Eusocial evolution without a nest: kin structure of social aphids forming open colonies on bamboo

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Abstract

Living in nests is an almost universal feature of eusocial animals. In some aphids, however, sterile soldier castes have evolved in open colonies without a nest. To clarify the factors promoting the evolution of eusociality in these colonies, we used newly developed microsatellite markers to compare the kin structure of the open colonies of two aphid species: the non-eusocial *Astegopteryx bambucifoliae* and the eusocial *Pseudoregma alexanderi*.

Our samples, from over 1,000 hectares, contained 99 clones of *A. bambucifoliae* and 19 of *P. alexanderi*. Clonal mixing occurred in both species: average pairwise relatedness within a colony was 0.54 in *A. bambucifoliae* and 0.71 in *P. alexanderi*. Each clone of *A. bambucifoliae* occurred in a unique location, whereas those of *P. alexander*i occurred in multiple locations and more than 90% of individuals came from just four clones. There was significant genetic variation among different colonies in the same clump (stem-cluster) in *A. bambucifoliae* but not in *P. alexanderi*, indicating that *P. alexanderi* colonies in a single clump are genetically homogenized, functioning as a large colony. In *P. alexanderi*, the proportion of sterile soldiers to normal first-instar nymphs was significantly different across the four clones.

Our results indicate that the lack of input of migrants from the primary host, combined with feeding on a large, stable host plant, are the key ecological factors that

favour the evolution of eusociality, enabling the production of genetically homogenised, large, and long-lived colonies. After eusociality evolves, the optimal strategy of soldier production might vary between different clones.

Keywords social aphids, eusociality, microsatellite, relatedness, cooperation

Declarations

Competing interests The authors declare no competing interests.

Significance Statement

Nest-living has often been considered to be a necessary condition for the evolution of eusociality. In a small number of aphid species, however, sterile soldier castes have evolved in open colonies without a nest. To understand why these aphids are unique, we examined the kin structure and genetic relatedness of individuals within open colonies of a eusocial and a non-eusocial aphid species. We found that clonal mixing occurred in both species, but the colonies of the eusocial species on bamboo are more genetically homogenized, functioning as a large colony. Our results suggest that ecological conditions that promote genetically homogenized, large and long-lived colonies are critical for the evolution of eusociality in these aphids. We propose that the open colonies of social aphids provide an ideal model system in which to study the evolution of altruism.

1. Introduction

Understanding how complex sociality has evolved and is maintained is a fundamental question in evolutionary biology. Two key factors need to be addressed: the costs and benefits of helping and being helped, and the genetic relatedness of the actors. The mode of reproduction, such as monogamy or parthenogenesis, can enhance genetic relatedness (Hughes et al. 2008; Boomsma 2009), and the ecological context, such as the intensity of parasitism or the availability of food, can influence the benefit-to-cost ratio of helping behaviour (Korb and Heinze 2008; Korb and Heinze 2016). Living in a nest can influence both of these factors. Confinement in a structure may make colony defence a more viable proposition; the structure itself, for example a plant gall, might provide a food source (Stone and Schönrogge 2003); and the construction and maintenance of the structure might select for adaptations for altruism in the colony members. Independently, enclosure in a structure will tend to keep close kin together and potentially increase relatedness within the colony. Nest-living has therefore been considered by some authors to be a necessary condition for the evolution of eusociality (Foster and Northcott 1994; Queller and Strassmann 1998; Hölldobler and Wilson 2009).

Within the aphids, social behaviour has evolved only within those clades that were ancestrally gall-formers on plants (Foster and Northcott 1994; Stern 1994; Stern 1998). The gall is induced by the feeding behaviour of the aphid foundress, whose clonal descendants are provided with a defendable fortress that is also a source of food. Gall-living provides a selective context for the evolution of altruistic behaviour such as gall-cleaning (removing honeydew), gall repair and, in some but not all species, colony defence. In about 40 out of the 5,000 species of aphids, a morphologically specialized and sterile soldier caste has evolved (Pike and Foster 2008; Aoki and Kurosu 2010; Abbot and Chapman 2017). These species are genuinely eusocial, showing overlap of generations, cooperative care of the young, and reproductive altruism (Costa 2006).

Arguably the most interesting of all the eusocial aphids are those species that have sterile soldiers and live in open colonies on plant surfaces. These are the only known eusocial animals that do not occupy some kind of nest. Sterile soldiers in open colonies

have evolved at least twice independently, in the subfamilies Eriosomatinae and Hormaphidinae (Stern and Foster 1996; Abbot 2015). In both clades, the origin of eusocial behaviour in open colonies on the secondary host is considered to be evolutionarily independent from the origin of eusociality in the aphid generations that live in galls on the primary tree host, where sexual reproduction occurs (Stern 1998). Very little is known about either the genetic relatedness between colony members or the costs and benefits of helping behaviour within these open aphid colonies.

Most of the few studies of clonal mixing in relation to sociality in aphids have focussed on the gall generations. These have shown that clonal mixing does occur within the galls, that the level of mixing is very variable, and that the dispersing aphids can adjust their social and reproductive behaviour depending on whether they are in their own gall or that of another clone (Abbot et al. 2001; Johnson et al. 2002; Wang et al. 2008; Abbot 2009; Miller et al. 2015). By contrast, there has been only one study of the genetic structure of soldier aphids in open colonies (Hattori et al. 2015). This was on a very small scale (about 10 m x 5 m) and showed that there was some clonal mixing in five open colonies of *Ceratovacuna japonica* using AFLP markers (Hattori et al. 2015). Further genetic analysis on a wider spatial scale is needed to reveal the clonal diversity in a population, the kin structure and genetic relatedness within a colony, and the evolutionary forces that determine the proportion of sterile soldiers. These aphids potentially provide a useful general model for investigating the relationship between spatial structure and cooperation, which has recently been extensively studied in microorganisms (Datta et al. 2013; Van Dyken et al. 2013; Nadell et al. 2016).

The aim of our study is to use newly developed microsatellite markers to provide a detailed account of the genetic relatedness amongst individual social aphids living in open colonies. We studied two cerataphidine species with contrasting levels of sociality on the secondary host: *Astegopteryx bambucifoliae* (Takahashi), which is non-eusocial (Figure 1a, c); and *Pseudoregma alexanderi* (Takahashi), which is eusocial (Figure 1b and d). Both species belong to genera that have eusocial colonies on the primary woody host (*Styrax*) and form colonies on the secondary host (bamboos etc.) where they may fight other aphids in the colony for feeding sites. In addition, in contrast to species of

Astegopteryx, aphids of the genus *Pseudoregma* (including *P. alexanderi*) living on their secondary host have evolved a novel kind of sterile first-instar horned soldier that defends the colonies, which can therefore be described as eusocial. Both species were studied on the same secondary host, the giant bamboo *Dendrocalamus latifolia*.

The key questions we intend to answer here are: (i) what is the level of clonal mixing in these open colonies of social aphids; (ii) does the level of clonal mixing correlate with the level of aphid sociality; (iii) what can we infer from our observations about how sociality may have evolved and be maintained in the absence of nest-living**?**

2. Materials and methods

2.1. Study species

Astegopteryx bambucifoliae

This aphid forms eusocial colonies in galls, which include sterile second-instar soldiers and may contain up to 5,000 aphids, on the primary woody host *Styrax suberifolia* (Kurosu and Aoki 1991a). Viviparous winged aphids disperse from the galls from August to November to form open colonies on the undersides of the leaves of their secondary host plants, *Bambusa* spp. (*dolichoclada*, *edulis*, *oldhami*, *stenostachya*), *Dendrocalamus latiflorus*, and *Phyllostachys lithophia* (Liao 1976).

