58	0.61*
FA2	-0.60	0.33	0.13***	0.07*	0.07	0.15*
FB1	0.05	-0.09*	-0.33	-0.37	0.40	0.51
FB2	-0.78	-0.16*	0.56	-0.06	0.04	0.13
<i>Ac. octospinosus</i>						
FA1	-0.19	0.04	-0.29	0.38	0.81	0.06
FA2	-0.32	-0.01	0.90	-0.01	0.06	0.12
FB1	0.62**	0.18	-0.31	-0.10*	0.35**	0.50**
FB2	-0.51**	0.04	0.47	0.07*	0.09**	0.85**
<i>At. colombica</i>						
FA1	-0.58	-0.57	0.08	0.39	0.57	0.59
FA2	0.59	-0.32	0.35	-0.49	0.21	0.67
FB1	-0.33	-0.21	0.94**	0.36*	0.52***	0.72***
FB2	0.01	-0.14	0.09**	0.48*	0.82***	0.24***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

factor 1 and 2 (Table 3), and all three age groups were differentiated from each other at a high confidence level of 79.1–100% (χ^2 -test, $P < 0.001$). In *T. isthmicus*, three activities, repose, fungus care, and walking outside the nest, and in *S. amabilis* three activities, fungus care, defense, and walking outside the nest, were significantly correlated with factors 1 and 2, and three age groups were discriminated at the low confidence level of 60%. In *Ac. octospinosus* and *At. colombica*, however, no activities were significantly correlated with either factor 1 or 2, and the three age groups could not be separated at a confidence level of 60%. These results suggest that while worker age may correlate with polyethism in lower attines, it probably does not for either *Ac. octospinosus*

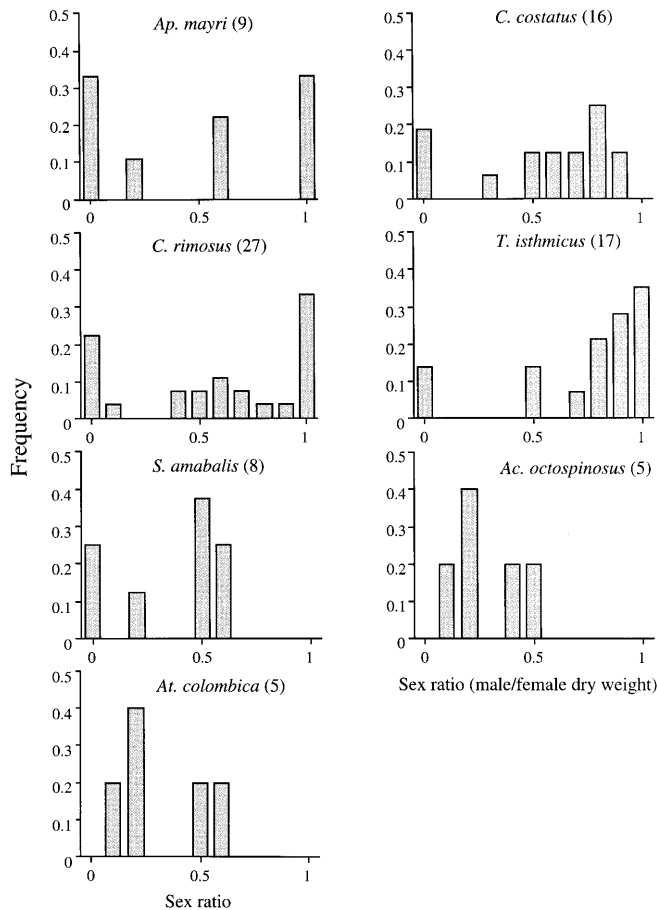
or *At. colombica*. A few significant effects of age on polyethism were observed in *T. isthmicus* and *S. amabilis*.

