Reconstructing the relatedness of cooperatively breeding queens in the Panamanian leaf-cutting ant *Acromyrmex echinatior* (Hymenoptera: Formicidae)

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Abstract

The evolution of permanent inquiline social parasites in ants has been conjectured to be facilitated by secondary polygyny, i.e. the re-adoption of new queens into existing mature colonies. This idea was first formulated by Wasmann, Wheeler, and Emery more than a century ago. Emery predicted that inquilines should be the sister-lineages of their hosts, which prompted Alfred Buschinger to propose that they evolve by sympatric speciation. However, these scenarios hinge on two vital conditions that have not been quantitatively documented: 1. That host sister-species are secondary polygynous and primarily recruit close kin, and 2. That such adoptions are prone to occasional mistakes that would select for the condition-dependent expression of exploitative traits and reproductive isolation by disruptive selection. Here we use a long-term data set on the leaf-cutting ant *Acromyrmex echinatior* (FOREL, 1899), known to have a closely related inquiline social parasite *A. insinuator* (SCHULTZ, BEKKEVOLD & BOOMSMA, 1998), to address the first of these conditions. We estimating the frequency of secondary polygyny and the degree to which cooperatively breeding queens are related. We find that the overall frequency of polygynous colonies is ca. 8% and that polygynous colonies typically have two queens. Most queen pairs are first degree relatives, consistent with colonies adopting one or two daughters either before or just after becoming orphaned. However, we also document a few pairs of cooperatively breeding queens that are unrelated and estimate that this social structure may apply to ca. 20% of the polygynous colonies, and thus ca. 1% of all colonies. Our findings show that the breeding system of *A. echinatior* matches the polygyny characteristics that are believed to facilitate the emergence of socially parasitic queen morphs.

key words: polygyny, social parasitism, inquilines, leaf-cutting ants

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Introduction

The evolutionary origin of inquiline ant social parasites ranks among the best documented cases of adaptive (sympatric) speciation (BOOMSMA & NASH 2014). The rationale is that such inquilines, which have convergently evolved in many ant genera (HUANG & DORNHAUS 2008), emanate from disruptive selection on alternative queen-morphs that coexist in the same colonies (EMERY 1909, HEINZE & KELLER 2000, SAVOLAINEN & VEPSÄLÄINEN 2003, BUSCHINGER 2009, RABELING & BACCI JR 2010, BOOMSMA & al. 2014). This implies that cooperative breeding of multiple queens in the same perennial colony is a prerequisite for the evolution of permanent inquilines. Lasting polygyny is essentially absent in most social insect lineages with permanent caste differentiation (corbiculate bees, vespine wasps, and higher termites) but common in the ants (BOOMSMA & al. 2014), consistent with permanent inquilinism appearing to be restricted to the ants. Cooperative breeding of multiple ant queens in the same colony is almost always a consequence of monogynous colonies re-adopting offspring queens back into the colony after they have been inseminated by unrelated males just outside their natal nest (HÖLLDOBLER & WILSON 1977, ELMES & KELLER 1993, BOOMSMA & al. 2014). In such cases kinship ties via the maternal line likely explain how cooperative queen breeding can be evolutionary stable (HÖLLDOBLER & WILSON 1977, NONACS 1988, KELLER 1993, KELLER 1995, BOOMSMA & al. 2014). Selection for polygynous cooperative breeding would be stronger when the alternative strategy, dispersal and *de novo* solitary colony founding, carry high mortality costs and are thus unlikely to be successful (BOURKE & HEINZE 1994). However, these dispersal constraints are counterbalanced by increasing sib-competition (WEST & al. 2002, BOOMSMA & al. 2014), so it is impossible for natural selection to remove all tendencies to disperse (HAMILTON & MAY 1977).

It has been proposed that ant polygyny arises from exclusive single-queen ancestry (BOOMSMA 2007, HUGHES & al. 2008) when some newly-inseminated queens occasionally succeed in re-entering their natal colonies. Any traits that favour this form of social promiscuity are also expected to select for some queens entering unrelated colonies, where they may be admitted in spite of the absence of maternal kinship (BOOMSMA & al. 2014). This produces a classic dichotomy between vertical transmission selecting for loyalty to kin and horizontal transmission selecting for parasitic exploitation of unrelated conspecifics (MAY & ANDERSON 1979, FRANK 1998).

Disruptive selection is likely to accumulate behavioural and morphological adaptations for a more efficient parasitic life-style in one of the queen morphs (RABELING & BACCI JR 2010, BOOMSMA & NASH 2014, RABELING & al. 2014). These adaptations normally include smaller body size, cryptic nest-intruding behaviours, cuticular chemistry that precludes easy recognition by non-kin. Ultimately, assortative mating could then arise and gradually sever gene-flow, so that inquiline populations become irreversibly reproductively isolated (BUSCHINGER 2009, RABELING & BACCI JR 2010). Over the years, substantial genetic evidence for adaptive sympatric origins of ant inquilines from their directly ancestral host lineages has accumulated (HUANG & DORNHAUS 2008, BUSCHINGER 2009), and some recent studies have reconstructed this process in considerable detail (SAVOLAINEN & VEPSÄLÄINEN 2003, RABELING & al. 2014, SCHÄR & NASH 2014).

An often-implicit assumption for sympatric speciation of ant inquilines is that evolutionary derived, polygynous species are more vulnerable to the emergence of inquiline social parasites over evolutionary time than monogynous sister lineages. This is because regular cooperative breeding of queens may lead to worker-controlled reproductive skew based on queen age (OZAN & al. 2013), i.e. differential contributions to the colony’s brood of reproductives rather than sterile workers (HEINZE & KELLER 2000, BOOMSMA & al. 2014). However, no permanent inquiline species are known to have evolved in the polygynous *Formica fusca* (LINNAEUS, 1758; *Serviformica*) ants studied by OZAN & al. (2013), in contrast to other genera such as *Myrmica* and *Acromyrmex* in which such social parasites have originated multiple times.