The open colonies of this aphid on the secondary host are not eusocial. None of the early-instar nymphs are sterile and we never observed them attacking predators, although all instars can use their frontal horns to attack each other for feeding sites (Aoki and Kurosu 1985). There is one brief report of soldier behaviour in *Astegopteryx bambucifoliae*: a total of eight first-instar aphids were observed to have the tips of their rostra in contact either with some unidentified insect eggs or scymnine pupae, but no evidence was presented that the stylets had penetrated (Aoki and Kurosu 1989). These observations are suggestive, but do not provide robust support that this species has altruistic defensive behaviour that deters or kills predators.

Winged individuals emerge from bamboo colonies from February to May and disperse to *Styrax suberifolia* to give rise to the females and males that produce eggs, but a small

number of aphids remain on bamboo and continue parthenogenetic reproduction (Takahashi 1923).

Pseudoregma alexanderi

Most species of this genus form eusocial colonies on the primary host (*Styrax spp.)* with sterile second-instar soldiers, as in *Astegopteryx* (Aoki and Kurosu 2010). However, in Taiwan, galls of *P. alexanderi* have never been recorded on a primary host plant. In contrast to *A. bambucifoliae*, therefore, *P. alexanderi* in Taiwan is permanently confined to the secondary host.

The open colonies of *P. alexanderi* on the shoots and stems of the secondary host, where wingless viviparous females reproduce throughout the year, are eusocial. The clones produce two types of first-instar nymphs: sterile soldiers and normal fertile nymphs. The soldiers are distinguishable from the normal nymphs of the same instar by their larger body size, sclerotized exoskeletons, enlarged and thickened forelegs, and well-developed sharp frontal horns, and they do not moult and grow to adulthood. They defend their colonies by clasping predators with enlarged forelegs and piercing their skins with a pair of sharp horns on a head. The horns and piercing behaviour of soldiers might be derived from head-butting behaviour between colony members, which is found in almost all of the cerataphidine aphids on the secondary host (Aoki and Kurosu 1985; Foster 1996; Howard et al. 1998; Morris and Foster 2008). First-instar normal nymphs can disperse by being carried on the wind. In spring, winged sexuparae adults emerge but they have never been recorded on a primary host.

2.2. Sampling

Both aphid species were sampled at Sun Moon Lake, Nantou, Taiwan: *A. bambucifoliae* on 18 November 2012 and *P. alexanderi* on 18 November 2012 and 23 April 2014. The host plant *D. latiflorus* forms a dense cluster of stems (a clump). Aphids were collected from nine (AB01-AB09) and seven (PA01-PA07) clumps of *D. latiflorus* for *A. bambucifoliae* and *P. alexanderi* respectively over an area of more than 1,000 hectares (Figure 1e). In the present study, we defined a “colony” as a group of aphids on the same

leaf for *A. bambucifoliae* and on the same developing shoot for *P. alexanderi*, in order to facilitate comparison between the two species. For each clump, two colonies were collected from different stems, except for clump PA04, in which only one colony of *P. alexanderi* was collected. In total, we collected 18 colonies of *A. bambucifoliae* and 13 of *P. alexanderi*. The numbers of aphids in the colonies that we sampled were roughly similar in the two species, ranging from 52 to 882 (mean 344.5) in *A. bambucifoliae* and from 56 to 831 (mean 235.3) in *P. alexanderi*. Note that it was not possible to record data blind because our study involved focal animals in the field.

All the aphids from each colony were put into 95% ethanol and the total number of aphids in each colony was counted under a dissecting microscope. For *P. alexanderi*, the numbers of soldier first-instar nymphs were also counted for each colony. From each colony, 15 – 24 individuals from four age classes (first-instar, second or third instar, third or fourth instar, and wingless adult in *A. bambucifoliae*; first-instar soldier, first-instar normal, from second to fourth instar, and wingless adult in *P. alexanderi*) were selected for DNA extraction and genotyping. In total, 364 individuals of *A. bambucifoliae* and 251 of *P. alexanderi* were genotyped. In addition, from two clumps (PA03 and PA07), where we found multiple clones of *P. alexanderi* by the first genotyping, 112 first-instar (57 soldier and 55 normal) nymphs were subsequently genotyped without breaking their exoskeleton to associate morphological characters with a genotype.

2.3. DNA extraction

DNA was extracted using a glass milk extraction method in a microtitre plate as described in (Amos et al. 2016). In the first genotyping, each aphid was crushed with a plastic pestle or a pipette tip in 40 μl of lysis solution (10 mM Tris HCl (pH 8.0), 1 mM EDTA, 1% SDS) with proteinase K and incubated at 56°C for 12 hours. The DNA was adsorbed to flint glass particles in the presence of a 3× excess of 6□M NaI. After two ethanol washes, the DNA was eluted in 50□μl of low TE buffer. In the additional genotyping in which the exoskeleton was not crushed, we punctured the body of a nymph with an insect pin and incubated it in the lysis solution with proteinase K described above

at 56 for 12 hours to extract DNA and clear inside the body. After three hours, the exoskeleton was picked up and the remaining solution was subjected to DNA extraction and genotyping. The exoskeleton was examined as described below in section 2.6.

2.4. Microsatellite isolation

Extracted DNA from a wingless adult of *A. bambucifoliae* and *P. alexanderi* were sequenced using Illumina Miseq. From the sequence data, we searched for microsatellites using custom C++ scripts, focusing on two motifs: $(AC)_n$ and $(AT)_n$ as described in (Amos and Filipe 2014). Then we used the web software Websat (Martins et al. 2009) to design primer sequences for each candidate microsatellite. We tested each locus for polymorphism using panels of 8-16 individuals from different colonies, and successfully isolated eight primer pairs for *A. bambucifoliae* and nine primer pairs for *P. alexanderi* that showed polymorphism.

2.5. Genotyping

Polymerase Chain Reactions (PCRs) were performed using Type-it Microsatellite PCR Kit (Qiagen) in 10 µl reactions. PCR conditions were as follows: an initial denaturing step of 5 min at 95°C, followed by 35 cycles of 95°C denaturation for 30 sec, 60°C annealing for 90 sec, 72°C extension for 60 sec, followed by a final extension step for 30 min at 60°C. One primer from each pair was labelled with a fluorescent dye (6-FAM / HEX / NED) as described in Table 1. After amplification, the PCR product was mixed with The GeneScan 500 LIZ Size Standard (Applied Biosystems) and subjected to fragment analysis. Allele calling was performed by GeneMapper software (Applied Biosystems) and checked manually. Samples with ambiguous genotype were re-run twice and the consensus genotype was taken forward for analysis. To reduce the possible impact of genotyping errors / *de novo* mutations, two individuals with unique multilocus genotypes that were found only once and differed from another genotype by only one single allele were excluded from the analysis. In total, 360 individuals of *A. bambucifoliae* and 244 of *P. alexanderi* were subjected to further statistical analysis.

2.6. Morphological examination of first-instar nymphs in *P. alexanderi*

Exoskeletons picked up from the proteinase K solution were washed with distilled water and mounted on a slide using Mount-quick Aqueous (Daido Sangyo, Japan). The mounted individuals were photographed using a digital camera (Nikon 1 V2) attached to a light microscope. Their morphological characters (body length, fore-femur length, horn length) were measured using ImageJ software.

2.7. Data analysis

Different clones were identified using their multilocus genotypes (MLGs) using the R package *poppr* (Kamvar et al. 2013). Deviations from Hardy-Weinberg Equilibrium (HWE) at each locus were tested based on 1000 Monte Carlo permutations of alleles implemented in R package *pegas* v0.9 (Paradis 2010), using only one individual per MLG. Following others (Gilbert et al. 2007; Vantaux et al. 2011; Kronauer et al. 2013), we use mean pairwise relatedness as a measure both of the potential for altruism and of the genetic similarity between groups of aphids. Pairwise relatedness values among all individuals were calculated following Queller and Goodnight (Queller and Goodnight 1989) using Kingroup (Konovalov et al. 2004) software. Since the pairwise data are non-independent, we could not directly compare average relatedness values between the different age or morph categories (Prugnolle and de Meeus 2002). Instead, we performed permutation tests (Coulon et al. 2006) by generating 1000 random resampling sets without replacement, such that each individual occurs only once in a given resampled set. Resampled individuals were chosen from the same colony. Under the null hypothesis, the difference of mean relatedness should follow a normal distribution centred on zero; under the alternative hypothesis (i.e. category-biased dispersal), the difference should be significantly different from zero. SPAGeDI (Hardy and Vekemans 2002) software was used to test whether the pairwise regression relatedness among the aphids decreased with the distance between the aphids. An analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed to test for population differentiation in order to show if a pair of colonies on a bamboo clump are demographically distinct. If significant variation occurs between the two colonies, then we inferred that they are genetically distinct units.

