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Camponotus fellah queens are singly mated

Danielle P. Mersch[1,2] · **Christine La Mendola[2]** · **Laurent Keller[2]**

Abstract The ant *Camponotus fellah* has been used in several behavioral and life history studies. An important factor that affects the genetic structure and division of labour within a colony is whether queens are singly or multiply mated. To determine whether queens are singly mated in *C. fellah*, as is the case in some other *Camponotus* species, we developed nine polymorphic microsatellite markers and sequenced 16 workers each from 20 colonies at six loci. Data in all colonies were compatible with queen monoandry. All the workers of one of the colonies had identical genotypes suggesting that they were clonally produced or that the queen was inbred. We, therefore, genotyped the mother queen as well as 31 more workers of the same colony at the same six loci plus the three remaining loci. These data revealed that the queen was homozygous at eight of the nine loci and that she mated with a male having a shared allele at all but one of the loci. Thus, the queen was apparently not only inbred but also probably mated with a brother.

Keywords *Camponotus fellah* · Microsatellites · Monoandry · Ant · Genetic structure

✉ Danielle P. Mersch
dmersch@mrc-lmb.cam.ac.uk

✉ Laurent Keller
laurent.keller@unil.ch

1 MRC-Laboratory of Molecular Biology, Francis
   Crick Avenue, Cambridge Biomedical Campus,
   Cambridge CB1 0QH, UK

2 Department of Ecology and Evolution, University
   of Lausanne, 1015 Lausanne, Switzerland

Introduction

The genetic structure of ant colonies varies widely between species and can impact colony immunity, reproductive output and division of labor (Crozier and Page 1985; Julian and Cahan 1999; Heinze and Keller 2000; Julian and Fewell 2004; Rheindt et al. 2005; Oldroyd and Fewell 2007; Smith et al. 2008). Several studies have shown that in colonies where queens are multiply mated workers from different patrilines vary in their tendency to specialize in different tasks, thereby potentially affecting the efficiency of division of labor and colony homeostasis (Robinson 1992; Rheindt et al. 2005; Schwander et al. 2005; Wiernasz et al. 2008; Waddington et al. 2010). Increased within-colony genetic diversity due to multiple patrilines and/or matrilines may also improve resistance to parasites and pathogens (Hughes and Boomsma 2004; Reber et al. 2008; Schmidt et al. 2011) thereby enhancing the lifetime and reproductive output of a colony. Thus, knowledge of the genetic makeup of colonies provides insights into what factors might shape colony function.

Studies in several species of the genus *Camponotus* (*C. herculeanus*, Gertsch et al. 1995; *C.* ligniperdus, Gertsch et al. 1995, Gadau et al. 1998; *C. floridanus*, Gadau et al. 1996; *C. nawai*, Satoh et al. 1997; *C. consobrinus*, Fraser et al. 2000; C. ocreatus, Goodisman and Hahn 2004 and *C. festinatus*, Goodisman and Hahn 2005) revealed that queens are generally singly mated with occasional instances of polyandry. A species of the genus *Camponotus* that has not yet been investigated is *C. fellah*. This species has been extensively used to study nestmate recognition (Boulay et al. 2000a, b; Katzav-Gozansky et al. 2004; Katzav-Gozansky et al. 2008), more recently to unravel the impact of social isolation (Boulay et al. 1999; Koto et al. 2015) and to decipher the organizational principles of division

of labor (Mersch et al. 2013; Greenwald et al. 2015; Kafsi et al. 2016). The colonies exhibit pronounced division of labor with workers organized in distinct social groups that specialize in different tasks (Mersch et al. 2013). The propensity of a worker to join a social group, and preferentially execute some tasks might thus be dependent on a worker's patriline. We developed and tested a set of nine microsatellite loci to determine whether *C. fellah* queens are singly mated, or whether workers come from different fathers.

Materials and methods

Collection of ants

Adult workers were collected from 20 lab-reared colonies (A–T) by opening the nest and then collecting workers randomly both near the brood and outside the nest. Each lab colony was established from a single queen collected during a mating flight in 2007 on the campus of the Tel Aviv University, Tel Aviv, Israel, which is within the natural range of the species (Ionescu-Hirsch 2009).