The secondary nature of polygyny is important because forms of primary polygyny (pleometrosis), i.e. cooperative colony foundation, are also relatively common in ants, and have led to other forms of social parasitism involving aggressive colony usurpation. This type of social parasitism has evolved in all lineages of social Hymenoptera, both basal ones with merely behavioural reproductive division of labour, and evolutionary derived ones in which all colony members belong to a single morphologically differentiated caste phenotype for life (BOOMSMA & GAWNE 2017). Early students of inquiline and slave-making ants did not always clearly separate between usurpation of incipient colonies by competing foundress queens and parasitism by dulosis (slave-making), but appear to have agreed that parasitism is derived from intraspecific queen adoption (EMERY 1909, WASMANN 1909, WHEELER 1910). Their main conceptual struggle was to explain dulosis due to the many secondary host switches in this type of social parasitism. However, while secondary polygyny might be the logical precursor state for the sympatric evolution of permanent inquilines (cf. BOOMSMA & NASH, 2014), there is no reason to predict that aggressive usurpation during colony founding should necessarily evolve directly from within host populations, i.e. according to the strict version of what came to be known as Emery's rule (EMERY 1909, HUANG & DORNHAUS 2008, LOPEZ-OSORIO & al. 2011).

The conditions that might allow the emergence of early (intraspecific) inquiline traits and the final adaptations of established inquiline species have been reasonably well studied (HEINZE & KELLER 2000, HUANG & DORNHAUS 2008, BUSCHINGER 2009, BOOMSMA & al. 2014, RABELING & al. 2014), but evidence for the intermediate steps in this process remains rare. The most critical transition appears to be the switch from the routine adoption of newly inseminated colony daughters to the derived establishment of a parasite lineage that relies completely on the exploitation of non-related colonies. In the present study we provide to our knowledge the first case study documenting secondary polygyny of a kind that is consistent with the putative necessary condition for making the transition to inquilinism in sympatry. Our study draws on almost 20 years of field surveys in Gamboa, Panama collecting hundreds of colonies of *Acromyrmex echinatior,* which is host to a rare sister-species inquiline *A. insinuator* (SCHULTZ, BEKKEVOLD & BOOMSMA, 1998; Schultz & al. 1998, Sumner, Aanen, & al. 2004) and is known to have a low frequency of polygynous colonies (BEKKEVOLD & al. 1999).

The leaf-cutting ant genus *Acromyrmex* is notorious for the convergent evolution of inquiline social parasites (SCHULTZ & al. 1998, DE SOUZA & al. 2007, RABELING & al. 2015), in spite of monogyny remaining the default colony structure. This contrasts with many other ant lineages (e.g. *Temnothorax, Myrmica*, *Crematogaster*) in which polygynous colonies have become the norm. When inquiline queens enter colonies that already have multiple host queens, overall relatedness among workers is low and kin-recognition efficiency possibly compromised (HEINZE & KELLER 2000, FOITZIK & HEINZE 2001). *Acromyrmex* leaf-cutting ants thus provide an interesting system to evaluate whether and to what extent colonies preferentially accept close kin as new reproductives, and whether occasional exceptions can also be found. We use multiple types of genetic marker analyses to reconstruct the most likely types of facultative polygyny that occur in *A. echinatior*. The present study greatly expands a previous one by BEKKEVOLD & al. (1999) which documented facultative polygyny in *A. echinatior* for the first time.

Material and methods

Sampling of polygynous colonies

During 15 field trips to Gamboa, Panama in the period 1993-2010, 197 colonies of *Acromyrmex echinatior* (identified according to SCHULTZ, BEKKEVOLD & BOOMSMA, 1998) were completely excavated so that at least one queen could be collected. For the purpose of the present study, we screened the field records for colonies in which more than a single potential mother-queen, characterized by a relatively dull and dark cuticle and acquiescent behaviour, had been observed. In 13 of the colonies two mother-queens were recorded during collection, and in two further colonies two or three potential mother-queens were discovered after the colonies had been set up in the lab (for details see Table 1 & Appendix S1, available as digital supplementary material to this article, at the journal's web pages). Of these 15 observationally polygynous colonies, we genotyped all queens in seven cases and one of two queens in an eighth colony, using four highly polymorphic microsatellite loci *Ech1390, Ech3385, Ech4126,* and *Ech4225* (ORTIUS-LECHNER & al. 2000). A single colony (Ae144) contained a third queen, which was homozygous at all loci, so we inferred she was a gynandromorph, which are known to occur in *Acromyrmex* leaf-cutting ants (Wheeler, 1931), and we omitted her from all further analyses. We also genotyped workers and in some cases males and/or gynes in 12 of the observationally polygynous colonies (for the remaining colonies no offspring samples had been kept; see Table 1 and Appendix S1 for details).

Detecting polygyny through offspring genotypes

For other projects (Appendix S2), we genotyped 27 colonies with one observed queen each and, in addition to the 197 queenright colonies already mentioned, another 6 colonies that had been collected without a queen. Among the 33 observationally not polygynous queens with genotyped offspring, we found two colonies (Ae028, Ae266) with genotypes that could not be explained by a single multiply mated mother queen (BEKKEVOLD & al. 1999), suggesting that these colonies had contained another fecund mother-queen as well (Table 1). We realize that the detection of second queens based on offspring genotypes is prone to false positives when these genotypes could represent unrelated stray workers from neighbouring colonies. Stray workers would seem most likely for samples from lab colonies because nest boxes may be close to neighbouring colonies on the same shelf. However, any such stray workers would be rare and unlikely to be related to each other, and their mothers should be unrelated to the queen(s) of the colony they were found in. These expectations were not met in the two colonies that had some workers whose genotypes could not be explained by a single queen despite there being no observation of a second queen (Ae028, Ae266). In both colonies, we found many offspring workers were incompatible with a single queen (18/40 workers in Ae028, 7/15 males and 5/20 gynes in Ae266), and in both cases, these workers were related among each other, suggesting they were likely offspring of the same mother who was in fact related to the single queen found in the colony. This would be an extremely unlikely coincidence for occasional stray workers and an impossible match for the sexuals, hence we explained these two cases as being consistent with a cryptic second mother queen.

Because the genotyped individuals of colonies Ae028 and Ae266 were collected well after excavation in the field, it is possible that these second queens were adopted after the colonies had become established in the laboratory (Table 1). Winged gynes and males sometimes start mating flights in the laboratory rearing rooms so we cannot exclude that some of these gynes can be adopted by their sister workers after insemination (NEHRING & al. 2015). However, our laboratory note-books indicate that only colony Ae266 had produced gynes in previous years in the laboratory, so the alternative explanation that a second queen was present in the field colony but missed upon collection seems more likely.