For the morphological characters of soldier nymphs in *P. alexanderi*, bimodality was tested using Hartigan's dip test (Hartigan and Hartigan 1985) implemented in the R package *diptest*.

3. Results

3.1. Size and disposition of the aphid colonies

Both of the aphid species feed on the same host plant, the Taiwan giant bamboo *Dendrocalamus latiflorus*, but the size and disposition of the colonies of the two species are markedly different. *A. bambucifoliae* feeds on the underside of the bamboo leaves, forming relatively small, discrete colonies (Figure 1c), whereas *P. alexanderi* feeds on shoots and stems, where the individual colonies tend to coalesce into super-colonies, often of immense size (Figure 1d). Although we did not measure aphid density, measurements on a related species with similar habits (*Pseudoregma baenzigeri*) suggest there would be around 10 adults or 120 first-instar nymphs per square cm, equivalent to 28,000 adults or 340,000 first-instar nymphs for every metre of 9-cm diameter bamboo stem (Aoki et al. 2007). The super-colonies that we studied were at this, or even greater, densities.

3.2 Clonal diversity and genetic differentiation

The number of alleles and the heterozygosities (observed and expected) for each locus are given in Table 1. The number of alleles per locus ranged from 5 to 12 (mean 7.9) in *A. bambucifoliae* and from 2 to 7 (mean 3.4) in *P. alexanderi*. When using only one individual per MLG, deviations from Hardy-Weinberg equilibrium (HWE) were significant ($P < 0.05$) in four of eight loci in *A. bambucifoliae* and in six of nine loci in *P. alexanderi*. In *A. bambucifoliae*, all of the eight loci exhibited excess homozygosity, suggesting that the deviations from HWE were due to inbreeding. By contrast, eight of the nine loci in *P. alexanderi* exhibited excess heterozygosity, which might be caused by population bottlenecks. A total of 99 different clones were recognized from eight microsatellite markers in the study population of *A. bambucifoliae* and 19 different clones from nine markers in *P. alexanderi*. In *A. bambucifoliae*, each clone was restricted to a

single sampling site. By contrast, in *P. alexanderi* three clones were found at multiple (two to six) widely dispersed sites. 89% (16/18) of the *A. bambucifoliae* colonies and 77% (10/13) of the *P. alexanderi* colonies consisted of multiple clones (two to 20 clones in *A. bambucifoliae*, two to six in *P. alexanderi*), although half (5/10) of the mixed-clone *P. alexanderi* colonies and 25% (4/16) of the mixed-clone *A. bambucifoliae* colonies were almost monoclonal, with more than 80% of the colony members being of a single genotype.

3.3 Relatedness within and between the colonies on a single clump

Average pairwise relatedness between individuals within a colony ranged from 0.04 to 1.0 (mean = 0.54, n = 18) in *A. bambucifoliae* and from 0.04 to 1.0 (mean = 0.71, n = 13) in *P. alexanderi*, which is not significantly different between the two species (Welch's t-test, t = -1.27, df = 23.4, P = 0.22, Figure 2 and Table 2). The relatedness value is significantly higher than predicted by a random distribution in 89% (16/18) of *A. bambucifoliae* colonies and 77% (10/13) colonies of *P. alexanderi* (permutation test, $P < 0.05$, Table 2). Average pairwise relatedness between individuals in the different colonies of the same clump ranged from -0.31 – 0.62 (mean = 0.13, n = 9) in *A. bambucifoliae* and from 0.07 – 1.0 (mean = 0.69, n = 6) in *P. alexanderi*, which is significantly different between the two species (Welch's t-test, t = -2.94, df = 7.6, P = 0.02, Figure 3 and Table 2).

Table 3 reveals significant variation in genetic variance among different bamboo clumps (16%) and among different colonies on the same clump (38%) in *A. bambucifoliae*, indicating a dispersal process in which related colonies do not usually end up as neighbours. By contrast, in *P. alexanderi*, there was significant variation in genetic variance among different bamboo clumps (63%) but not among different colonies on the same clump (1.4%), suggesting much more limited dispersal and a tendency for colonies to grow and split within a clump. If dispersal is primarily associated with one age class and if dispersers occasionally enter an unrelated colony, we would expect that age class to exhibit reduced pairwise relatedness. In fact, average pairwise relatedness was not significantly different among pairs of the four age-classes in *A. bambucifoliae*

(permutation test, $P > 0.05$; electronic supplementary material, Figure S1). The SPAGeDI analysis showed that pairwise relatedness did not decrease significantly with distance between samples for either species (*A. bambucifoliae*: $r^2 = 0.0007$, y = -0.03 - 0.007x, one-tailed $P = 0.34$; n = 360 individuals and 57470 pairs; *P. alexanderi*: $r^2 = 0.0002$, y = -0.15 + 0.01x, one-tailed $P = 0.48$; n = 245 individuals and 25439 pairs), suggesting that dispersal distances are either much smaller or much larger that the spatial scale of sampling in our study.

3.4 Differences in the proportion and morphology of soldiers among genotypes in *P. alexanderi*

We were interested in whether different clones were more or less likely to produce soldiers. To test this, we additionally genotyped 57 soldier and 55 normal first instar nymphs from the two clumps, PA03 and PA07. When included with 25 soldiers and 20 normal first instar nymphs that had already been genotyped, 96% (150/157) of the genotyped nymphs consisted of the four clones, “A”, “B”, “C”, and “D” described above, and all of the four clones contained soldiers. However, the proportion of soldiers to normal first-instar nymphs was significantly different among the four clones in both of the clumps (Fisher’s exact test, $P = 0.006$ in PA03 and $P = 0.01$ in PA07, Table 4). For the two clones (“A” and “C”) that occurred in both of the clumps, fewer soldiers of genotype A and more of genotype C were found than was expected (Table 4).

To test whether the soldiers of *P. alexanderi* are dimorphic, as has been shown in a previous study (Stern et al. 1996), we performed Hartigan’s dip test (Hartigan and Hartigan 1985) implemented in the R package *diptest.* The fore-femur length of the soldiers in both of the two mixed-clone clumps was bimodal, as judged by Hartigan’s dip test: ($P = 0.006$ in clump PA03 and $P = 0.003$ in clump PA07, Figure 4). Horn length was also bimodal, but the dip test is only significant in clump PA07 ($P = 0.42$ in PA03 and $P = 0.01$ in PA07, Figure 4). Even when analysed separately for each clone, fore-femur and horn length were bimodal in most clones, except for “A” in PA03 and “D” in PA07 (Figure S2).

4. Discussion

We report here the first detailed study of the clonal structure of social aphids living in open colonies and provide some insights into how eusociality might, uniquely within animals, have arisen in the absence of a nest. We studied two aphid species, which have colonies that feed on bamboos: the non-eusocial *Astegopteryx bambucifoliae* and the eusocial *Pseudoregma alexanderi*, which has sterile horned soldiers. We will first consider the clonal structure of the two species at three different spatial scales.

4.1. Clonal structure within aphid colonies

The clonal structure within the colonies of the two species was relatively similar. 89% (16/18) and 77% (10/13) of the colonies of *A. bambucifoliae* and *P. alexanderi* respectively consisted of multiple clones (two to 20 in *A. bambucifoliae*, two to six in *P. alexanderi*). Average pairwise relatedness within the colonies was 0.54 in *A. bambucifoliae* and 0.71 in *P. alexanderi* (Figure 2). Clonal mixing has been found in many open-colony-forming, non-social aphids (Loxdale and Brookes 1990; Guillemaud et al. 2011; Vantaux et al. 2011) and our results are consistent with this.

