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 on bamboo: the non-eusocial colonies of *Astegopteryx bambucifoliae* and the eusocial colonies of *Pseudoregma alexanderi* on *Dendrocalamus latiflorus*.

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 and feeding on a large, stable host plant are important ecological factors that might favour the

evolution of eusociality, enabling the production of genetically homogenised, large, and long-lived colonies. After eusociality evolves on the secondary host, 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 eusocial and non-eusocial open colonies of two aphid species on bamboo. We found that clonal mixing occurred in both species, but the eusocial colonies 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 important 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 (Starr, 1991). 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; and the construction and maintenance of the structure might select for adaptations for altruism in the colony members (Stone and Schönrogge 2003). Independently, enclosure in a structure will tend to keep close kin together and potentially increase relatedness within the colony. Therefore, although the role of the nest in the lives of social insects is complex and nuanced (McGlynn, 2012; Bengston and McGlynn, 2021), most authors consider that nest-living is a necessary condition for the initial evolution of eusociality, in both fortress defenders (for example aphids) and life-insurers (such as ants) (e.g. 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) (Aoki 1980, Benton and Foster 1991, Kutsukake et al. 2012, Uematsu et al. 2018, Kutsukake et al. 2019a, Shibao et al 2021), gall repair (Kurosu et al. 2003, Pike and Foster 2004, Kutsukake et al. 2009, Kutsukake et al. 2019b) 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 eusocial, showing

83 overlap of generations, cooperative care of the young, and reproductive altruism (Costa
84 2006).

85 Arguably the most puzzling of all the eusocial aphids are those species that have
86 sterile soldiers and live in open colonies on plant surfaces without modifying their
87 structures. These are the only known eusocial animals that do not occupy some kind of
88 nest. Sterile soldiers in open colonies have evolved at least twice independently, in the
89 subfamilies Eriosomatinae and Hormaphidinae (Stern and Foster 1996; Abbot 2015). In
90 both clades, the origin of eusocial behaviour in open colonies on the secondary host is
91 considered to be evolutionarily independent from the origin of eusociality in the aphid
92 generations that live in galls on the primary tree host, where sexual reproduction occurs
93 (Stern 1998). Very little is known about either the genetic relatedness between colony
94 members or the costs and benefits of helping behaviour within these open aphid colonies.

95 Most of the few studies of clonal mixing in relation to sociality in aphids have
96 focussed on the gall generations. These studies have shown that clonal mixing does occur
97 within the galls, that the level of mixing is very variable, and that the dispersing aphids
98 can adjust their social and reproductive behaviour depending on whether they are in their
99 own gall or that of another clone (Abbot et al. 2001; Johnson et al. 2002; Wang et al.
100 2008; Abbot 2009; Miller et al. 2015). By contrast, there has been only one study of the
101 genetic structure of soldier-producing aphids in open colonies (Hattori et al. 2015). This
102 showed that there was some clonal mixing in five open colonies of *Ceratovacuna*
103 *japonica* using AFLP markers. But this study was on a very small scale (about 10 m x 5
104 m), much smaller than the dispersal distance of individual aphids, and of a small sample
105 size. Further genetic analysis of the open colonies of soldier-producing aphids on a wider
106 spatial scale is needed to reveal the clonal diversity in a population, the kin structure and
107 genetic relatedness within a colony, and the evolutionary forces that determine the
108 proportion of sterile soldiers.

109 The aim of our study is to use newly developed microsatellite markers to provide
110 a detailed account of the genetic relatedness amongst individual social aphids living in
111 open colonies. We studied two cerataphidine species with contrasting levels of sociality
112 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 that form colonies on the secondary host (bamboos etc). The crucial difference between the two species is that aphids of the genus *Pseudoregma* (including *P. alexanderi*) living in open colonies on the secondary host have evolved a novel kind of sterile first-instar horned soldier that defends the colonies, which can therefore be described as eusocial on the secondary host: aphids of the genus *Astegopteryx* (including *A. bambucifoliae*) have not evolved specialized sterile soldiers on the secondary host (Figure S1). Both species were studied on the same secondary host, the giant bamboo *Dendrocalamus latiflorus*.

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 1991). 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 (Coccinellidae)

143 pupae, but no evidence was presented that the stylets had penetrated (Aoki and Kurosu
144 1989). These observations are suggestive, but do not provide robust support that this
145 species has altruistic defensive behaviour that deters or kills predators.
146 Winged individuals emerge from bamboo colonies from February to May and disperse to
147 *Styrax suberifolia* to give rise to the females and males that produce eggs, but a small
148 number of aphids remain on bamboo and continue parthenogenetic reproduction
149 (Takahashi 1923).
150
151 *Pseudoregma alexanderi*
152 Most species of this genus form eusocial colonies on the primary host (*Styrax*
153 spp.) with sterile second-instar soldiers, as in *Astegopteryx* (Aoki and Kurosu 2010). This
154 species has been found in Taiwan (Liao 1976), China (Zhang et al. 2013), Philippines
155 (Calilung, 1985), Nepal (Sharma 1968) and India (Ghosh 1988) on bamboos. However,
156 galls of *P. alexanderi* have never been recorded on a primary host plant. In contrast to *A.*
157 *bambucifoliae*, therefore, *P. alexanderi* in Taiwan is permanently confined to the
158 secondary host plant, *Dendrocalamus latiflorus* (Figure S1).
159 The open colonies of *P. alexanderi* on the shoots and stems of the secondary
160 host, where wingless viviparous females reproduce throughout the year, are eusocial.
161 The clones produce two types of first-instar nymphs: sterile soldiers and normal fertile
162 nymphs. The soldiers are distinguishable from the normal nymphs of the same instar by
163 their larger body size, sclerotized exoskeletons, enlarged and thickened forelegs, and
164 well-developed sharp frontal horns, and they do not moult and grow to adulthood (Aoki
165 1978). They defend their colonies by clasping predators with enlarged forelegs and
166 piercing their skins with a pair of sharp horns on a head (Aoki et al. 1981). The horns and
167 piercing behaviour of soldiers might be derived from head-butting behaviour between
168 colony members, which is found in almost all of the cerataphidine aphids on the
169 secondary host (Aoki and Kurosu 1985; Foster 1996; Howard et al. 1998; Morris and
170 Foster 2008). The soliders in a colony of *P. alexanderi* shows bimodal distribution for
171 morphological characters (Aoki and Miyazaki 1978, Stern et al. 1996). First-instar normal
172 nymphs can disperse by being carried on the wind (Aoki et al. 1981). In spring, winged