Genetic analysis

PCR primers were developed for *C. fellah* microsatellite loci from a partial genomic library that was derived from 10 μg of genomic DNA extracted with the BioSprint 96 robot (QIAGEN #900852) from one worker, and sent to Ecogenics (http://www.ecogenics.ch/) for 454 sequencing

and primer design. To determine which of the designed primers were polymorphic we tested nine primers on two workers from each of eight colonies (A–H). Six out of the nine microsatellites had five or more alleles in the workers tested (Table 1). We used these six microsatellites to assess queen-mating frequency in 20 colonies using 16 workers per colony. However, because there was a very high level of homozygosity in colony S, we increased the sample size to 47 workers and also genotyped the queen using one of her legs. In this colony we also genotyped all individuals at the three remaining microsatellites (7275, 7207, 7300).

We extracted DNA from each individual using the Bio-Sprint 96 DNA Blood Kit for tissue sample from QIA-GEN and eluted the DNA in a final volume of 200 μl. We performed PCR amplifications for each ant sample in a total volume of 20 μl. To reduce the amount of work and sequencing, we multiplexed primers 1284, 6826 and 5134 in one reaction, and primers 3186, 3675, 4380 in the other. Each reaction consisted of 4 μl of genomic DNA, 4 μl of 10×PCR buffer (QIAGEN), 1.92 μl of 25 mM MgCl₂, 0.32 μl of 25 mM dNTPs, 1 μl of each forward and reverse primer (10 μM), 0.3 μl of 5 U/μl Taq polymerase (QIA-GEN), 3.2 μl of 5×enhancers (Qsolution from QIAGEN), and 0.26 μl H₂O. The PCR consisted of an initial denaturing step of 5 min at 95 °C, followed by 35 cycles that each consisted of 30 s of denaturation at 95 °C, 30 s of annealing at 55 °C, and 60 s elongation at 72 °C. A final elongation step was performed at 72 °C for 10 min. We analyzed PCR products with the ABIprism3100 DNA sequencer and the GeneMapper Software (Applied Biosystems).

Table 1 Characteristics of the nine microsatellite loci from *Camponotus fellah* colonies

Locus	Primer sequence (5′–3′) (F: forward; R: reverse)	T_a (°C)	Repeat type	Size of cloned amplicon (bp)	No. of alleles	Amplicon size range
1284	F: GTCGGAATGTTGCACCTACG R: CAGACCGCGAGAGGAGATAC	53.8	(AG)	226	5	230–237
3186	F: AATTGAACGTTTCGTCCGCC R: AGTCTTTACGCCCTCCTACG	51.8	(AC)	190	8	194–206
3675	F: TTGTAGAGCGACGACGAGAG R: TGAGAGGTGAGAGCACGAAG	53.8	(CGT)	203	8	221–252
4380	F: TGGCTACAGTTGTTGTGCAG R: TTTGTCGCGGCTCGTAAATC	54.8	(AC)	115	10	126–148
5134	F: TACAATCGAGTGGACGGGAG R: CAGATGAAAGGCGAACGGTG	53.8	(CGA)	247	9	246–299
6826	F: GTACGCGGTCAGAATTACGC R: ACCTGATTTCTGGTTGGCAC	53.8	(AG)	176	14	178–207
7207	F: AGGCATTCGATACTGTCGGC R: TACGACAAGGATGCTACCGC	53.8	(TGC)	163	2	184–187
7275	F: CGCATCGGGAGCATAAACAG R: AAAGCTGGCATGCAGTTACG	53.8	(CA)	102	5	118–123
7300	F: AATTTGCGGAGAATCCGTGG R: CATCGGCAAAGTCGTCCTTC	51.8	(CT)	185	3	201–205

All forward primers are preceded with an 18-bp M13-tag: 5′-TGTAAAACGACGGCCAGT-3′

Data analysis

We estimated linkage disequilibrium between each pair of loci using the Genepop software (Raymond and Rousset 1995; Rousset 2008). For each colony, we determined the number of worker patrilines. This was simplified by the fact that all colonies were lab raised with all workers being the offspring of a single queen. Further, in ants, males are haploid because of the haplodiploid sex determination system of Hymenoptera. Thus, at each locus, a worker inherits one allele from her father and one of the two alleles of the queen. If two males share the same alleles at all genotyped loci, it is impossible to distinguish their offspring. The theoretical probability of such non-detection errors was estimated as:

$$\prod \sum q_i^2$$

with q_i as the frequency of the i allele (Boomsma and Ratnieks 1996). The sum is over all alleles at a locus, and the multiplication is over all loci.