4.2 Clonal structure of aphid colonies on the same bamboo clump.

There are interesting and significant differences between the clonal structures of the colonies of the two species at this spatial scale. The populations of the eusocial aphid *P. alexanderi* on large clumps of bamboo are much more genetically homogeneous than are those of the non-eusocial aphid *A. bambucifoliae*. In *P. alexanderi*, but not in *A. bambucifoliae*, the average pair-wise relatedness of aphids from the same small colony is not significantly different from the relatedness of two aphids from widely separate colonies on a large bamboo clump. In other words, a clump of bamboo typically contains several different clones of *A. bambucifoliae* but a single super-colony of very restricted genetic diversity in *P. alexanderi*. Of the two species, therefore, an individual *P. alexanderi* roaming around a bamboo clump is much more likely to encounter a clone-mate to whom altruism might profitably be directed.

4.3 Clonal structure across different bamboo clumps

There are significant differences between the two species at this larger spatial scale. In the non-eusocial *A. bambucifoliae*, each clone was restricted to a single bamboo clump, whereas in *P. alexanderi*, three of the four major clones were found at multiple sites (Table 2), which were more than 1km apart from each other.

4.4 Causes of the differences in clonal structure

The contrasting life-cycles and dispersal modes of the two species almost certainly underlie these differences in clonal structure. In *A. bambucifoliae*, colonies are founded by individual winged aphids flying from the primary host (*Styrax*) to the secondary host (bamboo) and it seems that there is little direct dispersal by aphids between separate bamboo plants. In Taiwan, the secondary host generations of *P. alexanderi* have lost all connection with the sexually-produced generations on the primary host and are maintained on the bamboo plants entirely by parthenogenesis. Younger instars of *P. alexanderi* can disperse by walking or floating on the air to found new colonies on distant bamboo plants. However, the colonies of *P. alexanderi*, the eusocial species, tend to coalesce into super-colonies on a clump of bamboo, which are both larger and more genetically homogeneous than the colonies on bamboo of the non-eusocial species, *A. bambucifoliae*, which are regularly restocked by genetically distinct aphids flying from the primary host and do not coalesce into large super-colonies.

It has been suggested that it is the evolution of soldiers on open colonies on the secondary host that has caused the loss of the gall generation, because the increased importance of the open-colony generation in the aphid's life cycle eventually causes the extinction of the gall generation on the primary host (Kurosu and Aoki 1991b). We suggest that it is much more likely that the arrow of causality is in the opposite direction: the rarity of the gall generation favours soldier production by enhancing the genetic integrity of the colony members on the secondary host and the efficacy of altruistic defence. A comparative phylogenetic approach will be needed to estimate the ancestral ecological condition and establish whether the rarity of galls is a cause, a consequence, or both of soldier evolution.

4.5 Consequences of differences in colony kin structure on soldier evolution in open colonies.

Relatedness within a colony does not by itself determine the evolution of altruism, but it does set the threshold that the benefit to cost ratio of helping must exceed before altruism can evolve. Clonal mixing occurs in both species, but the average pair-wise relatedness within a colony of both species was higher than 0.5, the average relatedness between parent and offspring, and between siblings in a monogamous diploid family. These values, however, can be lower than relatedness values in colonies of some non-social aphids (0.90 in *Aphis fabae fabae*, 0.79 in *A. fabae cirsiiacanthoidis*) (Vantaux et al. 2011), although other studies that use different measures of genetic relatedness within a colony show higher degree of clonal mixing (Yao and Akimoto 2009; Loxdale et al. 2011).

Why has *P. alexanderi* opted to produce a sterile, highly adapted soldier caste, whereas *A. bambucifoliae* has not? Our genetic observations show that the major difference between the two species is that the eusocial *P. alexanderi* lives in populations that are relatively homogeneous genetically, even across different stems in a bamboo clump, whereas the non-eusocial *A. bambucifoliae* lives in colonies, each of which tends to be genetically distinct. A soldier aphid in *P. alexanderi* will therefore be much more likely to be defending a clone-mate, however far it has roamed on the bamboo clump, than is a defending first-instar of *A. bambucifoliae.* A defensive individual of *A. bambucifoliae* is quite likely to find itself defending a non-clone mate and the benefit/cost ratio for an average act of altruism will therefore be much lower than for a soldier of *P. alexanderi*.

4.6 Other ecological factors that might affect soldier evolution in open colonies

A second potentially important difference between the two species is simply that the super-colonies of *P. alexanderi* are much larger than the colonies of *A. bambucifoliae.* We regularly observed dense colonies of *P. alexanderi* on the bamboo stems, which from comparisons with related *Pseudoregma* species (Shibao 1998; Aoki et al. 2007) must

have exceeded over 10,000 individuals. Theory suggests that, in any social organism, investment in costly soldiers is more likely to evolve in larger colonies (Aoki and Kurosu 2003; Aoki and Kurosu 2004; Aoki and Imai 2005). For example, in eusocial Hymenoptera, colony size is positively related to the degree of reproductive division of labour (Bourke 1999; Ferguson-Gow et al. 2014). In eusocial aphids, the proportion of soldiers in *Pseudoregma* species on the secondary host is extremely low in early colonies and then increases as they mature (Sakata et al. 1991; Schutze and Maschwitz 1991; Tanaka and Ito 1994; Shibao 1998), suggesting that soldier production is especially beneficial in a large colony.

A third factor that might be critical in the evolution of altruism in these open colonies is the nature of the host-plant and of the part of the plant upon which the aphids feed. Bamboos are long-lived perennial plants that grow and spread rapidly, producing a high biomass. Colonizing bamboo therefore enables long colony lifetimes and large colony sizes and it ensures the provision of vacant patches for dispersing aphids. In addition, it has been suggested that bamboo is a relatively poor food-source and that therefore investing in defensive behaviour is a wise strategy (Stern and Foster 1996), because the colonies are compelled to grow slowly. There is little direct evidence for this idea, but it is telling that the two species of horned aphids within the clade that have lost the soldier morph on the secondary host have both shifted from bamboo to herbaceous broad-leaved grasses (Stern 1998). This factor might be especially important for *P. alexanderi*, which grows on the hard woody stems and shoots of bamboo, rather than for *A. bambucifoliae*, which feeds on the relatively more nutritious leaves. This idea needs to be tested experimentally.

4.7. Maintenance of eusociality in open colonies

Social colonies that are not corralled within a nest face the additional problem of being especially vulnerable to invasion by free-rider unrelated conspecifics from another colony. This could be especially tricky for aphids which seem unable to distinguish clone mates from non-clone mates, in a variety of contexts (Aoki et al. 1991; Shibao 1999; Abbot et al. 2001). If this lack of kin recognition is genetically based, it might be a

consequence of Crozier's Paradox (Crozier 1986; Field et al. 2018). This posits that recognition cues will not evolve if individuals act as both hosts and invaders and if the costs of rejection are higher than the costs of acceptance, both of which might be true for these social aphids. There is some evidence that aphids are able to rely on the context in which they encounter another aphid. For example, first-instars of *Pemphigus* species appear to know when they are not in their home gall and behave appropriately (Abbot et al. 2001; Abbot 2009). An immature *P. alexanderi*, having been carried on the wind to a distant bamboo clump, might also "know" that it is no longer near its home clone, and behave accordingly. This is perhaps less likely in the non-eusocial *A. bambucifoliae*, which do not disperse over long distances between different bamboos and are more likely to encounter an alien colony after a relatively short and imperceptible excursion from their home clone.