173 adults that give rise to the females and males (sexuparae) emerge but they have never
174 been recorded on a primary host.
175
176 **2.2. Sampling**
177 Both aphid species were sampled at Sun Moon Lake, Nantou, Taiwan: *A. bambucifoliae*
178 on 18 November 2012 and *P. alexanderi* on 18 November 2012 and 23 April 2014. The
179 host plant *D. latiflorus* forms a dense cluster of stems (a clump). Aphids were collected
180 from nine (AB01-AB09) and seven (PA01-04 in 2012 and PA05-07 in 2014) clumps of
181 *D. latiflorus* for *A. bambucifoliae* and *P. alexanderi* respectively over an area of more
182 than 1,000 hectares (Figure 1e). In the present study, we defined a "colony" as a group of
183 aphids on the same leaf for *A. bambucifoliae* and on the same developing shoot for *P.*
184 *alexanderi*, in order to facilitate comparison between the two species. For each clump,
185 two colonies were collected from different stems, except for clump PA04, in which only
186 one colony of *P. alexanderi* was collected. In total, we collected 18 colonies of *A.*
187 *bambucifoliae* and 13 of *P. alexanderi*. The numbers of aphids in the colonies that we
188 sampled were roughly similar in the two species, ranging from 52 to 882 (mean 344.5) in
189 *A. bambucifoliae* and from 56 to 831 (mean 235.3) in *P. alexanderi*. Note that it was not
190 possible to record data blind because our study involved focal animals in the field.
191 All the aphids from each colony were put into 95% ethanol and the total number
192 of aphids in each colony was counted under a dissecting microscope. For *P. alexanderi*,
193 the numbers of soldier first-instar nymphs were also counted for each colony.
194 From each colony, 15 – 24 individuals from four age classes (first-instar, second or third
195 instar, third or fourth instar, and wingless adult in *A. bambucifoliae*; first-instar soldier,
196 first-instar normal, from second to fourth instar, and wingless adult in *P. alexanderi*) were
197 selected for DNA extraction and genotyping. In total, 364 individuals of *A. bambucifoliae*
198 and 251 of *P. alexanderi* were genotyped. In addition, from two clumps (PA03 and PA07),
199 where we found multiple clones of *P. alexanderi* by the first genotyping, 112 first-instar
200 (57 soldier and 55 normal) nymphs were subsequently genotyped without breaking their
201 exoskeleton to associate morphological characters with a genotype.
202

203

204 **2.3. DNA extraction**

205 DNA was extracted using a glass milk extraction method in a microtitre plate as described
206 in Amos et al. (2016). In the first genotyping, each aphid was crushed with a plastic pestle
207 or a pipette tip in 40 µl of lysis solution (10 mM Tris HCl (pH 8.0), 1 mM EDTA, 1%
208 SDS) with proteinase K and incubated at 56°C for 12 hours. The DNA was adsorbed to
209 flint glass particles in the presence of a 3× excess of 6 M NaI. After two ethanol washes,
210 the DNA was eluted in 50 µl of low TE buffer. In the additional genotyping in which the
211 exoskeleton was not crushed, we punctured the body of a nymph with an insect pin and
212 incubated it in the lysis solution with proteinase K described above at 56°C for 12 hours
213 to extract DNA and clear inside the body. After three hours, the exoskeleton was picked
214 up and the remaining solution was subjected to DNA extraction and genotyping. The
215 exoskeleton was examined as described below in section 2.6.

216

217 **2.4. Microsatellite isolation**

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

226

227 **2.5. Genotyping**

228 Polymerase Chain Reactions (PCRs) were performed using Type-it Microsatellite PCR
229 Kit (Qiagen) in 10 µl reactions. PCR conditions were as follows: an initial denaturing
230 step of 5 min at 95°C, followed by 35 cycles of 95°C denaturation for 30 sec, 60°C
231 annealing for 90 sec, 72°C extension for 60 sec, followed by a final extension step for 30
232 min at 60°C. One primer from each pair was labelled with a fluorescent dye (6-FAM /

233 HEX / NED) as described in Table 1. After amplification, the PCR product was mixed
234 with The GeneScan 500 LIZ Size Standard (Applied Biosystems) and subjected to
235 fragment analysis. Allele calling was performed by GeneMapper software (Applied
236 Biosystems) and checked manually. Samples with ambiguous genotype were re-run twice
237 and the consensus genotype was taken forward for analysis. To reduce the possible impact
238 of genotyping errors / *de novo* mutations, two individuals with unique multilocus
239 genotypes that were found only once and differed from another genotype by only one
240 single allele were excluded from the analysis. In total, 360 individuals of *A. bambucifoliae*
241 and 244 of *P. alexanderi* were subjected to further statistical analysis.
242
243 **2.6. Morphological examination of first-instar nymphs in *P. alexanderi***
244 Exoskeletons picked up from the proteinase K solution were washed with distilled water
245 and mounted on a slide using Mount-quick Aqueous (Daido Sangyo, Japan). The
246 mounted individuals were photographed using a digital camera (Nikon 1 V2) attached to
247 a light microscope. Their morphological characters (body length, fore-femur length, horn
248 length) were measured using ImageJ software (Schneider et al., 2012).
249
250 **2.7. Data analysis**
251 All statistical analyses were conducted using the software R (R Core Team 2022).
252 Different clones were identified using their multilocus genotypes (MLGs) and the R
253 package *poppr* (Kamvar et al. 2013). Deviations from Hardy-Weinberg Equilibrium
254 (HWE) at each locus were tested based on 1000 Monte Carlo permutations of alleles
255 implemented in R package *pegas* v0.9 (Paradis 2010), using only one individual per MLG.
256 Following others (Gilbert et al. 2007; Vantaux et al. 2011; Kronauer et al. 2013), we use
257 mean pairwise relatedness as a measure both of the potential for altruism and of the
258 genetic similarity between groups of aphids. Pairwise relatedness values among all
259 individuals were calculated following Queller and Goodnight (Queller and Goodnight
260 1989) using Kingroup (Konovalov et al. 2004) software. Since the pairwise data are non-
261 independent, we could not directly compare average relatedness values between the
262 different age or morph categories (Prugnolle and de Meeus 2002). Instead, we performed