We discarded from our patriline analyses 8 out of the 351 workers genotyped. Two samples from colony B were contaminated during the PCR and six workers from four different colonies (colony B: 2 workers, colony G: 2 workers, colony L: 1 worker, colony R: 1 worker) had genotypes incompatible with the genotype inferred for their queen. Because colonies were all lab raised and headed by a single queen, we suspect that these workers were intruders that had escaped from other colonies. Aggression toward non-nestmates is often attenuated in the lab for two reasons. First, colonies, which are kept in proximity to each other, habituate to olfactory volatiles of other colonies, which reduces aggression (Katzav-Gozansky et al. 2008). Second, lab colonies are typically fed with the same diet, which can reduce inter-colony differences in hydrocarbon profiles (Richard et al. 2004, Ichinose et al. 2009).

Results

The six microsatellite loci were very polymorphic with an average of 7.11 alleles per locus (range: 2–14, Table 1), as estimated from all genotyped workers ($n = 365$). There was no sign of gametic linkage among the loci ($p > 0.05$ for all pair-wise comparisons of loci after sequential Bonferroni corrections), and all loci were in Hardy–Weinberg equilibrium ($p > 0.4$). Overall, the probability of not detecting a second male was extremely low (4.89×10^{-4}).

Our patriline analysis showed that for all colonies and all loci, except one (locus 3675 in colony I), worker genotypes were consistent with workers originating from a queen mated to a single male (Table 2). In colony I,

Table 2 Summary of all worker genotypes and inferred male and queen genotypes

Colony	Locus	Worker genotypes (# workers)	Inferred male	Inferred queen
A	3675	224::230 (7) 221::224 (9)	224	221::230
	1284	230::236 (8) 231::236 (8)	236	230::231
	4380	128::128 (6) 128::132 (10)	128	128::132
	5134	261::261 (9) 246::261 (7)	261	246::261
	6826	188::188 (7) 188::190 (9)	188	188::190
	3186	197::197 (11) 197::203 (5)	197	197::203
B[a]	3675	221::221 (7) 221::228 (5) 221::224 (2)[b]	221	221::228
	1284	232::236 (10) 236::236 (4)	236	232::236
	4380	132::146 (6) 132::132 (7) 128::132 (1)[b] 128::132 (1)[b]	132	132::146
	5134	246::261 (12) 246::246 (1)[b] 246::272 (1)[b]	246 or 261	246::246 or 261::261
	6826	178::188 (8) 178::184 (4) 180::184 (2)[b]	178	184::188
	3186	201::203 (5) 199::201 (7) 203::205 (2)[b]	201	199::203
C	3675	221::224 (16)	221 or 224	221::221 or 224::224
	1284	230::230 (8) 230::236 (8)	230	230::236
	4380	130::144 (8) 144::148 (8)	144	130::148
	5134	246::270 (9) 249::270 (5)	270	246::249
	6826[c] (1)	186::190 (7) 186::196 (8)	186	190::196
	3186	197::203 (9) 203::203 (7)	203	197::203

Table 2 (continued)

Colony	Locus	Worker genotypes (# workers)	Inferred male	Inferred queen
D	3675 [c](2)	240::240 (5) 224::240 (9)	240	224::240
	1284 [c](1)	232::237 (10) 237::237 (5)	237	232::237
	4380 [c](1)	130::146 (9) 130::142 (6)	130	142::146
	5134 [c](1)	249::299 (5) 249::249 (10)	249	249::299
	6826	184::194 (12) 194::203 (4)	194	184::203
	3186 [c](1)	196::203 (7) 196::196 (8)	196	196::203
E	3675	228::252 (11) 224::228 (5)	228	224::252
	1284	230::232 (12) 232::236 (4)	232	230::236
	4380	130::130 (4) 130::146 (12)	130	130::146
	5134	246::246 (10) 246::249 (6)	246	246::249
	6826	188::196 (7) 196::196 (9)	196	188::196
	3186	197::205 (5) 197::197 (11)	197	194::205
F	3675	221::249 (9) 224::249 (7)	249	221::224
	1284	231::231 (4) 231::236 (12)	231	231::236
	4380	130::130 (10) 126::130 (6)	130	126::130
	5134	246::249 (10) 246::261 (6)	246	249::261
	6826 [c](1)	194::198 (7) 184::198 (8)	198	184::194
	3186	203::205 (7) 203::203 (9)	203	203::205
G	3675	221::228 (14) 221::224 (2)[b]	221 or 228	221::221 or 228::228
	1284	231::236 (8) 232::236 (6) 230::230 (2)[b]	236	231::232
	4380	128::128 (6) 128::130 (9) 130::130 (1)[b]	128	128::130
	5134	246::272 (10) 246::249 (6)	246	249::272
	6826	186::198 (8) 186::207 (6) 188::188 (1)[b] 188::199 (1)[b]	186	198::207
	3186	201::203 (10) 203::203 (4) 197::201 (1)[b] 201::205 (1)[b]	203	201::203