Our results, admittedly from a small sample size, show that all of the four major clones of *P. alexanderi* contain soldiers in the mixed-clone clumps, suggesting that there is no obligate cheater clone that never produces soldiers. However, our results do not rule out the existence of conditional cheater clones that produce fewer altruistic soldiers when coexisting with other clones, as found in *Pemphigus* aphids (Abbot et al. 2001; Abbot 2009). In fact, the proportion of soldier and normal first-instar nymphs in the two mixed-clone clumps were significantly different among the clones, and the difference was consistent in both clumps, in which clone "A" produced fewer soldiers than expected, while clone "C" produced more (Table 4). This result suggests that there is genetic variation in soldier investment among clones, which could potentially adjust their investment of soldiers according to the presence of other clones in the same colony, although our observations provide no direct evidence for such an adjustment.

The morphological characters of the soldiers of *P. alexanderi* in a colony had a bimodal distribution (Figure 4). One possible mechanism for the soldier dimorphism is that they consist of two clones with genetically distinct morphologies, but genotyping showed that a single genotype produced both of the major and minor soldiers (Figure S2). In other social insects, genetic diversity has a positive effect on group-level fitness by increasing phenotypic variation that leads to more efficient division of labour

(Mattila and Seeley 2007; Oldroyd and Fewell 2007). Soldiers that have more specialized morphology tend to behave more aggressively in *Ceratovacuna japonica* (Hattori et al. 2017). Our results might suggest that the soldier dimorphism in *P. alexanderi*, which has not been found in related *Pseudoregma* species (Shingleton and Foster 2001), is beneficial for a colony by increasing phenotypic variation of the soldiers, even if it is monoclonal. Interestingly, the most frequently observed clone "C" invested highly in the production of soldiers and is dimorphic. It would be interesting to see if these characteristics enhance the ecological success of a clone.

4.8. Future directions

The open colonies of social aphids provide an ideal model system in which to study the evolution of reproductive altruism. The critical parameters of the costs and benefits of soldier production and of relatedness can be measured and manipulated with relative ease. These parameters include predation pressure, colony growth-rate, colony size, soldier ratio and within-colony relatedness. It would be exciting to combine genetic analysis with manipulative experiments in *P. alexanderi* to determine the variability of the proportions of soldiers among clones and how this is influenced by the environment. There is a wonderful range of social aphids with open colonies, including the fairly distantly related *Colophina* species (Pemphigidae: Eriosomatinae) (Aoki 1977a,b; Uematsu et al. 2021). A comparative study of a range of these species on different host plants and with different degrees of sociality would greatly help us to understand the ecological and genetic factors that are important for the evolution of sociality in these unique and fascinating animals.

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Data Availability Statement

Additional data and details of statistical analyses are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.2547d7wtm) .

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Figure legends

Figure 1. Study species and sampling sites. (*a*) *Astegopteryx bambucifoliae*. (*b*) *Pseudoregma alexanderi*. A soldier nymph (left) and normal first-instar nymphs (right). (*c*, *d*) A colony of *A. bambucifoliae* (*c*) and *P. alexanderi* (*d*). (*e*) Sampling sites of *A. bambucifoliae* (AB01-AB09) and *P. alexanderi* (PA01-PA07). A dot shows the location of a bamboo clump where aphids were collected.

Figure 2. Within-colony pairwise genetic relatedness (n = 18 for *A. bambucifoliae* (*a*); n = 13 for *P. alexanderi* (*b*)). Box plots show the range of values, the first and third quartiles and medians. Open circles show outliers and filled circles show means.

Figure 3. Average values of within-colony relatedness and between-colony relatedness in the same clump in *A. bambucifoliae* and *P. alexanderi*. Box plots show the range of values, the first and third quartiles and medians. Open circles show outliers and filled circles show means.

Figure 4. Frequency distributions of the morphological characters (horn length and fore-femur length) in the two clone-mixed clumps (PA03 (*a*) and PA07 (*b*)) of *P. alexanderi*. Blue bars show the distribution in the first-instar normal nymphs and orange bars in the first-instar soldier nymphs. *P*-values of the dip test are shown.

Figure 5. Comparison of the genetic structure between *A. bambucifoliae* (a) and *P. alexanderi* (b) within and between bamboo clumps. Colours in the pie charts indicate different clones. In *A. bambucifoliae*, winged adults frequently migrate from a distant gall-harbouring primary host plant, then mix with other resident clones in a bamboo clump. In *P. alexanderi*, the dispersing morphs are young nymphs that walk around between local clumps or migrate to long distance by walking or being borne by the wind. *P. alexanderi* has no primary host plant in this locality and can thus propagate only by

parthenogenetic reproduction. As a result, colonies on the local site are genetically homogenised and major clones can spread to a distant bamboo clump in this species.

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Table 1. Summary statistics of the polymorphic microsatellite loci with the primer sequences in (*a*) *A. bambucifoliae* and (*b*) *P. alexanderi*.

(*a*) *A. bambucifoliae*

Locus	Allele size range (bp)	Primer sequence (5'_3')	N_A	H_E	H_O	P
AbMSAC1	107-121	Fwd-TATACACACCCACATACCCACC Rev-TAGATGGCCCTTAACGAGTTTT	8	0.667	0.734	0.097
AbMSAC2	131-150	Fwd-CTAAGGGGAGGGAAATCACG Rev-AAGTGAACCTTCCACGAAAATG	7	0.755	0.64	0.019
AbMSAC3	176-188	Fwd-GACGATAGCCCTTCTTCGG Rev-ATTTGCAGCTCTCAAGATTTCC	6	0.701	0.702	0.884
AbMSAC4	85-136	Fwd-ACCACTGCTACGAGATGTTTGT Rev-AAAAGGAGAGCTGACGTGG	12	0.737	0.712	0
AbMSAC5	175-190	Fwd-CGTAAACGGCCCCAAAAC Rev-AGTCGATGAGATAGCATAAGAACG A	8	0.774	0.618	0
AbMSAT6	228-259	Fwd-CGTATGGACGTTTGAGATCGTA Rev-AAGTGGAATAGAATCCCGCATA	12	0.825	0.668	0.011
AbMSAT7	249-259	Fwd-CCAGAGAACTCGTCGTCGTAAT Rev-TCTTCCGTTAAATTCGGTTCAC	5	0.652	0.618	0.759
AbMSAT15	134-142	Fwd-TGTCTGTCATCCATTGTCGAA Rev-TGTAGATATGTGGGCCTCTTCA	5	0.527	0.470	0.147

(*b*) *P. alexanderi*

Locus	Allele size range (bp)	Primer sequence (5'_3')	N_A	H_E	H_O	P
PaMSAC1	102-108	Fwd-AATTAGACGAGTTTTGTACGGGC	4	0.67	0.73	0

		Rev-TGAAGACAGAGAGTGAAGCCG				
PaMSAT2	176-180	Fwd-ACTTTTCGGATTTTGCCAGTC	3	0.41	0.16	0
		Rev-AGGGGTTTTCAGTAGGCGTTAT				
PaMSAC3	125-145	Fwd-AATTAGGCGGAATCGTAAACAG	3	0.54	0.81	0.096
		Rev-GCCGTTATGGGTAGGAGTAGAG				
PaMSAT4	194-198	Fwd-CGATTATTTTGAAGACTGCCTC	3	0.55	0.93	0
		Rev-CCACGACGAAAGAACACATTA				
PaMSAC6	151-173	Fwd-TGTCAACTCTCGGCTATATTGG	7	0.76	0.96	0
		Rev-TCTGTGTGAGGCTGTGTGTATG				
PaMSAC2	108-110	Fwd-ATTCAAACGATGTGTGCGATAC	2	0.48	0.81	0.023
		Rev-CCCAACAGCCTATATGTAAAACAG				
PaMSAC5	136-150	Fwd-TCCCACTAATAACACATGCACAC	5	0.72	0.98	0
		Rev-ATTCGGAGTAAAACAGCACGAG				
PaMSAC11	196-198	Fwd-ACGTTCTTGGCATTGATTTCAG	2	0.44	0.620	0.695
		Rev-GGTCTTTGATGTTGCCGAATAG				
PaMSAT6	175-177	Fwd-ACGTCGCCGGTTAGTCAAT	2	0.47	0.73	0.26
		Rev-AAATCAGGTTGCTGGAGTGG				

N_A = number of alleles, H_O= observed heterozygote frequency, H_E = expected heterozygote frequency assuming Hardy–Weinberg equilibrium (HWE), and *P* refers to significance of deviation from HWE following Bonferroni correction.