263 permutation tests (Coulon et al. 2006) by generating 1000 random resampling sets
264 without replacement, such that each individual occurs only once in a given resampled set.
265 Resampled individuals were chosen from the same colony. Under the null hypothesis, the
266 difference of mean relatedness should follow a normal distribution centred on zero; under
267 the alternative hypothesis (i.e. category-biased dispersal), the difference should be
268 significantly different from zero. SPAGeDI (Hardy and Vekemans 2002) software was
269 used to test whether the pairwise regression relatedness among the aphids decreased with
270 the distance between the aphids. An analysis of molecular variance (AMOVA) (Excoffier
271 et al. 1992) was performed to test for population differentiation in order to show if a pair
272 of colonies on a bamboo clump are demographically distinct. If significant variation
273 occurs between the two colonies, then we inferred that they are genetically distinct units.
274 For the morphological characters of soldier nymphs in *P. alexanderi*, bimodality was
275 tested using Hartigan's dip test (Hartigan and Hartigan 1985) implemented in the R
276 package *diptest*.
277
278 **3. Results**
279 **3.1. Size and disposition of the aphid colonies**
280 Both of the aphid species feed on the same host plant, the Taiwan giant bamboo
281 *Dendrocalamus latiflorus*, but the size and disposition of the colonies of the two species
282 are markedly different. *A. bambucifoliae* feeds on the underside of the bamboo leaves,
283 forming relatively small, discrete colonies (Figure 1c), whereas *P. alexanderi* feeds on
284 shoots and stems, where the individual colonies tend to coalesce into a large colony, often
285 of immense size (Figure 1d). Although we did not measure aphid density, measurements
286 on a related species with similar habits (*Pseudoregma baenzigeri*) suggest there would be
287 around 10 adults or 120 first-instar nymphs per square cm, equivalent to 28,000 adults or
288 340,000 first-instar nymphs for every metre of 9-cm diameter bamboo stem (Aoki et al.
289 2007). The colonies that we studied were at this, or even greater, densities.
290
291 **3.2 Clonal diversity and genetic differentiation**

292 The number of alleles and the heterozygosities (observed and expected) for each locus
293 are given in Table 1. The number of alleles per locus ranged from 5 to 12 (mean 7.9) in
294 *A. bambucifoliae* and from 2 to 7 (mean 3.4) in *P. alexanderi*. When using only one
295 individual per MLG, deviations from Hardy−Weinberg equilibrium (HWE) were
296 significant ($P < 0.05$) in four of eight loci in *A. bambucifoliae* and in six of nine loci in *P.*
297 *alexanderi*. A total of 99 different clones were recognized based on MLGs by using eight
298 microsatellite markers in the study population of *A. bambucifoliae* and 19 different clones
299 by using nine markers in *P. alexanderi*. In *A. bambucifoliae*, each clone was restricted to
300 a single sampling site. By contrast, in *P. alexanderi* three clones were found at multiple
301 (two to six) widely dispersed sites. 94% (230/244) of the genotyped individuals of *P.*
302 *alexanderi* consisted of the four major clones, described as "A", "B", "C", and "D" (Table
303 2). 89% (16/18) of the *A. bambucifoliae* colonies and 77% (10/13) of the *P. alexanderi*
304 colonies consisted of multiple clones (two to 20 clones in *A. bambucifoliae*, two to six in
305 *P. alexanderi*), although half (5/10) of the mixed-clone *P. alexanderi* colonies and 25%
306 (4/16) of the mixed-clone *A. bambucifoliae* colonies were almost monoclonal, with more
307 than 80% of the colony members being of a single genotype.
308
309 **3.3 Relatedness within and between the colonies on a single clump**
310 Average pairwise relatedness between individuals within a colony ranged from 0.04 to
311 1.0 (mean = 0.54, n = 18) in *A. bambucifoliae* and from 0.04 to 1.0 (mean = 0.71, n = 13)
312 in *P. alexanderi*, which is not significantly different between the two species (Welch's t-
313 test, t = -1.27, df = 23.4, P = 0.22, Figure 2 and Table 2). The relatedness value is
314 significantly higher than predicted by a random distribution in 89% (16/18) of *A.*
315 *bambucifoliae* colonies and 77% (10/13) colonies of *P. alexanderi* (permutation test, $P <$
316 0.05, Table 2). Average pairwise relatedness between individuals in the different colonies
317 of the same clump ranged from -0.31 – 0.62 (mean = 0.13, n = 9) in *A. bambucifoliae* and
318 from 0.07 – 1.0 (mean = 0.69, n = 6) in *P. alexanderi*, which is significantly different
319 between the two species (Welch's t-test, t = -2.94, df = 7.6, P = 0.02, Figure 3 and Table
320 2).