After dismissing that these two additional cases of polygyny were false positives, we concentrated our further analyses on estimating the possible non-detection error, i.e. the likelihood of having obtained false negatives because additional queens were overlooked. Such polygynous colonies that were assessed as monogynous are most likely when we had to base our estimate of queen number on offspring genotypes only, or when we observed two queens but the offspring genotypes only supported one mother (Ae043, Ae141, Ae168). Support for a second queen producing offspring is only obtainable when there is at least one marker locus for which the total set of offspring genotypes has at least three different maternal alleles, after adjusting for multiple paternity because *A. echinatior* queens are always inseminated by multiple males (see below). Colonies for which we only had offspring genotypes are therefore challenging even with highly variable co-dominant marker loci, particularly when the two putative mother-queens are full-sisters related by 75%.

To approximately quantify the likelihood of non-detection errors, we simulated 1,000 pairs each of full-sister queens, half-sister queens, mother and daughter, and unrelated queens, using the Kingroup software (KONOVALOV 2004), based on the observed population-wide allele frequencies that were deduced from the genotypes of 24 queens from monogynous colonies that had been genotyped over the years (Appendix S2). We then counted how many of the queen-pairs had a maximum of two alleles in common for each of the four marker loci, so their proportional presence could be used to adjust our overall likelihood estimates of the three possible scenarios of positive queen relatedness (full-sisters, half-sisters, mother and daughter) against a null hypothesis of no relatedness. We did not evaluate non-sampling error (BOOMSMA & RATNIEKS 1996) i.e. the probability of not detecting a second queen due to low offspring sample size, because the number of offspring genotyped was at least 32 in colonies deduced to be monogynous (Table 1).

Inferring genetic colony structures

Genotypes of queens that had not been directly genotyped were deduced from worker offspring both manually and by using the program COLONY (JONES & WANG 2009), the latter to check our manual inferences and to quantitatively assign maternity because COLONY allows for likelihood-based deduction of ambiguous alleles before assigning parentage. The results obtained were generally congruent across methods, with only a few exceptions. In two colonies for which we could document that two queens had contributed to the worker castes, we also found some offspring among the laboratory samples that could not be daughters of either of these queens and inferred they must have been rare escape workers from neighbouring colonies, i.e. false positives erroneously suggesting more than two queens (colony Ae020: 6/151; Ae047: 1/145). To reduce the likelihood of mistakenly diagnosing colonies as being polygynous, we always analysed maternity shares and included only workers with likelihoods of correct maternity assignment of at least 90% into the COLONY analyses. This also allowed us to evaluate the significance of reproductive skew with a binomial test. Resampling procedures suggested that, depending on the number of offspring workers genotyped (n), we would detect reproductive skew > 80% (for n = 30), > 74% (n = 60), > 68% (n = 90) and > 67% (n = 120). Finally, we analysed inbreeding (F_{IS})as the proportion of observed heterozygous workers and gynes relative to the expected heterozygosity based on each colony's allele frequencies across the four marker loci. There is some evidence that queens of *A. insinuator* do occasionally mate with brothers (BEKKEVOLD & BOOMSMA 2000), so checking whether the host might have such tendencies is relevant.

Reconstructing queen relatedness

We analysed genetic relatedness (R) among queens with the program Relatedness (QUELLER & GOODNIGHT 1989, GOODNIGHT & QUELLER 1999) against the background of the population’s allele frequencies (see above), focusing on the estimation of relatedness between pairs of queens in the same colony. We derived standard errors for relatedness by jackknifing over loci for the estimates of individual queen pairs, and by jackknifing across queen-pairs for the estimation of the average relatedness between cooperatively breeding queens. Based on the 1,000 simulated queen pairs (see above) of full-sisters, half-sisters, mother and daughter, and unrelated individuals, we inferred how much the values of R overlapped between these four hypothetical queen-relatedness scenarios to estimate the power of our R-estimates in discriminating among the specific scenarios of queen relatedness.

The power of relatedness estimates for discriminating between different kinship scenarios can vary widely and maximum likelihood approaches have proven to be the most appropriate tools to achieve inferences of this kind without bias (ARÉVALO & al. 1998, BLOUIN 2003). We therefore used Kingroup (KONOVALOV 2004) for a direct analysis of the likelihood that two cooperatively breeding queens were unrelated, a mother and daughter, half-sisters, or full-sisters, based on the observed allele frequencies in the genotypes of the queen-pairs. For each of the four alternative hypotheses, we also calculated the overall likelihood that all queen-pairs in our sample represented the same kinship scenario, by multiplying the likelihoods of all individual queen pairs. Some queen-pairs had at least one locus where they did not share at least one allele, which excluded the possibility of a full-sisters or mother-daughter relationship, so we inevitably obtained an overall likelihood of zero for these two specific scenarios. We resolved this by excluding these zero cases and re-calculating the overall relative likelihoods of being sister or mother-daughter pairs for all other queen-pairs.

To generate population-wide estimates, we also calculated likelihoods of the sister-queen scenarios at a finer scale, to account for the fact that *A. echinatior* is polyandrous and a colony's offspring are thus a mix of full-sisters and half-sisters. If cooperatively breeding queens were randomly drawn sisters from a colony's offspring, the expected relatedness would thus be expected to lie somewhere between full-sister (R = 0.75) and half-sisters (R = 0.25).

Results

Relative abundance of polygynous colonies

We found more than a single queen upon collection in 13 of the 197 queenright *Acromyrmex echinatior* colonies collected in Gamboa, Panama (Ae012, Ae020 Ae022, Ae043, Ae047, Ae109, Ae134, Ae141, Ae168, Ae263, Ae394, Ae406, Ae480). For ten of these colonies we had offspring genotypes, which supported two fertile queens in seven but not in the three remaining colonies (Ae043, Ae141, and Ae168; Sumner, Hughes, & al. 2004), in which all workers could be explained as offspring of a single queen (Table 1). We further found a second queen in two colonies after they had been established in the laboratory (Ae144 and Ae154). Molecular offspring data confirmed maternity by at least two fecund queens in both colonies (Table 1; DIJKSTRA & BOOMSMA 2007). The second queen could have been present in the field colony but have been overlooked upon collection, because collectors might not have scrutinized all fungus gardens by thorough fragmentation after the colony was confirmed to have a queen. Later queen discoveries also happened sometimes when a colony was registered as queenless in the field but later turned out to have a queen as established lab colony (DIJKSTRA & BOOMSMA 2008). Estimates of the frequency of polygyny purely from field observations about observed queen numbers are thus likely to underestimate the true number of polygynous colonies. Our subsample of 12 colonies with observed polygyny and genotyped offspring produced nine positively confirmed cases of functional polygyny and three ambiguous cases, yielding a minimal population-wide prevalence of polygyny of 9/197 = 4.6 %.