Table 2. Colony structure of (*a*) *A. bambucifolia*e and (*b*) *P. alexanderi* colonies that were collected and partly genotyped.

(*a*) *A. bambucifoliae*

Clump	Colony	Within-colony pairwise relatedness	Between-colony pairwise relatedness in the same clump	No. of individuals genotyped	No. of clones	Colony size	Area (cm^2)	Density (no. of individuals / cm^2)
AB01	1	0.53^{***}	0.62	14	3	233	97.4	2.39
	2	0.81^{***}		15	3	128	95.2	1.34
AB02	3	1^{***}	-0.31	20	1	437	131.3	3.33
	4	0.85^{***}		20	4	543	47.9	11.34
AB03	5	0.85^{***}	-0.08	14	2	305	135.9	2.24
	6	0.47^{***}		23	4	274	79.6	3.44
AB04	7	0.3^{**}	0.26	22	7	75	54.1	1.39
	8	0.29^{**}		22	5	52	48.8	1.07
AB05	9	1^{***}	0.16	20	1	430	96.7	4.45
	10	0.62^{***}		23	2	608	107.8	5.64
AB06	11	0.04	0.02	24	20	241	183.9	1.31
	12	0.06		21	16	882	274.6	3.21
AB07	13	0.93^{***}	0.28	24	3	439	161.2	2.72
	14	0.19^{**}		24	9	548	155.9	3.52
AB08	15	0.41^{***}	0.12	23	5	66	19.7	3.35
	16	0.76^{***}		21	6	252	42.4	5.94
AB09	17	0.19^{**}	0.07	17	11	456	220.5	2.07
	18	0.34^{**}		17	9	233	162.2	1.44

(*b*) *P. alexanderi*

Clump	Colony	Within-colony pairwise relatedness	Between-colony pairwise relatedness in the same clump	No. of individuals genotyped	No. of clones	Colony size	Major clones found	No. of soldiers
PA01	1	1^{***}	1.00	17	1	95	C	6
	2	1^{***}		20	1	56	C	13
PA02	3	0.95^{***}	0.93	20	2	97	D	2
	4	0.91^{***}		20	5	140	D	12
PA03	5	0.22^{*}	0.23	20	6	207	A,B,C	13
	6	0.31		19	4	221	A,B,C	24
PA04	7	0.85^{**}		15	4	70	C	7
PA05	8	1^{***}	0.97	20	1	237	C	8
	9	0.94^{***}		20	3	831	C	122
PA06	10	0.94^{***}	0.91	20	3	159	C	25
	11	0.88^{***}		20	4	68	C	8
PA07	12	0.04	0.08	20	5	444	A,C,D	18
	13	0.08		20	3	434	A,C,D	26

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Table 3. Analysis of molecular variance (AMOVA) table for populations of (*a*) *A. bambucifoliae* and (*b*) *P. alexanderi*.

(*a*) *A. bambucifoliae*

Sources of variation	df	% of variation	*Phi*-statistics	P^a
Between tree	8	15.8	0.158	0.003
Between colonies within tree	9	37.7	0.447	< 0.001
Within colonies within tree	342	46.5	0.535	

(*b*) *P. alexanderi*

Sources of variation	df	% of variation	Phi-statistics	P^a
Between tree	6	62.8	0.628	0.001
Between colonies within tree	6	1.4	0.038	0.123
Within colonies within tree	232	35.8	0.642	

[a]*P*-values are based on 9999 permutations.

Table 4. Clonal composition of first-instar nymphs in the two clone-mixed clumps.

Clone	Clump			
	PA03[a]		PA07[a]	
	Soldier	Normal	Soldier	Normal
A	4	14	11	12
B	21	11	0	0
C	11	5	25	12
D	0	0	7	17
Others	2	1	1	3

[a]The frequency distribution significantly differed between soldier and normal nymphs (Fisher's exact test, $P = 0.006$ in PA03 and $P = 0.01$ in PA07).

Figure 1.

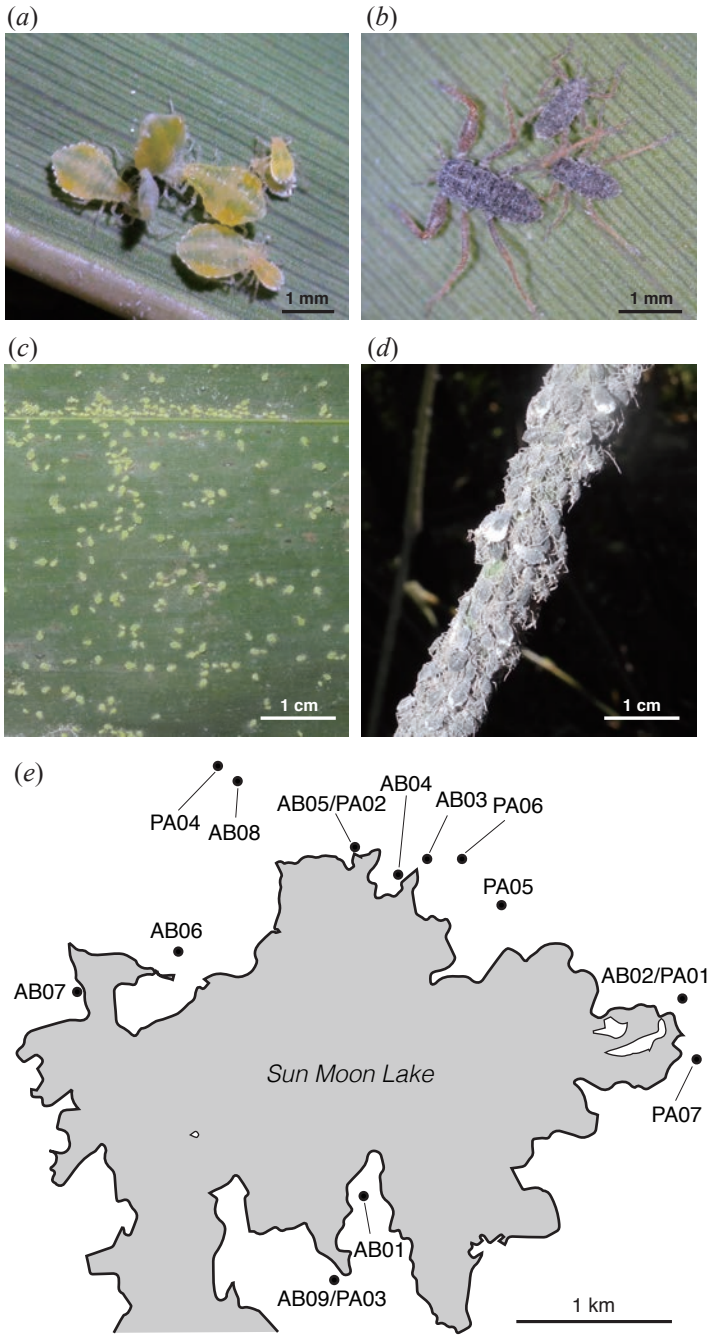

(a)
1 mm
(b)
1 mm
(c)
1 cm
(d)
1 cm
(e)
PA04
AB08
AB05/PA02
AB04
AB03
PA06
PA05
AB06
AB07
AB02/PA01
Sun Moon Lake
PA07
AB01
AB09/PA03
1 km

Figure 2.