321 Table 3 reveals significant variation in genetic variance among different bamboo
322 clumps (16%) and among different colonies on the same clump (38%) in *A. bambucifoliae*,
323 indicating a dispersal process in which related colonies do not usually end up as
324 neighbours. By contrast, in *P. alexanderi*, there was significant variation in genetic
325 variance among different bamboo clumps (63%) but not among different colonies on the
326 same clump (1.4%), suggesting much more limited dispersal and a tendency for colonies
327 to grow and split within a clump. If dispersal is primarily associated with one age class
328 and if dispersers occasionally enter an unrelated colony, we would expect that age class
329 to exhibit reduced pairwise relatedness. In fact, average pairwise relatedness was not
330 significantly different among pairs of the four age-classes in *A. bambucifoliae*
331 (permutation test, $P > 0.05$; electronic supplementary material, Figure S2). The SPAGeDI
332 analysis showed that pairwise relatedness did not decrease significantly with distance
333 between samples for either species (*A. bambucifoliae*: $r^2 = 0.0007$, y = -0.03 - 0.007x,
334 one-tailed $P = 0.34$; n = 360 individuals and 57470 pairs; *P. alexanderi*: $r^2 = 0.0002$, y =
335 -0.15 + 0.01x, one-tailed $P = 0.48$; n = 245 individuals and 25439 pairs), suggesting that
336 dispersal distances are either much smaller or much larger that the spatial scale of
337 sampling in our study.
338
339 **3.4 Differences in the proportion and morphology of soldiers among genotypes in *P.***
340 ***alexanderi***
341 To test whether different clones were more or less likely to produce soldiers, we
342 additionally genotyped 57 soldier and 55 normal first instar nymphs from the two clumps,
343 PA03 and PA07. When included with 25 soldiers and 20 normal first instar nymphs that
344 had already been genotyped, 96% (150/157) of the genotyped nymphs consisted of four
345 major clones, "A", "B", "C", and "D" described above, and all of the four clones
346 contained soldiers. However, the proportion of soldiers to normal first-instar nymphs was
347 significantly different among the four clones in both of the clumps (Fisher's exact test, P
348 $= 0.006$ in PA03 and $P = 0.01$ in PA07, Table 4). For the two clones ("A" and "C") that
349 occurred in both of the clumps, fewer soldiers of genotype A and more of genotype C
350 were found than was expected (Table 4).

To test whether the dimorphism of soldiers of *P. alexanderi*, shown in previous studies (Aoki and Miyazaki 1978, Stern et al. 1996), is genetically or environmentally determined, we performed Hartigan's dip test (Hartigan and Hartigan 1985) implemented in the R package *diptest* using the morphological characters of the genotyped soldier nymphs. 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 S3).

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

380 There are interesting and significant differences between the clonal structures of the
381 colonies of the two species at this spatial scale. The populations of the eusocial aphid *P.*
382 *alexanderi* on large clumps of bamboo are much more genetically homogeneous than are
383 those of the non-eusocial aphid *A. bambucifoliae*. In *P. alexanderi*, but not in *A.*
384 *bambucifoliae*, the average pair-wise relatedness of aphids from the same small colony is
385 not significantly different from the relatedness of two aphids from widely separate
386 colonies on a large bamboo clump. In other words, a clump of bamboo typically contains
387 several different clones of *A. bambucifoliae* but a single large colony of very restricted
388 genetic diversity in *P. alexanderi*. Of the two species, therefore, an individual *P.*
389 *alexanderi* roaming around a bamboo clump is much more likely to encounter a clone-
390 mate.
391
392 **4.3 Clonal structure across different bamboo clumps**
393 There are significant differences between the two species at this larger spatial scale. In
394 the non-eusocial *A. bambucifoliae*, each clone was restricted to a single bamboo clump,
395 whereas in *P. alexanderi*, three of the four major clones were found at multiple sites
396 (Table 2), which were more than 1km apart from each other.
397
398 **4.4 Causes of the differences in clonal structure**
399 The contrasting life-cycles and dispersal modes of the two species almost certainly
400 underlie these differences in clonal structure (Figure 5). In *A. bambucifoliae*, colonies are
401 founded by individual winged aphids flying from the primary host (*Styrax*) to the
402 secondary host (bamboo) and it seems that there is little direct dispersal by aphids
403 between separate bamboo plants. In Taiwan, the secondary host generations of *P.*
404 *alexanderi* have lost all connection with the sexually-produced generations on the
405 primary host and are maintained on the bamboo plants entirely by parthenogenesis.
406 Younger instars of *P. alexanderi* can disperse by walking or floating on the air to found
407 new colonies on distant bamboo plants (Aoki et al. 1981). However, the colonies of *P.*
408 *alexanderi*, the eusocial species, tend to coalesce into a large colony on a clump of
409 bamboo, which are both larger and more genetically homogeneous than the colonies on