As mentioned in the Materials and Methods section, two further polygynous colonies (Ae028 and Ae266) were detected by offspring genotyping (Table 2; Appendix S2). If we assume that our sample of 33 genotyped but observationally non-polygynous colonies across the years is representative of all field colonies and that both colonies Ae028 and Ae266 were functionally polygynous in the field, this would imply that only counting queens upon field excavation would underestimate the prevalence of polygyny by 2/33 = 6.1%.

An additional risk of obtaining false negatives arises when genetic markers are not variable enough to discover a second queen, in particular when queens are related. In our simulations, 556 of the 1,000 full-sister pairs, 926 of the 1,000 half-sister pairs, 842 of the 1,000 mother-daughter pairs, and 975 of the 1,000 unrelated queen-pairs had between them at least three different alleles for at least one of the four marker loci. This implies that 43% of the full-sister queen pairs would have all alleles in common, so they would be scored as a single queen when only offspring genotypes are available. In contrast, our set of genetic markers would only have missed 2.5% of any unrelated queen pairs, 7.4% of the half-sister queen-pairs, and 15.8% of the mother-daughter queen-pairs.

Relatedness estimates of queen pairs

To assess whether second queens are kin or whether colonies adopt strangers, we directly estimated the relatedness among queen pairs. In eleven polygynous *A. echinatior* colonies we could either genotype all queens (Ae144), or infer the genotypes of two queens from offspring (Ae28, Ae109, Ae154, Ae266), or do both (Ae012, Ae020, Ae047, Ae394, Ae406, Ae480). In seven of these cases (Ae012, Ae028, Ae047, Ae144, Ae154, Ae266, Ae480) the two queens shared at least one allele for each marker locus (Table 1). This implies they could be either mother-daughter or full-sister pairs. We could exclude such close relatedness for the queen pairs in the other four colonies (Ae020, Ae109, Ae394, Ae406), which must therefore have been half-sisters or unrelated.

The cross-colony average coefficient of relatedness between queen-pairs was $R = 0.29$ ($SE = 0.09$), which is significantly greater than zero (one-sample $t = 3.42$, $n = 11$, two-tailed $p < 0.01$). The pairwise relatednesses across these eleven queen-pairs ranged from -0.08 to 0.73, spanning the entire range from unrelated ($R = 0$) to full-sister relatedness (0.75; Table 1). Our simulation of queen pairs showed that the four alternative kinship scenarios are difficult to disentangle based on pairwise relatedness estimates alone, because R can vary substantially within each kinship scenario so that estimates of alternative scenarios overlap (Figure 1). However, unrelated queens had a median R = -0.01 (interquartile range -0.22 – 0.22), half-sister queens a median R = 0.25 (0.05 – 0.45), full-sisters queens a median R = 0.76 (0.57 – 0.86), and mother-daughter queens a median R = 0.49 (0.36 – 0.62), suggesting that our simulation approach produced unbiased results with correct averages.

All relationships mentioned above are possible in principle because most colonies in the analysis were large enough to have produced gyne offspring before we observed the second queen or took the offspring samples from which we deduced the presence of a second queen. For nine of the 17 suspected or confirmed polygynous colonies we had directly observed sexual offspring from the field (Appendix S1). In two of the remaining colonies (Ae043, Ae047), we observed two queens during the field collections and both of these colonies were large enough at the time of collection to have produced winged reproductives in earlier years (i.e. having > 1 L fungus garden volume; BEKKEVOLD & BOOMSMA 2000). Four out of the 17 colonies did not contain alates (winged reproductives) and may have been too small to produce any (Ae012, Ae020, Ae141, Ae266). In these cases the additional queens may either have come from outside (unlikely given the relatedness estimates) or they may have been adopted years before we collected the colony, if colony size would have been declining since.

Since relatednesses of paired queens could not be unequivocally derived from the overall estimates of R (see also BLOUIN 2003), we also used a likelihood approach to find the queen relationship that was most likely for each colony. This indicated that queen-pairs were most likely to be mothers and daughters (5 pairs) or sisters (4 pairs) (Tab. 1, Fig 2), but that two of the pairs were unlikely to be related at all. Finally, we reconstructed the best overall maternity matches across all colonies by combining the colony-specific likelihoods that we obtained. The assumption that all queen-pairs are sisters (either full-sisters or half-sisters) explained the observed genetic marker data very well, whereas the likelihoods of all queens being mother-daughter pairs or unrelated pairs were zero or very low (Fig. 3). The likelihood curve peaked at an average offspring relatedness value of R = 0.285, i.e. close to the expected value for queen-pairs being half-sisters (0.25) and suggesting that only ca. 7% of the queen pairs were in fact full-sisters.

As *Acromyrmex* queens are obligatorily polyandrous, daughters of the same queen are known to be either full-sisters or half-sisters. The likelihood of being full-sisters is then

$R_p = 0.19$ (the inverse of the genetically effective paternity $R_e = 5.3$ (NIELSEN & al. 2003, SUMNER, HUGHES, & al. 2004). When queen pairs are assumed to be daughters of the same mother, an estimate of average relatedness of *Acromyrmex echinatior* queen pairs based on this likelihood comes out at R = 0.345 (0.81 x 0.25 + 0.19 x 0.75). This is somewhat higher than the R = 0.285 that we found for our dataset, which might be consistent with a few queen-pairs being in fact unrelated or with the analysis failing to recognize a significant number of the full-sister queen-pairs (see discussion of false-negatives for full-sister detection). In this population-wide estimate, the relative effect of less related or unrelated queen-pairs on the overall average is strong because even a single queen-pair not sharing at least one allele per locus will render the likelihood of the average queen-pair being full-sisters or mother and daughters zero, because such queen-pairs would always share alleles. When we excluded the queen-pairs that did not share alleles at all four marker loci, the maximum likelihood peaked at ca. $R_p = 0.15$, which is rather close to the expected value of $R_p = 0.19$. However, this analysis considered mother-daughter relationships to be the most prevalent kin structure among cooperatively breeding queens (Fig 3).