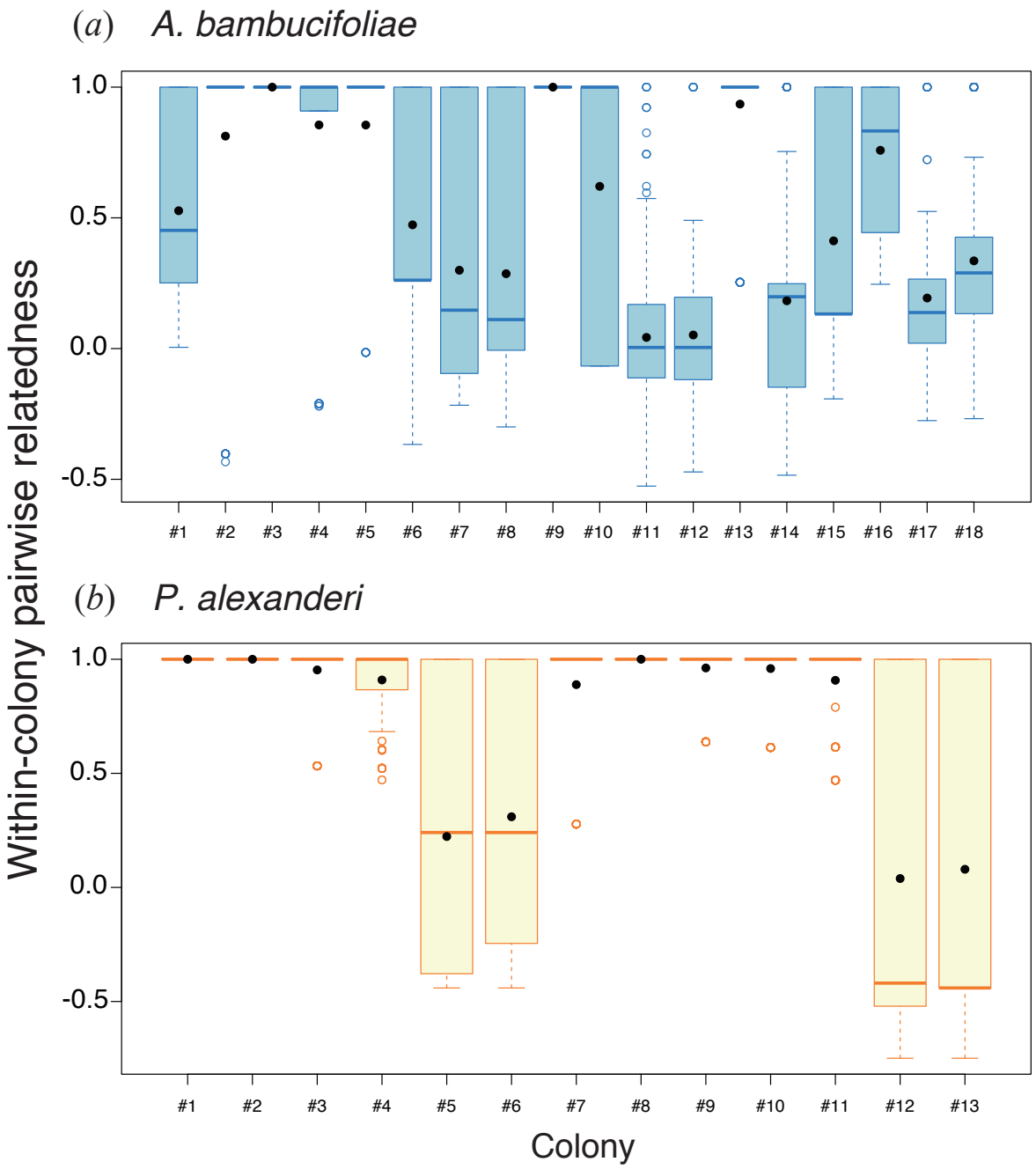

(a) A. bambucifoliae
1.0
0.5
0.0
-0.5
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13 #14 #15 #16 #17 #18
(b) P. alexanderi
1.0
0.5
0.0
-0.5
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13
Within-colony pairwise relatedness
Colony

Figure 3.

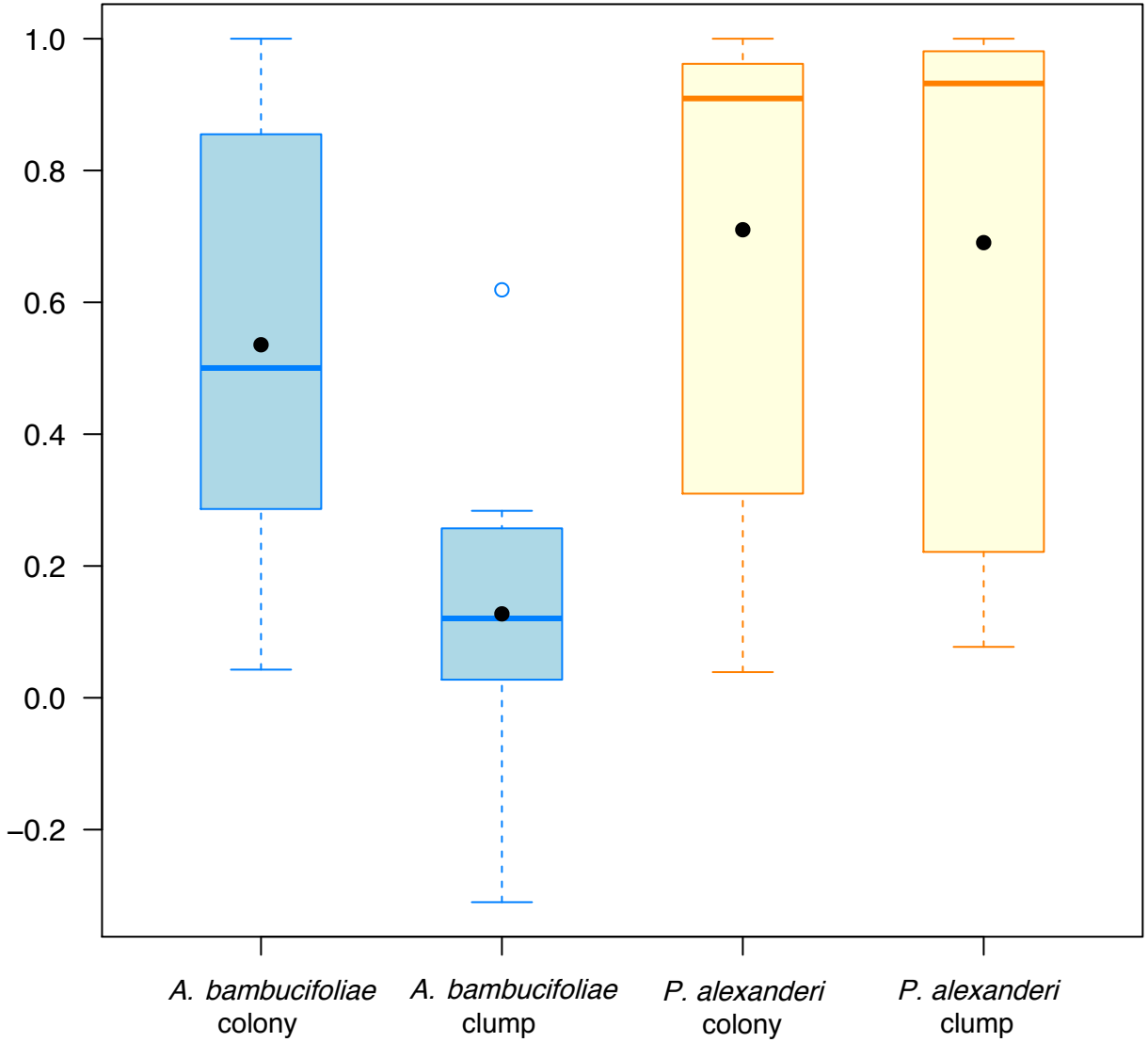
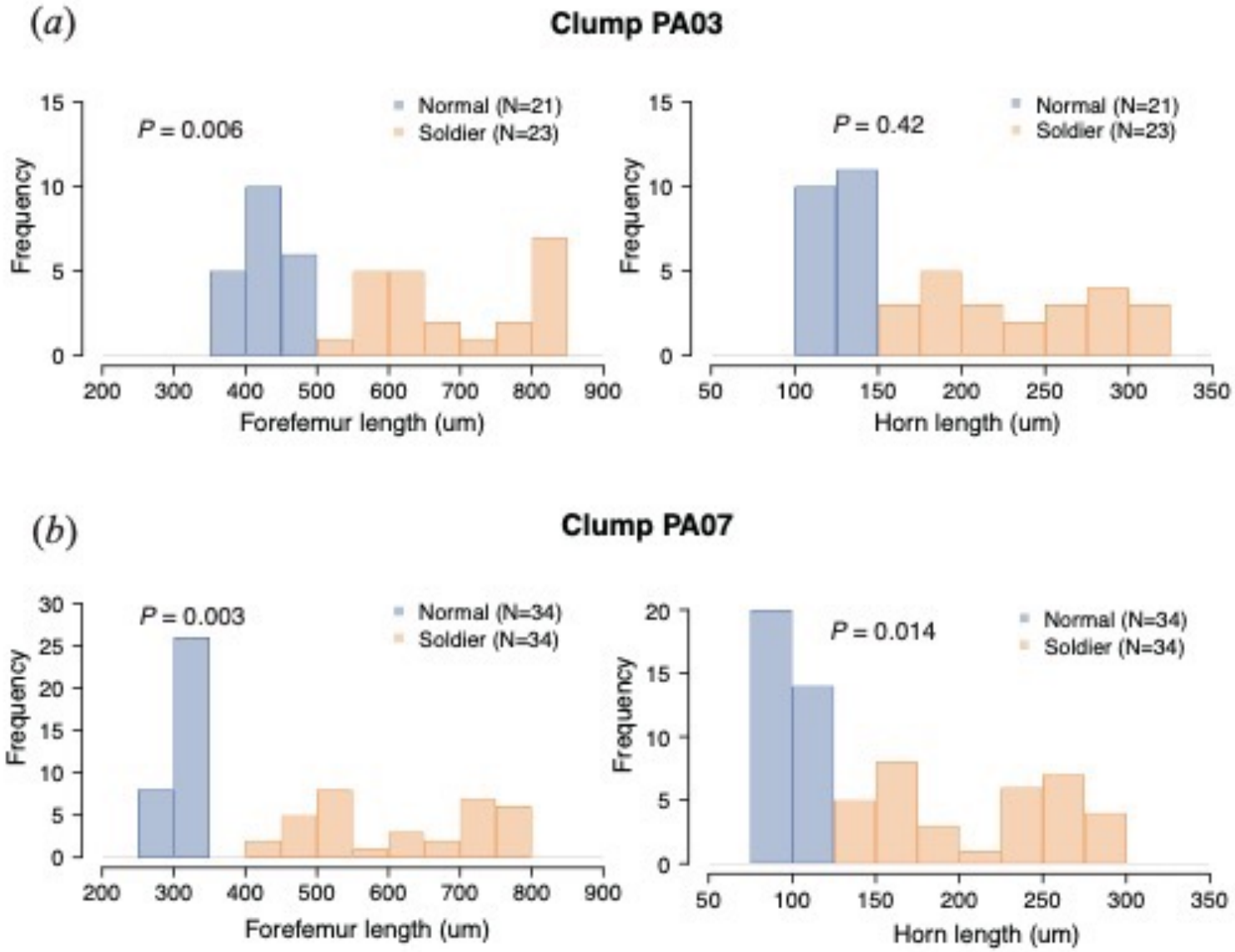