410 bamboo of the non-eusocial species, *A. bambucifoliae*, which are regularly restocked by
411 genetically distinct aphids flying from the primary host.
412
413 **4.5 Consequences of differences in colony kin structure on soldier evolution in open**
414 **colonies**
415 Relatedness within a colony does not by itself determine the evolution of altruism, but it
416 does set the threshold that the benefit to cost ratio of helping must exceed before altruism
417 can evolve. Clonal mixing occurs in both species, but the average pair-wise relatedness
418 within a colony of both species was higher than 0.5, the average relatedness between
419 parent and offspring, and between siblings in a monogamous diploid family. These values,
420 however, can be lower than relatedness values in colonies of some non-social aphids (0.90
421 in *Aphis fabae fabae*, 0.79 in *A. fabae cirsiiacanthoidis*) (Vantaux et al. 2011), although
422 other studies that use different measures of genetic relatedness within a colony show
423 higher degree of clonal mixing (Yao and Akimoto 2009; Loxdale et al. 2011).
424 Why has *P. alexanderi* opted to produce a sterile, morphologically
425 specialized soldier caste, whereas *A. bambucifoliae* has not? Our genetic observations
426 show that the major difference between the two species is that the eusocial *P. alexanderi*
427 lives in populations that are relatively homogeneous genetically, even across different
428 stems in a bamboo clump, whereas the non-eusocial *A. bambucifoliae* lives in colonies,
429 each of which tends to be genetically distinct. A soldier aphid in *P. alexanderi* will
430 therefore be much more likely to be defending a clone-mate, however far it has roamed
431 on the bamboo clump, than is a defending first-instar of *A. bambucifoliae.* A defensive
432 individual of *A. bambucifoliae* is quite likely to find itself defending a non-clone mate
433 and the benefit/cost ratio for an average act of altruism will therefore be much lower than
434 for a soldier of *P. alexanderi*.
435
436 **4.6 Other ecological factors that might affect soldier evolution in open colonies**
437 A second potentially important difference between the two species is simply that the
438 colonies of *P. alexanderi* are much larger than the colonies of *A. bambucifoliae.* We
439 regularly observed dense colonies of *P. alexanderi* on the bamboo stems, which from

440 comparisons with related *Pseudoregma* species (Shibao 1998; Aoki et al. 2007) must
441 have exceeded over 10,000 individuals. Some models suggest that, in any social organism,
442 a soldier caste is more likely to evolve in larger colonies (e.g. Oster and Wilson (1978);
443 Bourke, 1999; Ferguson-Gow *et al.*2014), but the evidence is mixed (Dornhaus, Powell
444 & Bengston, 2012). In the social aphids, there is good theoretical and empirical support,
445 from data on the development of individual colonies, for the idea that investment in
446 soldiers increases as colonies grow larger (e.g. Aoki and Kurosu 2003, 2004; Aoki and
447 Imai 2005; Sakata et al.1991; Schutze and Maschwitz 1991; Tanaka and Ito 1994; Shibao
448 1998). However, soldiers have been maintained in the open colonies of some
449 Cerataphidini species with relatively small colonies (fewer than 1,000 aphids) such as
450 *Ceratovacuna japonica* (Hattori et al. 2013) and *Pseudoregma panicola* (Aoki 1982). To
451 understand soldier evolution in these relatively small colonies, future studies should
452 investigate their genetic homogeneity on a large spatial scale. In addition, a phylogenetic
453 comparative approach that included genetic homogeneity, colony sizes and other traits of
454 many species of Cerataphidini could reveal whether a large colony favoured the initial
455 evolution of eusociality.
456 A third factor that might be critical in the evolution of altruism in these
457 open colonies is the nature of the host-plant and of the part of the plant upon which the
458 aphids feed. Bamboos are long-lived perennial plants that grow and spread rapidly,
459 producing a high biomass. Colonizing bamboo therefore enables long colony lifetimes
460 and large colony sizes and it ensures the provision of vacant patches for dispersing aphids.
461 In addition, it has been suggested that bamboo is a relatively poor food-source and that
462 therefore investing in defensive behaviour is a wise strategy (Stern and Foster 1996),
463 because the colonies are compelled to grow slowly. There is little direct evidence for this
464 idea, but it is telling that the two species of horned aphids within the clade that have lost
465 the soldier morph on the secondary host have both shifted from bamboo to herbaceous
466 broad-leaved grasses (Stern 1998). This factor might be especially important for *P.*
467 *alexanderi*, which grows on the hard woody stems and shoots of bamboo, rather than for
468 *A. bambucifoliae*, which feeds on the relatively more nutritious leaves. This idea needs to
469 be tested experimentally.

470

471 **4.7. Maintenance of eusociality in open colonies**

472 Social colonies that are not corralled within a nest face the additional problem of being
473 especially vulnerable to invasion by free-rider unrelated conspecifics from another colony.
474 This could be especially tricky for aphids which seem unable to distinguish clone mates
475 from non-clone mates, in a variety of contexts (Aoki et al. 1991; Shibao 1999; Abbot et
476 al. 2001). If this lack of kin recognition is genetically based, it might be a consequence
477 of Crozier's Paradox (Crozier 1986; Field et al. 2018). This posits that recognition cues
478 will not evolve if individuals act as both hosts and invaders and if the costs of rejection
479 are higher than the costs of acceptance, both of which might be true for these social aphids.
480 There is some evidence that aphids are able to rely on the context in which they encounter
481 another aphid. For example, first-instars of *Pemphigus* species appear to know when they
482 are not in their home gall and behave appropriately (Abbot et al. 2001; Abbot 2009). An
483 immature *P. alexanderi*, having been carried on the wind to a distant bamboo clump,
484 might also "know" that it is no longer near its home clone, and behave accordingly. This
485 is perhaps less likely in the non-eusocial *A. bambucifoliae*, which do not disperse over
486 long distances between different bamboos and are more likely to encounter an alien
487 colony after a relatively short and imperceptible excursion from their home clone.

488 Our results, admittedly from a small sample size, show that all of the four
489 major clones of *P. alexanderi* contain soldiers in the mixed-clone clumps, suggesting that
490 there is no obligate cheater clone that never produces soldiers. However, our results do
491 not rule out the existence of conditional cheater clones that produce fewer altruistic
492 soldiers when coexisting with other clones, as found in *Pemphigus* aphids (Abbot et al.
493 2001; Abbot 2009). In fact, the proportion of soldier and normal first-instar nymphs in
494 the two mixed-clone clumps were significantly different among the clones, and the
495 difference was consistent in both clumps, in which clone "A" produced fewer soldiers
496 than expected, while clone "C" produced more (Table 4). This result suggests that there
497 is genetic variation in soldier investment among clones, which could potentially adjust
498 their investment of soldiers according to the presence of other clones in the same colony,
499 although our observations provide no direct evidence for such an adjustment.