Possible effects of reproductive skew and sib-mating in queen-pairs

Both queens contributed to the offspring in ten out of twelve colonies where offspring workers or gynes were genotyped. We tested for maternity skew in workers and gynes separately and found evidence for unequal contributions in three of the colonies (Table 3). We also tested for a difference in skew between worker and gyne production within the five colonies for which both worker and gyne genotypes were available (Table 3). In one of these (colony Ae109), one of the queens contributed relatively more gynes than workers, but only ten gynes could be unequivocally attributed to either of the queens. Overall, these results appear to imply that reproductive skew is relatively minor, consistent with queen-pairs not forming direct dominance hierarchies. This implies that reproductive skew does not provide a significant overall complication for estimating the overall prevalence of functional polygyny in *A. echinatior*.

We found no indication for sib-mating because the colony-level inbreeding coefficients ranged from $F_{IS} = -0.27$ to $F_{IS} = 0.17$ (median -0.07). The only reasonable scenario for sib-mating to occur would require that gynes staying in their maternal colony at least sometimes refrain from a mating flight and mate with a brother inside the colony. Estimates of worker inbreeding coefficients did not covary with queen-queen relatedness, which is consistent with all queens mating outside the nest (Appendix S3). Also a high number of genetically distinct reconstructed mates for each queen (median 9.5, ranging from 4-18) seems impossible to match with a sib-mating scenario.

Combining alternative estimates for the prevalence of polygyny

Reviewing our combined analyses it seems clear that our first estimate of 4.6% polygyny across the population and sampling years, based on colonies for which both observational and molecular data supported two queens, is likely to be an underestimate. Considering colonies for which only observational or molecular data are available, and taking the estimated false-negatives specific to our methods into account, makes it possible to adjust this estimate, although at some danger of overestimating the true percentage.

Our best possible adjustments are as follows. We add two colonies, for which evidence for polygyny came only from offspring genotypes and a second queen might thus have been overlooked (6.1% of the genotyped observationally not polygynous colonies; Table 2). To further adjust this estimate for the detection efficiencies specific to the four alternative queen-relatedness scenarios, we multiplied the observed queen-queen relatedness-prevalences with their specific likelihood of overlooking a second queen when only offspring molecular data are available. The sum of these products (0.025 x 0.18 + 0.074 x 0.18 + 0.44 x 0.18 + 0.16 x 0.45) then produces the likelihood of a polygynous colony having been overlooked based on molecular data. This estimate (17%) implies an overall detection efficiency of 83% (100%-17%), which then produces a best estimate of 2/0.83 = 2.4 polygynous colonies among the 33 genotyped colonies that were observationally monogynous, which would imply that 7.3% of all observationally monogynous colonies are in fact polygynous. (Table. 2). Applying this proportion to the remaining 155 observationally monogynous colonies that were not genotyped would add thus another 11.3 polygynous colonies, so that a revised overall prevalence estimate accounting for both, average molecular non-detection error and false-negatives owing to related queens, is 11.2%, i.e. an estimated 22.7 out of the 203 excavated colonies being polygynous.

Finally, there were three colonies (Ae022, Ae134, Ae263, see Table 1) in which two queens were observed but no molecular data were available. Adding these would increase the overall polygyny prevalence estimate for the Gamboa population of *A.*

echinatior to 12.7% (25.7 out of 203 colonies, Table 2), but some of these queens might not have had offspring among the workers. Overall, therefore, we inferred that our combined data suggest that the frequency of colonies with two cooperatively breeding queens was probably intermediate between the scenarios that we explored. To facilitate discussion in round figures we will use a polygyny prevalence of 8%, which, combined with the breakdown of types of polygyny (Table 1), implies that ca. 92% of the *A. echinatior* colonies in Gamboa, Panama were monogynous, 3.5% were polygynous with a mother-offspring pair of queens, another 3% with a sister pair of queens, leaving 1.5% being polygynous without any relatedness between the pair of queens.

Discussion

Our analysis encompassed a total of 203 excavated colonies of *Acromyrmex echinatior*, of which 45 colonies were genotyped with microsatellite markers. It confirms and adds considerable detail to an earlier study (BEKKEVOLD & al. 1999) showing that polygyny in this leaf-cutting is a real but rather rare phenomenon (ca. 8% prevalence). It also became clear that colonies do not typically have more than two co-breeding queens, which are first degree relatives in the large majority of cases. This implies that workers may adopt a single reserve queen even though their mother queen is still fully functional, or two sister queens before or just after they become orphans. However, we also provide evidence that it is in fact possible that ca. 20% of the co-breeding queens are unrelated, suggesting that mistakes in the adoption of inseminated daughter queens can occur. To our knowledge this is the first study to provide robust estimates of relatedness structures among queens in an ant species where the prevalence of polygyny is low, which is difficult to achieve due to the large number of colonies that need to be sampled and genotyped.

Co-breeding queens in polygynous colonies are related

Our two independent types of analyses both showed that queen-pairs are likely to be first degree relatives, but emphasized different aspects. Direct relatedness analyses showed that queen-pairs heading polygynous colonies have an average relatedness of R = 0.29, suggesting mostly half-sister (R = 0.25) and occasional full-sister (R = 0.75) relationships, but not excluding mother-daughter (R = 0.5) combinations or occasional unrelated queen-pairs (R = 0). Alternative maximum likelihood analyses also identified a combination of full-sister and half-sister queen pairs as the most likely scenario for explaining the overall genetic marker patterns across colonies, but identified mother-daughter combinations as the most likely scenario after excluding colonies whose queens could not possibly be closely related.

In spite of these relatively consistent first-degree relative patterns, it is not straightforward how newly inseminated queens occasionally end up being adopted in their natal colonies. Mother queens from established colonies of the related *Acromyrmex subterraneus molestans* (SANTSCHI, 1925) have a high chance of being accepted into conspecific non-nestmate colonies, which is likely the reason for the substantial prevalence of polygyny in this species (SOUZA & al. 2005). Although it is not known to what extent these cooperatively breeding *A. subterraneus molestans* queens are related, it seems of interest that other subspecies of *A. subterraneus* are also polygynous (*Acromyrmex subterraneus subterraneus* (FOREL, 1893), see DE SOUZA & al. 2004; *A. subterraneus brunneus* (FOREL, 1912), see DELABIE 1989) and harbour an inquiline social parasite, *A. ameliae* (DE SOUZA & AL, 2007) that convergently evolved many similar traits as the *A. insinuator* inquiline of *A. echinatior* in Panama (DE SOUZA & al. 2007).