Table 2 (continued)

Colony	Locus	Worker genotypes (# workers)	Inferred male	Inferred queen
H	3675 [c](1)	221::221 (15)	221	221::221
	1284	231::236 (10) 231::231 (6)	231	231::236
	4380	128::130 (6) 130::132 (10)	130	128::132
	5134	246::261 (8) 246::246 (8)	246	246::261
	6826	186::186 (16)	186	186::186
	3186 [c](1)	203::203 (11) 203::205 (4)	203	203::205
I	3675[d]	240::240 (8) 224::224 (8)	[d]	[d]
	1284 [c](2)	230::236 (12) 232::236 (2)	236	230::232
	4380	128::135 (16)	128 or 135	128::128 or 135::135
	5134 [c](1)	246::246 (6) 246::267 (9)	246	246::267
	6826	192::192 (7) 192::199 (9)	192	192::199
	3186 [c](1)	201::203 (9) 203::206 (6)	203	201::206
J	3675	221::224 (16)	221 or 224	221::221 or 224::224
	1284	230::231 (10) 231::236 (6)	231	230::236
	4380	130::133 (9) 133::148 (7)	133	130::148
	5134	246::246 (16)	246	246::246
	6826	190::198 (7) 186::198 (9)	198	186::190
	3186	197::203 (16)	197 or 203	197::197 or 203::203
K	3675 [c](2)	221::221 (5) 221::236 (9)	221	221::236
	1284	231::236 (16)	231 or 236	231::231 236::236
	4380 [c](2)	144::144 (9) 130::144 (5)	144	130::144
	5134	246::261 (16)	246 or 261	246::246 or 261::261
	6826	184::201 (8) 184::192 (8)	184	192::201
	3186 [c](2)	203::205 (14)	203 or 205	203::203 or 205::205

Table 2 (continued)