The existence of dimorphic soldiers (Figure 4) suggests that the open colonies of *Pseudoregma alexanderi* are relatively sophisticated for a eusocial aphid. Our results show that both the large and small soldiers are, like aphid morphs more generally, differential expressions of the same genotype (Figure S3). The functional significance of this dimorphism, which was not found in two related *Pseudoregma* species (Shingleton and Foster 2001), might be in providing useful phenotypic variation in the defensive effectiveness of the soldiers (Stern et al. 1996, Hattori et al. 2017). 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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530

531 **Data Availability Statement**

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

534

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770
771

772 **Figure legends**

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

778

779 Figure 2. Within-colony pairwise genetic relatedness (n = 18 for *A. bambucifoliae* (*a*); n
780 = 13 for *P. alexanderi* (*b*)). Box plots show the range of values, the first and third quartiles
781 and medians. Open circles show outliers and filled circles show means.

782

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

787

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

792

793 Figure 5. Comparison of the genetic structure between *A. bambucifoliae* (a) and *P.*
794 *alexanderi* (b) within and between bamboo clumps. Colours in the pie charts indicate
795 different clones. In *A. bambucifoliae*, winged adults frequently migrate from a distant
796 gall-harbouring primary host plant, then mix with other resident clones in a bamboo
797 clump. In *P. alexanderi*, the dispersing morphs are young nymphs that walk around
798 between local clumps or migrate to long distance by walking or being borne by the wind.
799 *P. alexanderi* has no primary host plant in this locality and can thus propagate only by
800 parthenogenetic reproduction. As a result, colonies on the local site are genetically
801 homogenised and major clones can spread to a distant bamboo clump in this species.

802

803

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- AGTCGATGAGATAGCATAAGAACGA	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 Rev-TGAAGACAGAGAGTGAAGCCG	4	0.67	0.73	0

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.

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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

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

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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.

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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).

811

812

Figure 1.

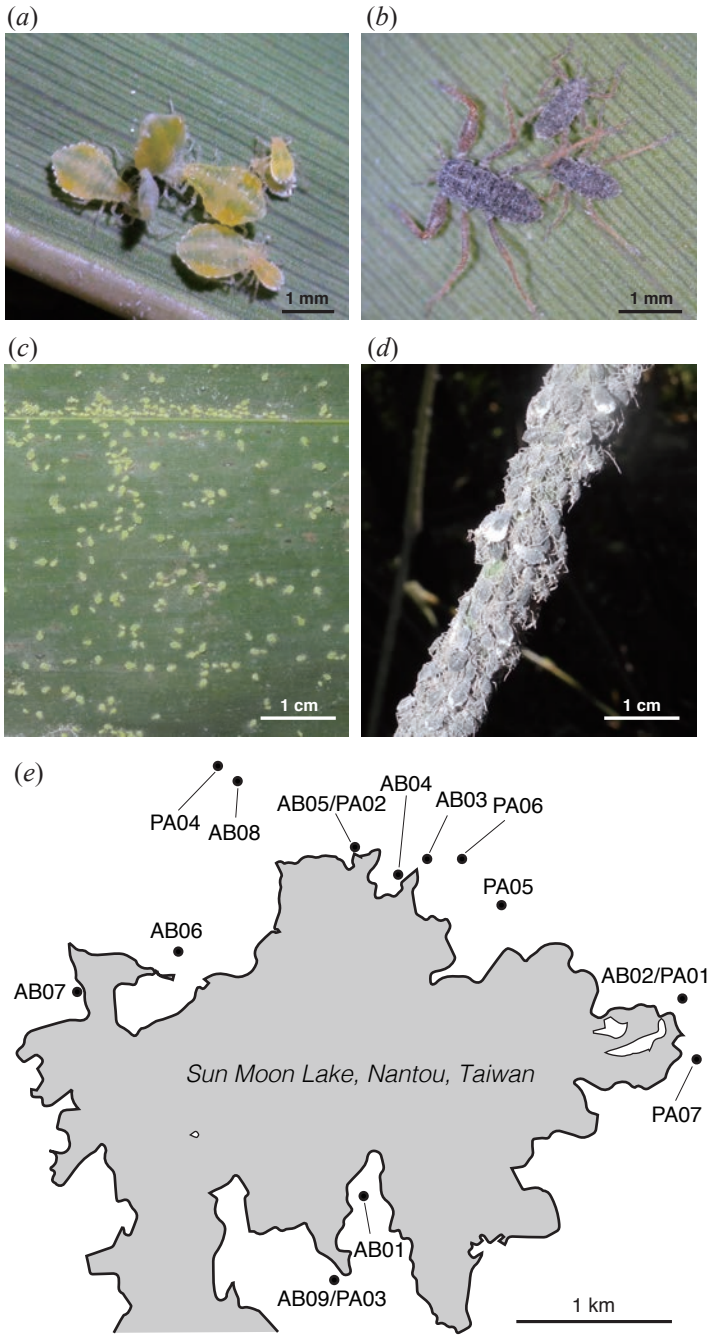

(a)
1 mm
(b)
1 mm
(c)
1 cm
(d)
1 cm
(e)
PA04
AB08
AB05/PA02
AB04
AB03
PA06
PA05
AB06
AB07
AB02/PA01
Sun Moon Lake, Nantou, Taiwan
PA07
AB01
AB09/PA03
1 km

Figure 2.