Rare field observations during the 15 years of field collections in the period 1993-2010 reported here have suggested that virgin reproductives of *A. echinatior* aggregate and mate at landmarks such as big trees, and that inseminated queens may fly away from these trees before shedding their wings and excavating a shallow cavity to deposit the fungus-garden fragment that they transported in their infrabuccal pocket (WEBER 1972, FERNÁNDEZ-MARÍN & al. 2004, POULSEN & al. 2009). As long-distance dispersal seems incompatible with finding the natal nest, it might be that colonies are more likely to become polygynous when they happen to be located near such mating landmarks, also because *A. echinatior* colonies typically kill non-nestmate queens (NEHRING & al. 2015), in contrast to *A. subterraneus molestans* colonies. The ants' nestmate recognition system would then act as a natural filter so that normally only a former nestmate gyne would be allowed to re-enter the colony. However, rare recognition errors can happen (NEHRING & al. 2013, LARSEN & al. 2014, NEHRING & al. 2015), consistent with few colonies adopting a non-related additional queen. An alternative hypothesis would be that adopted daughter queens never leave their natal colonies and mate in or very close to their nest. However, that hypothesis seems unlikely as it should imply that such queens mate with fewer males, likely brothers, so that their offspring would be inbred.

These implications were incompatible with our data sets when we manually checked for paternity nested within maternity and offspring homozygosity. The absence of sparsely inseminated queens also makes the scenario of some colonies acquiring a second queen after insemination of gynes in the lab a rather academic possibility.

If daughter queens are occasionally re-adopted, they would have some likelihood of breeding alongside their mother, which would explain that the mother-daughter scenario appeared to be most likely in a number of polygynous colonies. Once the old mother queen dies, such colonies would then either return to the monogynous state or remain polygynous when two newly mated daughter queens were adopted. Sister queen pairs might also arise in recently orphaned *Acromyrmex* colonies that raise "emergency gynes", i.e. turning all female brood into gynes before starting to produce worker sons from unfertilized eggs (DIJKSTRA & BOOMSMA 2007). As gynes are related to half-sister sons by only 0.125, inbreeding would be less detectable than in the brother-sister mating (R = 0.25) scenario rejected above. However, the explicitly outbred scenario of landmark-related adoption seems to remain the most parsimonious hypothesis.

While landmark-related adoption of daughter queens might occur due to an almost "passive" filtering process, it might well be adaptive since it allows versatile colonies at high quality nest sites to considerably extend their life-span. *A. echinatior* colonies might thus sometimes resemble termite colonies in which dying queens or kings can be replaced by their own offspring without much cost to colony efficiency (HARTKE & BAER 2011). However, in contrast to the termites producing replacement reproductives to mate with siblings, newly adopted *Acromyrmex* queens introduce new alleles into the colony when they are inseminated by unrelated males. This has the potential to introduce reproductive conflict between the worker offspring of the old and the new queen, similar to what happens during queen replacement in honeybees (WOYCIECHOWSKI & KUSZEWSKA 2012). While this conflict is expressed in the honeybee, in which queens never co-breed, it may never become an actual conflict in *Acromyrmex* colonies unless the required kin-discrimination mechanisms evolved to be sufficiently efficient (NEHRING & al. 2011).

Pleometrosis appears to be unlikely in A. echinatior

An alternative route to polygyny would be cooperative colony founding by unrelated queens. This has been observed in several ant lineages, but usually results in the elimination of all but one queen when the first workers hatch (STRASSMANN 1989, BERNASCONI & STRASSMANN 1999). Pleometrosis has been documented in a relative of *A. echinatior*, the desert-living species *Acromyrmex versicolor* (PERGANDE, 1894), which often founds colonies by groups of unrelated queens (HAGEN & al. 1988, RISSING & al. 1989). Co-founding *A. versicolor* queens appear to survive for at least half a year, i.e. well into the stage when they already have a considerable workforce (CLARK & FEWELL 2014), but it remains unclear whether this ever results in lasting polygyny of mature colonies. As in *Lasius* ants in which pleometrosis enhances early colony survival (SOMMER & HÖLLDOBLER 1995, HOLMAN & al. 2010), cooperative colony founding in *A. versicolor* improves the chance of successful fungus garden initiation, which may be beneficial in a desert environment where many foundresses compete for rare habitat patches with suitable resources (RISSING & al. 1986, RISSING & POLLOCK 1987, CAHAN 1999). However, incipient colonies with two or more queens are almost never observed in the Gamboa population of *A. echinatior* (FERNÁNDEZ-MARÍN & al. 2004; we only know of a single case among over two hundred founding colonies that were collected; J. Howe, unpublished observations), likely because founding queens are normally aggressive against intruders (HOWE & al. 2016). It thus appears that queen adoption in mature colonies is the prime viable route towards polygyny in *A. echinatior*, although the mating at landmark scenario does not completely exclude that two sister queens might find each other and initiate a colony together.

Adoption of unrelated queens and the evolution of social parasitism

While the majority of cohabiting queens had been adopted by their own mother colonies, two out of eleven polygynous colonies likely contained unrelated queens. In these instances, new queens apparently succeeded in becoming adopted in an alien mature colony despite the typically efficient recognition and expulsion of non-nestmate queens (NEHRING & al. 2015). Such adoption of an unrelated queen may double the productivity of the joint colony and create a cooperative win-win situation. However, when joint productivity is constrained, the immigrating unrelated queen may be under selection to cheat by investing less in somatic colony growth (i.e. producing worker brood) and more in colony reproduction. This is because an unrelated immigrant queen gains no indirect fitness benefits from the offspring of the resident queen. She would benefit disproportionally from diverting resources towards the production of her own reproductive offspring. This scenario reflects what has been hypothesized for the sympatric evolution of permanent inquiline social parasites from within their host species, with facultative polygyny as necessary condition for the early evolution of intraspecific social parasitism (SAVOLAINEN & VEPSÄLÄINEN 2003, BUSCHINGER 2009, RABELING & BACCI JR 2010, BOOMSMA & NASH 2014). As our analysis consistent with outbreeding and not with any queens being inseminated by close relatives in the nest, supporting BUSCHINGER'S (2009) conjecture that sibmating of inquiline species evolves as a derived trait and is not part of a preadaptation syndrome of hosts.