Colony	Locus	Worker genotypes (# workers)	Inferred male	Inferred queen
L	3675 [c](1)	221::224 (10) 221::221 (5)	221	221::224
	1284	236::236 (9) 232::236 (6) 230::236 (1)[b]	236	232::236
	4380 [c](5)	128::132 (4) 128::128 (6) 132::133 (1) [b]	128	128::132
	5134	246::261 (16)	246 or 261	246::246 or 261::261
	6826	184::184 (8) 184::188 (7) 186::194 (1)[b]	184	184::188
	3186 [c](1)	203::203 (6) 199::203 (8) 197::199 (1)[b]	203	199::203
M	3675	221::221 (7) 221::224 (8) 221::--- (1)	221	221::224
	1284 [c](1)	231::236 (8) 231::231 (7)	231	231::236
	4380	130::130 (7) 130::146 (9)	130	130::146
	5134	246::275 (10) 246::246 (6)	246	246::275
	6826	194::196 (12) 196::207 (4)	196	194::207
	3186	197::201 (16)	197 or 201	197::197 or 201::201
N	3675 [c](1)	221::221 (9) 221::224 (6)	221	221::224
	1284	236::237 (6) 231::236 (9) 231::--- (1)	236	231::237
	4380	128::132 (10) 128::146 (6)	128	132::146
	5134 [c](1)	246::246 (15)	246	246::246
	6826	186::194 (16)	186 or 194	186::186 or 194::194
	3186 [c](1)	197::203 (5) 199::203 (10)	203	197::199
O	3675 [c](1)	221::221 (15)	221	221::221
	1284 [c](1)	232::232 (9) 231::232 (6) [f]	232	231::232
	4380 [c](1)	130::142 (6) 128::130 (9)	130	128::142
	5134 [c](1)	246::270 (6) 246::246 (9)	246	246::270
	6826 [c](1)	178::194 (9) 178::188 (6)	178	188::194
	3186 [c](1)	201::203 (3) 203::203 (12)	203	201::203
P	3675	221::221 (16)	221	221::221
	1284	231::236 (7) 231::232 (9) [f]	231	232::236
	4380	128::128 (16)	128	128::128
	5134	261::275 (16)	261 or 275	261::261 or 275::275
	6826	180::196 (10) 196::207 (6)	196	180::207
	3186	197::203 (8) 203::203 (8)	203	197::203
Q	3675	221::221 (16)	221	221::221
	1284	232::232 (6) 231::232 (10) [f]	232	231::232
	4380	128::130 (4) 130::130 (12)	130	128::130
	5134	249::249 (8) 249::272 (8)	249	249::272
	6826	184::203 (16)	184 or 203	184::184 or 203::203
	3186	199::201 (8) 199::199 (8)	199	199::201
R	3675	224::224 (15) 221::224 (1)[b]	224	224::224
	1284	230::236 (9) 230::231 (7)	230	231::236
	4380 [c](1)	128::128 (7) 128::146 (7) 126::128 (1) [b]	128	128::146
	5134	246::246 (8) 246::263 (7) 246::275 (1) [b]	246	246::263
	6826	188::196 (9) 184::196 (6) 188::207 (1)[b]	196	184::188
	3186	197::203 (15) 197::197 (1)[b]	197 or 203	197::197 or 203::203
S[e]	3675 [c](3)	221::221 (44)	221	221::221 [e]
	1284	236::236 (47)	236	236::236[e]
	4380	130::130 (47)	130	130::130[e]
	5134	246::246 (47)	246	246::246[e]
	6826	184::188 (47)	184	188::188[e]
	3186	203::203 (47)	203	203::203[e]
	7275	118::120 (23) 120::120 (24)	120	118::120[e]
	7207	184::184 (47)	184	184::184[e]
	7300	203::203 (47)	203	203::203[e]

Table 2 (continued)

Colony	Locus	Worker genotypes (# workers)	Inferred male	Inferred queen
T	3675 [c](1)	221::224 (16)	221 or 224	221::221 or 224::224
	1284	236::236 (10) 230::236 (6)	236	230::236
	4380	128::132 (14) 128::128 (2)	128	128::132
	5134	246::263 (16)	246 or 263	246::246 or 263::263
	6826	180::207 (7) 180::203 (9)	180	203::207
	3186	203::203 (11) 197::203 (5)	203	197::203

[a] Two samples were discarded because they had been contaminated

[b] Worker genotype is incompatible with the queen genotype and thus the worker(s) likely comes from another nest

[c] **(X)** number of samples that did not amplify

[d] All workers are homozygous for this locus, but there are two alleles in the colony, thus we could not infer the queen and male genotype

[e] We genotyped 47 workers and the queen for all nine primers

[f] Genotype profile is ambiguous, possibly homozygous

eight workers were homozygous for allele 224 at locus 3675 and the remaining worker homozygous for allele 240 (Table 2). Given that workers of this colony had normal genotypes at other loci, this pattern is most likely explained by the queen having a 224/240 genotype, and the male having a null allele at this locus.

In colony S, all 16 workers had identical genotypes. They were homozygous at five of the six loci while at locus 6826 they were all heterozygotes. This pattern could be explained by workers being clonally produced or by the mother being homozygous at the six loci and mated with a male having an identical allele at five of the six loci. To discriminate between these two hypotheses we genotyped all workers at three additional loci, analyzed another 31 workers from the same colony at the nine loci and also genotyped the queen. The 31 additional workers had identical genotypes as the initial 16 workers at the six first loci. For two of the additional loci all 47 workers had again identical homozygous genotypes, but for the third additional locus (locus 7275) 24 workers were homozygotes and 23 were heterozygotes. The queen was homozygote for all loci except locus 7275 for which she was heterozygote. Because workers were heterozygote at locus 6826 where the queen was homozygote, and varied in their genotypes for locus 7275, they were apparently sexually produced. The most parsimonious explanation for the genotypic composition of this colony is that

the queen was inbred and that she mated with a brother that shared the same allele at all but locus 6826.