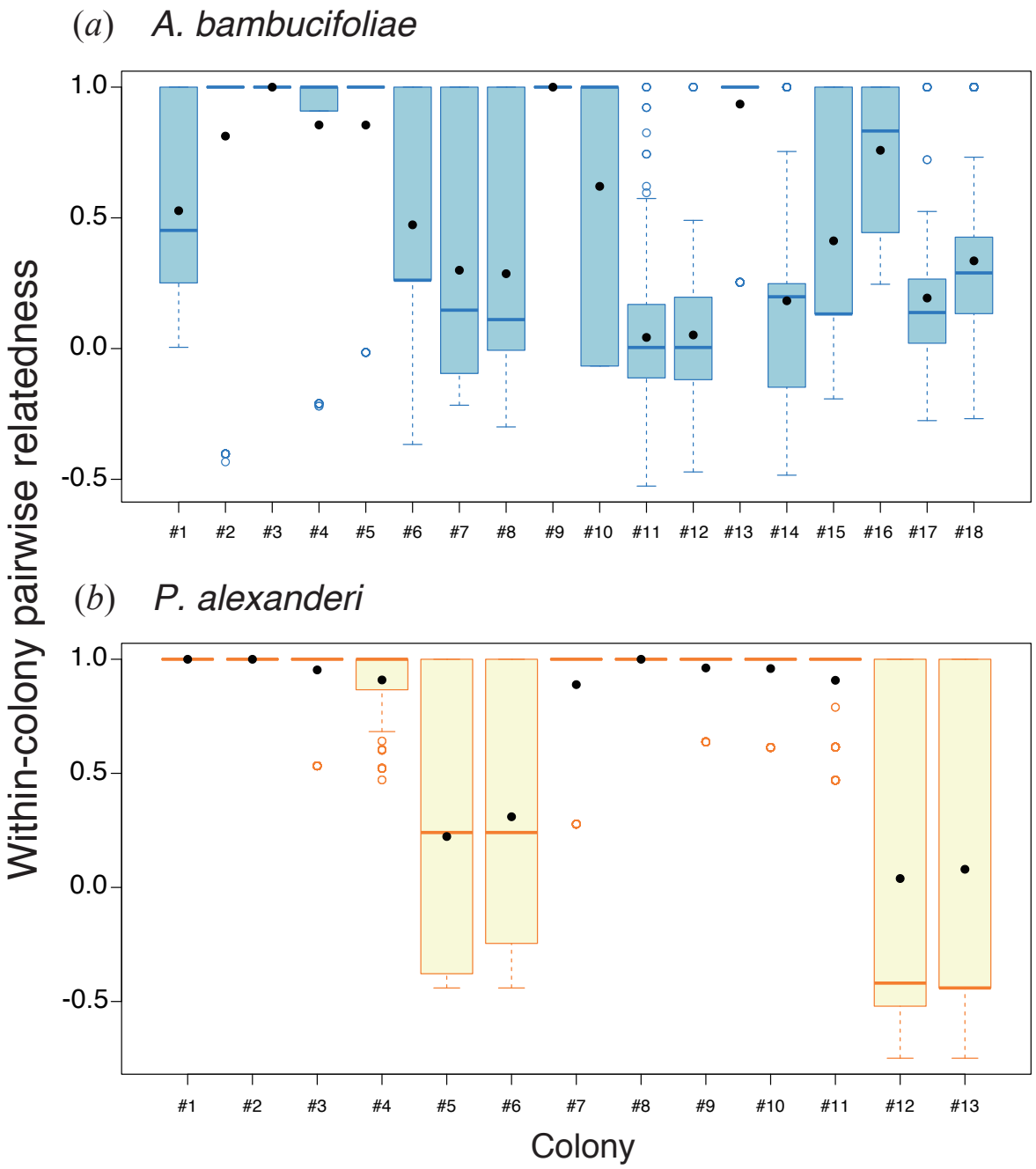

(a) A. bambucifoliae
1.0
0.5
0.0
-0.5
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13 #14 #15 #16 #17 #18
(b) P. alexanderi
1.0
0.5
0.0
-0.5
#1 #2 #3 #4 #5 #6 #7 #8 #9 #10 #11 #12 #13
Within-colony pairwise relatedness
Colony

Figure 3.