Our sample of merely two cases of a putative unrelated queen-pair was insufficient to detect forms of cheating because we could not tell which of the queens was the original resident. Detecting intraspecific cheating in the form of overproduction of reproductives may be subtle and would require large numbers of gynes and workers to be genotyped, as in an analysis of cheating patrilines in *A. echinatior* (HUGHES & BOOMSMA 2008). However, if exploiting the joint public good pays off, queens entering non-related colonies may come under selection to start expressing incipient inquiline traits such as body size reduction, loss of the worker caste, and chemical adaptations to avoid being recognized as a non-nestmate (HEINZE & KELLER 2000, BUSCHINGER 2009, RABELING & BACCI JR 2010, BOOMSMA & NASH 2014, SCHÄR & NASH 2014). If assortative mating arises during this process, e.g. through a shift in mating locality or mating time, reproductive isolation from the host may follow and a new social parasite species would evolve. This scenario matches what is so far known from the biology of the *A. echinatior* inquiline *A. insinuator* (SCHULTZ & al. 1998, BEKKEVOLD & BOOMSMA 2000, SUMNER, AANEN, & al. 2004, LAMBARDI & al. 2007, NEHRING & al. 2015). However, much of this remains conjecture, because of the low number of polygynous colonies that were available.

During the entire sampling period (1993-2010), sympatric colonies of the related species *Acromyrmex octospinosus* (REICH, 1793) were also routinely collected and transported to rearing rooms in Copenhagen, amounting to a total sample of 166 colonies. One of these colonies (Ao044) contained three potential mother-queens upon collection, but a single queen was sufficient to explain all offspring, suggesting that the additional queens were in fact unmated soldier-like helpers (NEHRING & al. 2012). In only one other colony (Ao273) did we find a second queen after it had been set up in a Copenhagen rearing room. In this case, the queen pair shared alleles in two out of the four marker loci, suggesting that the queens were half-sisters, and not full-sisters or mother and daughter, consistent with multiple insemination and effective paternity (*N*e = 5.3 - 6.8) being of similar magnitude as in *A. echinatior* (ORTIUS-LECHNER & al. 2003). These data suggest that functional polygyny in Panamanian *A. octospinosus* is possible but truly exceptional and perhaps an order of magnitude less common than in *A. echinatior*.

Queens of the inquiline *A. insinuator* manage to become adopted in colonies of *A. octospinosus* with about equal efficiency as in *A. echinatior* colonies, but they almost never suppress the host queen and realize their typical semelparous burst of reproduction that normally kills *A. echinatior* host colonies (SCHULTZ & al. 1998, BEKKEVOLD & BOOMSMA 2000, SUMNER & al. 2003, NEHRING & al. 2015). This pattern is consistent with Emery's rule for the evolution of inquiline social parasitism in ants (SUMNER, AANEN, & al. 2004, HUANG & DORNHAUS 2008, BUSCHINGER 2009, RABELING & al. 2014), predicting that new inquiline species initially evolve as sister species of their host (the strict version). Once they have become fully specialized on a parasitic life history, they may later colonize additional host species of the same genus, which can lead to further adaptive radiation in the inquiline lineage (captured by the loose version of Emery's rule). The barely successful colonization of *A. octospinosus* as a secondary host may thus represent the incipient stage of this further speciation process.

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Author contributions

JJB, MBD, VN and WOHH developed the rationale of this study over the last decade; MBD, SRS, VN and WOHH obtained the genotyping data; VN did most of the analyses after MBD and WOHH initiated some of them; JJB and VN wrote the paper with input from WOHH. All authors approved the final version of the manuscript.

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Tables

Table 1. Details for seventeen colonies of *Acromyrmex echinatior* (Ae) and two of *A. octospinosus* (Ao), which either had two or more observed queens that were mostly but not always available for genetic analysis, or produced workers whose genotypes we could only explain by deducing more than a single mother queen. For colonies where the genotypes of two queens were known, we highlighted the likelihood of them being related by: 1) listing the number of marker-loci for which they shared alleles (out of the maximum of four loci genotyped; 3 in one case), 2) estimating the pairwise relatedness ± SE via jackknifing over all loci (Goodnight & Queller 1999), and 3) providing the

relationship with the highest likelihood in a formal likelihood-analysis (Konovalov 2004). For some colonies, additional queens were observed once the colonies were established in the laboratory. Relatedness and formal likelihood analyses require population-wide background allele frequencies, which were not available for *A. octospinosus* (*). Some deduced offspring numbers are based on published results ([1] Sumner, Hughes, & al. 2004; [2] Dijkstra & Boomsma 2007)

Colony	Queen number			Number of offspring genotyped	Number of loci with shared alleles	Queen-queen Relatedness coefficient (SE)	Maximum likelihood relationship
	Observed in field (lab)	Available for genetic analysis	Deduced from offspring genotypes				
Ae012	2	2	2	66	4/4	0.26 (0.07)	mother-daughter
Ae020	2	2	2	151	3/4	-0.08 (0.35)	half-sisters
Ae022	4	0	-	0	-	-	-
Ae028	0 (1)	1	2	40	4/4	0.57 (0.22)	full-sisters
Ae043	2	0	1	32	-	-	-
Ae047	2	2	2	145	4/4	0.15 (0.13)	mother-daughter
Ae109	2	1	2	79	2/3	0.23 (0.25)	half-sisters
Ae134	2	0	-	0	-	-	-
Ae141	2	0	1[1]	0	-	-	-
Ae144	1 (3)	3	2[2]	0	4/4	0.23 (0.17)	mother-daughter
Ae154	1 (2)	0	2	47	4/4	0.73 (0.13)	mother-daughter
Ae168	2	0	1	32	-	-	-
Ae263	2	0	-	0	-	-	-
Ae266	1	0	2	20	4/4	0.52 (0.26)	full-sisters
Ae394	2	2	2	40	3/4	0.09 (0.31)	unrelated
Ae406	2	2	2	63	3/4	-0.07 (0.32)	unrelated
Ae480	2	2	2	40	4/4	0.34 (0.14)	mother-daughter
Ao044	3	0	1	44	-	-*	-*
Ao273	1 (2)	0	2	29	2/4	-*	-*

Table 2. Estimates of the prevalence of functional polygyny among the 203 analysed colonies of *Acromyrmex echinatior* collected in Gamboa, Panama (158 collected with at least one queen but not genotyped; 39 collected with one or more queens and genotyped; 6 collected without a queen and genotyped). Estimates are ranked from top to bottom starting with the most reliable estimation method (only counting colonies with both observational and molecular evidence for polygyny) and stepwise including less rigorous evidence.