Body size polyethism

Worker activities were not significantly correlated with either of the two body size factors in lower attines (Table 3). Three body size groups could not be separated from each other at the 60% confidence level (Table 3). In *T. isthmicus* and *S. amabilis*, one or two activities were significantly correlated with factors 1 and 2, and three body size groups were distinguished from each other at

Table 4 Alate production and sex ratio in seven attine species. The expected sex ratio was calculated from insemination frequency in each species (calculation based on Boomsma and Grafen 1991)

	Total number		Total dry weight(mg)		Sex ratio (F/M)	Expected sex ratio
	Alate females	Males	Alate females	Males		
<i>Ap. mayri</i>	29	35	137.3	95.7	1.43	3.0
<i>C. costatus</i>	181	136	114.6	39.0	2.94	3.0
<i>C. rimosus</i>	127	126	86.4	37.8	2.29	3.0
<i>T. isthmicus</i>	219	84	553.7	104.0	5.32	3.0
<i>S. amabilis</i>	88	309	481.1	382.4	1.26	2.0
<i>Ac. octospinosus</i>	34	98	1,700.0	3815.5	0.45	1.7
<i>At. colombica</i>	352	1,188	161,354.0	359,973.5	0.45	1.7

**Fig. 3** Frequency distribution of colony sex ratios in seven attine species. Sample sizes are shown in parentheses

the low confidence level of 60%. In *Ac. octospinosus* and *At. colombica*, however, four activities were significantly correlated with factors 1 and 2, and three body size groups could be separated at confidence levels of 73.0 and 81.0%, respectively (χ^2 -test, $P < 0.001$). Thus, body size appears to be one factor which may affect worker polyethism in *Ac. octospinosus* and *At. colombica*, but not in lower attines. A few significant effects of body size on worker polyethism were found in *T. isthmicus* and *S. amabilis*.

Sex ratio

The sex ratio in each species, estimated by the ratio of total dry weight of alate females to that of males (Table 4), was correlated with queen mating frequency. In *Ap. mayri*, *C. costatus*, *C. rimosus*, and *T. isthmicus*, most queens were estimated to mate with a single male, and the sex ratio was biased toward females. In contrast, sex ratios were biased toward males in *Ac. octospinosus* and *At. colombica*, species in which the mean mating frequency was estimated to be between two and three. In *S. amabilis*, which had a mating frequency of about 2.0, the sex ratio (1.32) was biased toward males, a frequency more than twice that theoretically expected under the condition of mating frequency=2 and monogyny (Benford 1978). In all species, the sex ratio varied considerably among colonies (Fig. 3), between-colony variation being greater in species with smaller colony size.

Discussion

The queen's mating frequency estimated in this study for *At. colombica* agreed closely with the estimate by previous researchers; however, that of *Ac. octospinosus* differed from the data of Boomsma et al. (1999). This difference may have been due to sample size differences. Whereas Boomsma et al. (1999) analyzed 30–60 individuals per colony, our study analyzed 15 individuals per colony. Boomsma and Ratnieks (1996) suggested that at least 20 individuals per colony are needed to analyze the effective paternity. Because our sample size was slightly smaller than this suggestion, the *Ac. octospinosus* mating frequency may be underestimated.

Queens of *S. amabilis*, *Ac. octospinosus*, and *At. colombica* showed higher average mating frequencies than those of lower attines and *T. isthmicus*. This is the first report that the mating frequency in Attini varies among genera, especially in the higher attines *T. isthmicus* and *S. amabilis*. Villensen et al. (1999) reported the mating frequencies of three lower attines and, as in our study, they deduced that lower-attine queens are monandrous. *Atta* is among a shortlist of social insects that are polyandrous (Boomsma and Ratnieks 1996). Of the five hypotheses mentioned earlier, bet-hedging under

a wide range of environmental condition is unlikely to account for the evolution of polyandry in arthropods, because polyandry cannot significantly raise the fitness of females above that of monandrous females unless the cost of mating is nearly nil (Yasui 1998). The polyandry of Attini may support the disease/pathogen hypothesis, given that the tribe is constantly exposed to various fungi, including pathogenic forms, as it chooses and cultivates its mutualistic partner. However, the present study shows that five species of these ants are monandrous, and so the disease/pathogen hypothesis cannot be a general hypothesis to explain the evolution of polyandry in these ants. Whether species forming larger colonies are more frequently exposed to harmful pathogens than those with smaller colonies is not presently known (Villensen et al. 1998).