Discussion

The genotypic data from a total of 343 workers from 20 colonies suggest that *C. fellah* queens mate with a single male, and thus all workers produced by the queen are full sisters. This, together with previous studies in *C. herculeanus* (Gertsch et al. 1995), *C. ligniperdus* (Gertsch et al. 1995; Gadau et al. 1998), *C. floridanus* (Gadau et al. 1996), *C. nawai* (Satoh et al. 1997), *C. consobrinus* (Fraser et al. 2000), C. ocreatus (Goodisman and Hahn 2004) and *C. festinatus* (Goodisman and Hahn 2005) indicate that queens are mostly singly mated in the genus *Camponotus*.

Estimates of patrilines can be inaccurate due to non-detection errors and small sample size (Pedersen and Boomsma 1999). Given the microsatellites were very polymorphic, the probability of non-detection of a second father was very low (<0.1%). Our sample size of 16 workers per colony enabled us to detect a second patriline with a high probability (>95%) if the second male contributed to about 20% of the workers (Boomsma and Ratnieks, 1996). In colony S we genotyped 47 workers enabling us to detect a second patriline with a probability of more than 90% even if the second male fathered only 5% of the workers (Boomsma and Ratnieks, 1996). However, also in this colony we only detected one patriline.

In one of the 20 colonies analyzed, worker and queen homozygosity was very high with only one heterozygote locus in workers and another one in the queen. Given the high polymorphism of the microsatellites, the most likely explanation for this high homozygosity is that the queen was inbred and that she mated with a brother. In ants, there is usually a low level of inbreeding due to the high synchronization of mating flight among colonies of the same populations (Tabot 1945; McCluskey 1965; Boomsma and Leusink 1981). Queens and males from *C. fellah* colonies typically leave their nest in the evenings of the first warm and dry spring days where individuals of many nests join in a mating flight (Hefetz A, personal communication). However, instances of inbreeding due to sib-matings have been reported in two Camponotus species (C. ocreatus, Goodisman and Hahn 2004 and C. *yamaokai*, Satoh et al. 1997) as well as several species of other genera (e.g., *Cardiocondyla batesii*, Schrempf et al. 2005; *Cardiocondyla nigra*, Schrempf 2014; *Cardiocondyla shuckardi*, Heinze et al. 2014; and *Formica exsecta*, Sundström et al. 2003; Vitikainen et al. 2015). Interestingly, the inbred queen that we found had apparently mated with a brother. A similar pattern has been uncovered in *Formica exsecta* where more inbred queens are also more likely to have mated with a

brother (Vitikainen et al. 2015). An association between the level of inbreeding of queens and their likelihood of mating with siblings could occur if there is a genetic basis influencing dispersal propensity. Females that are genetically less inclined to disperse may be more likely to mate with males of their nest and, therefore, become more inbred. Alternatively, the propensity to disperse may be directly influenced by the level of inbreeding (Vitikainen et al. 2015). Because more inbred queens may be smaller and less fit than outbred queens (Coltman et al. 1999; Keller and Waller 2002; McQuillan et al. 2012), they might disperse less (Wolf and Seppa 2016), thereby increasing their likelihood to mate with a sibling.

Mating has several costs that may select monoandry over polyandry (Daly 1978; Keller and Reeve 1995). For example, when mating with several males, females increase their risk of contracting sexually transmitted diseases (Knell and Webberley 2004; Peng et al. 2016), and also spent more time outside the nest exposed to predators (Lafaille et al. 2010; Alem et al. 2011). In addition, multiple mating results in sperm competition (Keller and Reeve 1995; den Boer et al. 2010) and associated conflict of interest between the sexes, which can reduce female lifespan and reproductive output (Chapman et al. 1995; Crudgington and Siva-Jothy 2000; Lessells 2005; Alonza and Pizzari 2013). Consequently, if mating with a single male can provide sufficient sperm to cover for the lifetime reproduction of the colony, and the benefits obtained from polyandry fail to compensate the endured mating costs, then queens might benefit more from being monoandrous.

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