Estimate	**Number**	**% of total**	**Cumulative number**	**Cumulative %**
Total colonies with and without queen observation	203			
Colonies in which multiple queens were both observed and confirmed by microsatellite analysis	9	4.4%	9	4.4%
Colonies in which only a single queen was observed, but where genetic marker data provided evidence for a 2nd queen	2	1.0%	11	5.4%
Adjustment for the previous row, based on the distribution of false-negatives in queen pairs of different theoretical relatedness	0.4	0.2%	11.4	5.6%
Adjustment for the likelihood (7.31%) of not detecting a second queen in the monogynous colonies where neither queens nor workers were genotyped	11.3	5.6%	22.7	11.2%
Colonies in which a second queen was observed, but without any genetic data being available to validate multiple maternity	3	1.6%	25.7	12.7%

Table 3. The majority contribution of one of the paired queens to the offspring workers and gynes in polygynous *A. echinatior* colonies based on genotypes for four microsatellite loci (see Table 1). The percentage of offspring produced by the focal queen, the total number of offspring analysed (n), and the p-values from binomial tests of whether deviation from 50/50 was significant are given. We also tested whether skew in gyne production differed from skew in worker production, using χ^2 tests (final column). Offspring that could not be unambiguously attributed to either of the two queens were omitted. * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.

Colony	**Workers**			**Gynes**			**Worker skew vs gyne skew**
	Focal queen	**n**	**p**	**Focal queen**	**n**	**p**	**p**
Ae012	55%	29	0.71	48%	25	0.99	0.80
Ae020	50%	109	0.99	33%	36	0.07	0.13
Ae028	52%	40	0.87	-	-	-	-
Ae047	64%	141	0.001***	-	-	-	-
Ae109	58%	69	0.23	10%	10	0.02*	0.01**
Ae154	60%	15	0.61	50%	32	0.99	0.74
Ae266	-	-	-	76%	21	0.03*	-
Ae394	57%	40	0.43	-	-	-	-
Ae406	56%	32	0.60	56%	32	0.60	0.99
Ae480	63%	40	0.15	-	-	-	-

Figure legends

Figure 1 Density functions illustrating the relatedness (R) values obtained in 2000 simulations of sampling full-sisters, half-sisters, mother-daughter combinations, and unrelated (random) queen-pairs, based on the observed population-wide allele frequencies for the four marker loci used. The peaks of the first three curves coincide with the expected values of 0, 0.25 and 0.5, but the full-sister curve is more erratic, consistent with the available diversity at marker loci not being quite high enough to resolve these cases. The upper and lower values of each of the curves further illustrate that there remains a low, but non-zero, likelihood of full-sister pairs producing a relatedness estimate of zero by chance and of mother-daughter pairs producing a relatedness estimate of one by chance.

Figure 2: Likelihoods of the four alternative types of relatedness relationship between paired queens for the 11 separate *Acromyrmex echinatior* queen pairs. Pairs could either be full-sisters or half-sisters (curves between R = 0.25 and R = 0.75), mother and daughter (black dot at R = 0.5), or unrelated (black dot at R = 0). See Table 1 for comparisons between these likelihood estimates and direct relatedness estimates.

Figure 3. Results of maximum likelihood analyses to evaluate the overall probability of all queen-pairs of *A. echinatior* to be full-sisters, half-sisters, mother-daughter, or unrelated. **A.** The likelihood that either of the four relatedness scenarios between paired queens were universally true for all eleven pairs for which queen genotypes were available, calculated as the product of the likelihoods for individual queen pairs (see Fig. 2). The filled circles are the probabilities of all queen pairs being unrelated (at R = 0; Relative Likelihood RL = 0.00006) and being mother and daughter (R = 0; RL = 0 since some queen pairs could definitely not be mother and daughter). For sister scenarios, likelihoods were simulated for a range of combinations with varying probabilities of two queens being half-sisters or full-sisters (i.e. having relatedness of one or zero through the father R_p), which produced a dome-shaped likelihood curve of open circles each representing an individual simulation and gave a maximal RL of 0.176 when $R_p = 0.05$ and almost the same maximal RL of 0.173 for $R_p = 0.10$. These values are lower than those predicted from the earlier observed effective queen mating

frequency of 5.3 (SUMNER & al. 2004b) and suggest that ca. 20% of all queen-pairs would be expected to be full-sisters ($R_p = 0.19$; dotted vertical line). In these simulations, the likelihood of all queen-pairs being full-sisters (R = 0.75) was zero (RL = 0) because this scenario was definitely impossible for some queen-pairs. **B.** The same results plotted with a logarithmic vertical axis to illustrate that likelihood values differ only slightly towards the left of the dotted vertical line and to show that there is still a positive, but very low, likelihood of queens being unrelated, whereas the likelihood of all queen-pairs being full-sisters or mother-daughter combinations is truly zero, so they could not be plotted. **C**. A log likelihood plot similar to the B-panel, but only including the seven colonies where queen-pairs shared at least one allele at each of the four marker loci, so they could in theory be full-sisters or mother-daughter combinations, whereas being unrelated or half-sisters seemed unlikely but not impossible because shared alleles may also be due to chance. Here the likelihood curve (open circles) peaks closer to the expected value based on $R_p = 0.19$, and mother-daughter combinations became more likely while being unrelated remains possible but with very low likelihood.

Appendices

Appendix S1: Details for the potentially polygynous colonies discussed in this study

Appendix S2: List of all colonies that were genotyped for the current or previous projects, with references to previously published genotyping data and the molecularly derived queen number according to the listed sources.

Appendix S3: The estimated inbreeding coefficients (F_{IS}) varied among colonies but did not covary with the estimated relatedness among pairs of queens. Random mating would produce an F_{IS} of zero; while evidence for inbreeding would require significantly positive F_{IS} values. Negative F_{IS} values could suggest disassortative mating if they were significant. Each data point represents a colony and colours codes reflect the most likely relatedness scenarios for queens. There is no significant correlation between queen relatedness (R) and the inbreeding coefficient (F_{IS}) among female offspring (ANOVA $F_{1,11} = 0.41$, $p = 0.54$).