1.0
0.8
0.6
0.4
0.2
0.0
−0.2
A. bambucifoliae colony
A. bambucifoliae clump
P. alexanderi colony
P. alexanderi clump

Figure 4.


(a)
Clump PA03
P = 0.006
Normal (N=21)
Soldier (N=23)
Frequency
Forefemur length (um)
P = 0.42
Normal (N=21)
Soldier (N=23)
Frequency
Horn length (um)
(b)
Clump PA07
P = 0.003
Normal (N=34)
Soldier (N=34)
Frequency
Forefemur length (um)
P = 0.014
Normal (N=34)
Soldier (N=34)
Frequency
Horn length (um)

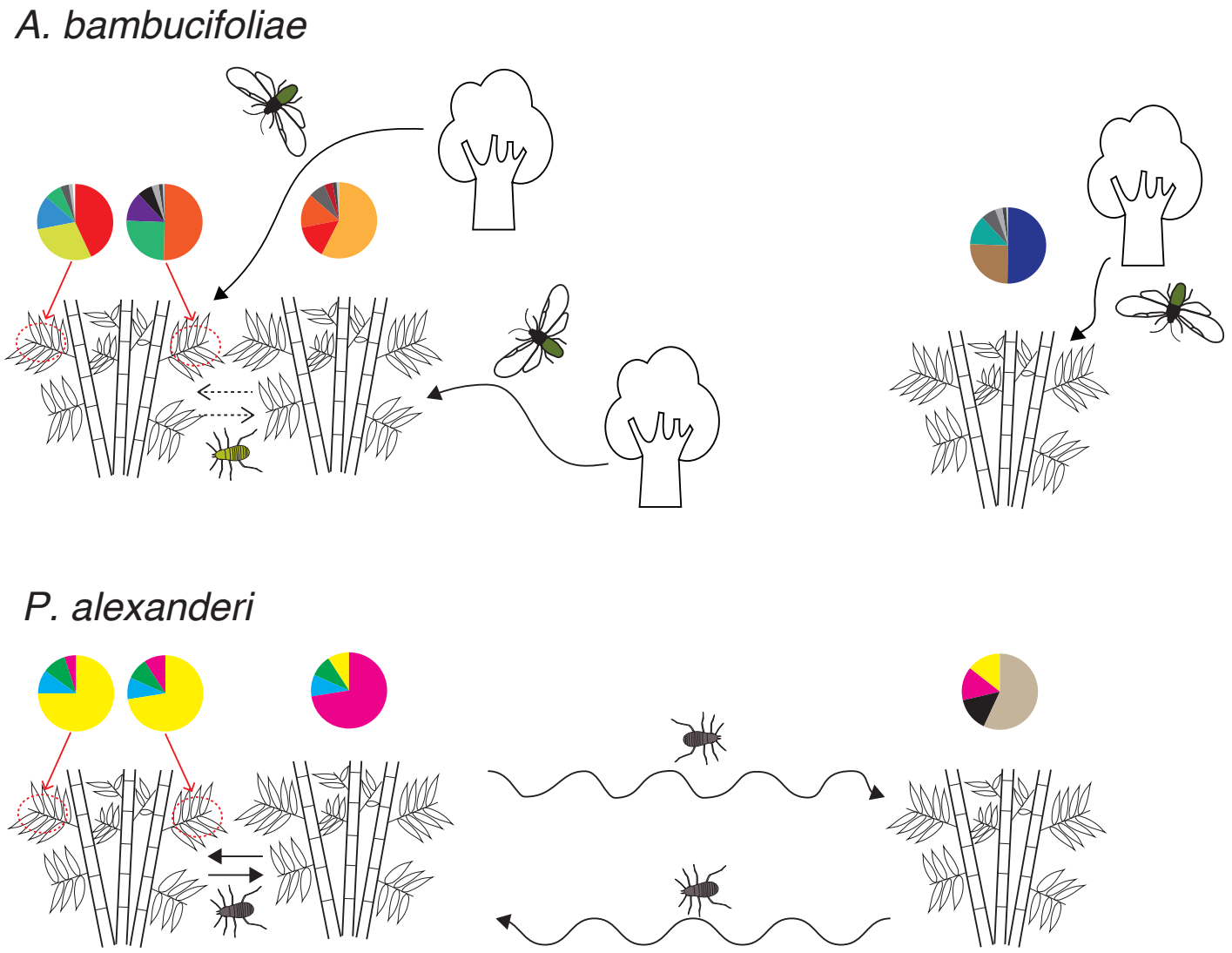



Figure 5.