Crozier (1977) suggested that the production of diploid males is probable in ants. According to Crozier and Pamilo (1996), several ant species such as *Solenopsis invicta* (Ross and Fletcher 1985, 1986), *Rhytidoponera chalybaea* and *R. confusa* (Ward 1978), *Leptothorax ambiguus* (Herbers and Grieco 1994), and *Formica* ants (Pamilo and Rosengren 1984; Pamilo et al. 1994) have produced diploid males. Crozier and Page (1985) have emphasized that the reduction in the variance of diploid male production, under the heterozygosity sex determination model, favors polyandry when sexuals are produced late in colony growth. Boomsma et al. (1999) indicated that at least 7% of the matings in *Ac. octospinosus* had matched sex-determining loci, and thus would produce diploid males. However, their paternity share was very low and most diploid males may be removed as eggs or larvae. That is, the costs of producing a diploid male would be only a minor selective effect on the mating frequency in *Ac. octospinosus*. Therefore, the diploid-male hypothesis may not explain the difference in mating frequency between lower and higher attines.

Both remaining hypotheses, the sperm supply and the caste system hypothesis, appear to be consistent with the results from the present study. The caste system hypothesis was initially suggested by Crozier and Consul (1976) and has since been tested by many authors in honeybees (see Page and Robinson 1991), but has never been confirmed in ants. Comparing the relatedness of small- and large-bodied workers, the results may directly support the caste system hypothesis that division of labor in polyandrous attines is significantly more dependent on morphological than on age polyethism (Oster and Wilson 1978; Wilson 1983), because morphological polyethism requires a developmental differentiation which usually has a genetic basis. Nevertheless, the caste system hypothesis is unlikely to explain the origin of polyandry, because morphological differentiation among workers should take a long time to evolve. Furthermore, Woyciechowski and Warakomska (1994) showed that even in honeybees, genetic diversity between workers had no effect on the division of labor, and several authors have indicated that there is no convincing evidence that honeybee colonies with greater genetic diversity

have higher survival or reproduction than those with low diversity (Oldroyd et al. 1991, 1993, 1994; Fuchs and Schade 1994). Therefore, it is difficult to explain the evolution of polyandry in eusocial Hymenoptera using this hypothesis.

In contrast, the sperm supply hypothesis could apply to the evolution of polyandry, since insemination by a single male of the species *At. texana*, *At. sexdens*, *At. colombica*, *Ac. octospinosus* and *Ac. versicolor* (Kerr 1961; Moser 1967; Weber 1972; Cole 1983; Hölldobler and Wilson 1990; Reichardt and Wheeler 1996; Fjerdingstad and Boomsma 1998; Fjerdingstad et al. 1998) cannot fill a female's spermatheca, and monandrous queens therefore may be at a disadvantage in generating large colonies. However, in both *At. colombica* (Fjerdingstad and Boomsma 1998) and *Ac. versicolor* (Reichardt and Wheeler 1996), queens store only the number of sperm which can be supplied by a single male, a fact which cannot be explained by the sperm supply hypothesis for these polyandrous species (Boomsma et al. 1999).

Another ultimate factor for the evolution of polyandry may be the change in habitat from the ground surface, that is dead twigs and under stones which place limits on ant colony sizes, to deep under ground where ant colonies are often able to grow to enormous sizes (Hölldobler and Wilson 1990) because of the abundant space to build nest chambers.

In the five attine species which were detected to be monandrous based on the analysis of relatedness by CAP-PCR DNA fingerprinting, the sex ratio was biased to females as theoretically expected under worker control (Trivers and Hare 1976). In *Ac. octospinosus* and *At. colombica*, which were estimated to be polyandrous, however, the sex ratio (female/male) was about 0.45, whereas it is expected to be about 1.0 even under complete worker control (Boomsma and Grafen 1990).

Male-biased sex ratios have been reported in *A. mellifera* (Michener 1974) and *E. burchelli* (Rettenmeyer 1963). Nonacs (1986), Frank (1987), and Page et al. (1993) attributed such male-biased sex ratios to local resource competition (LRC) and/or Hamilton's (1975) idea that the investment in the worker force accompanying the queen ought to be added to female investment in the species which found new colonies by budding or fission of natal colonies (Franks and Hölldobler 1987). Since queens of *Atta* and *Acromyrmex* show independent colony founding but not budding or fission (Weber 1972; Hölldobler and Wilson 1990), Hamilton's idea does not explain the male-biased sex ratio of these ant genera. Leaf-cutting ants form huge colonies whose territories often cover several hectares of habitat and show severe competition for food resource. This suggests that LRC among colonies founded by new females may select for male-biased sex ratios in these ant species. However, this remains to be tested because there is little direct evidence that related queens of these ants settle near and compete more with each other than with non-related queens (Weber 1972), and even the sex ratio may not be correctly estimated for *Atta*.

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