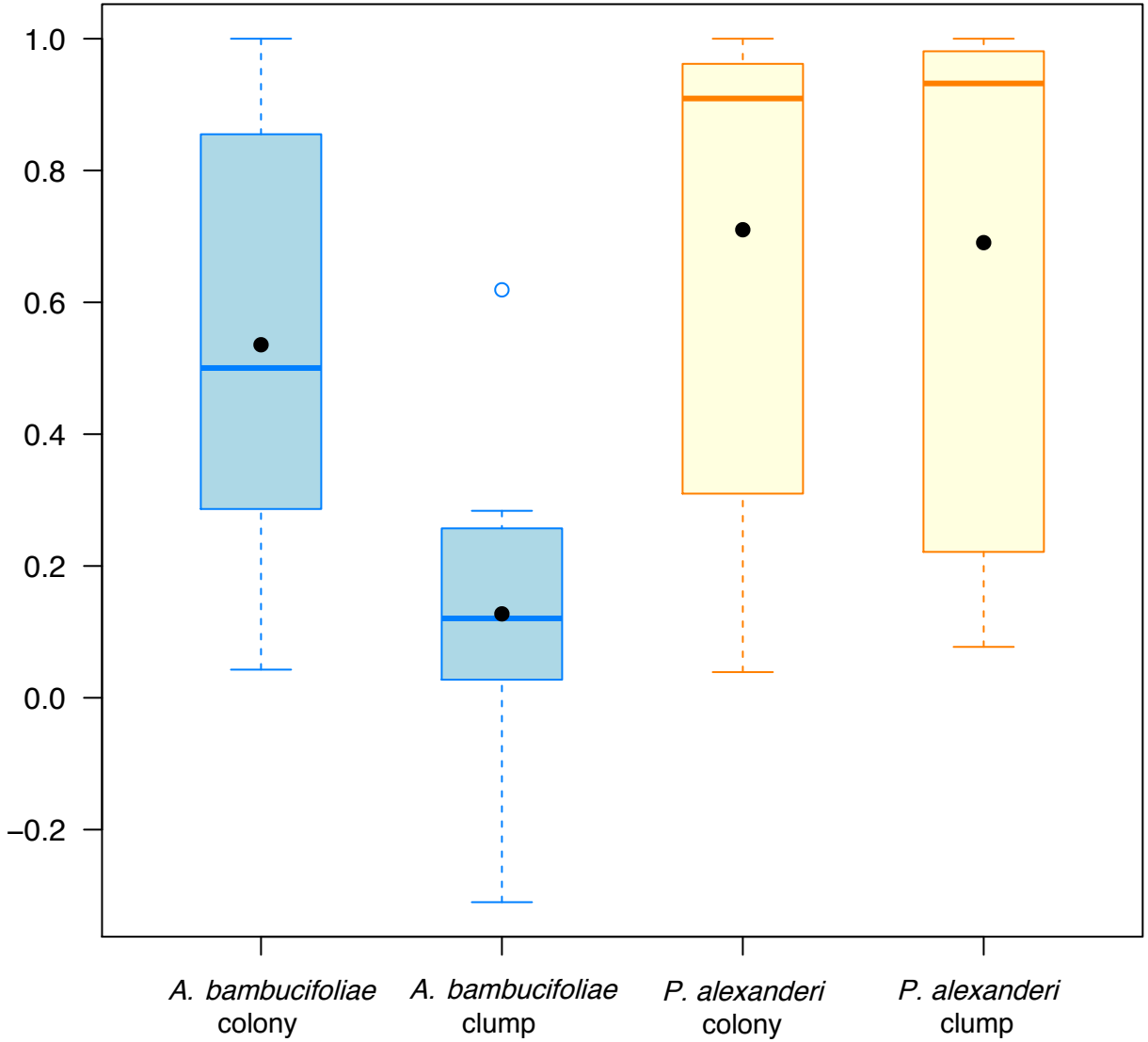
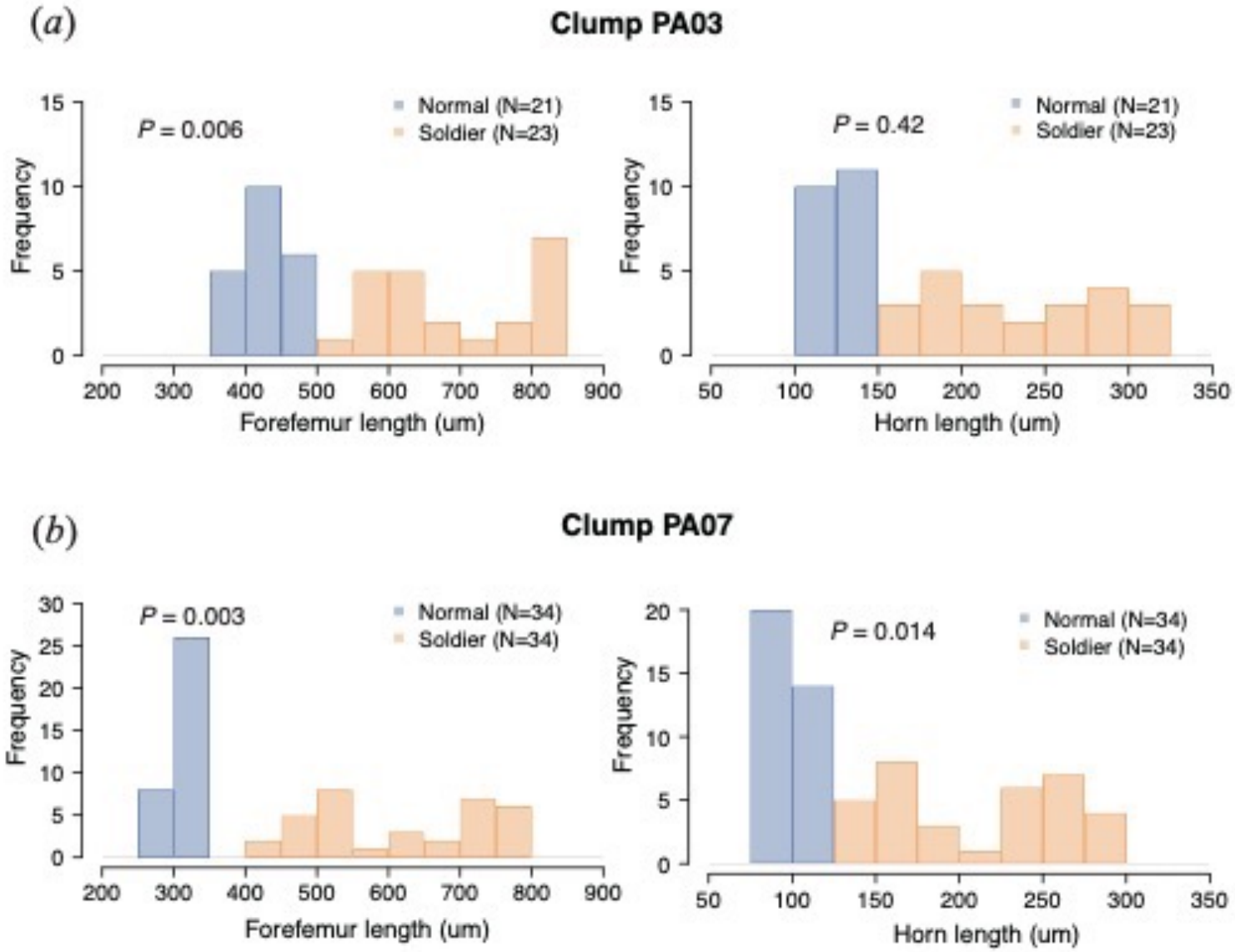

1.0
0.8
0.6
0.4
0.2
0.0
−0.2
A. bambucifoliae colony
A. bambucifoliae clump
P. alexanderi colony
P. alexanderi clump

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)

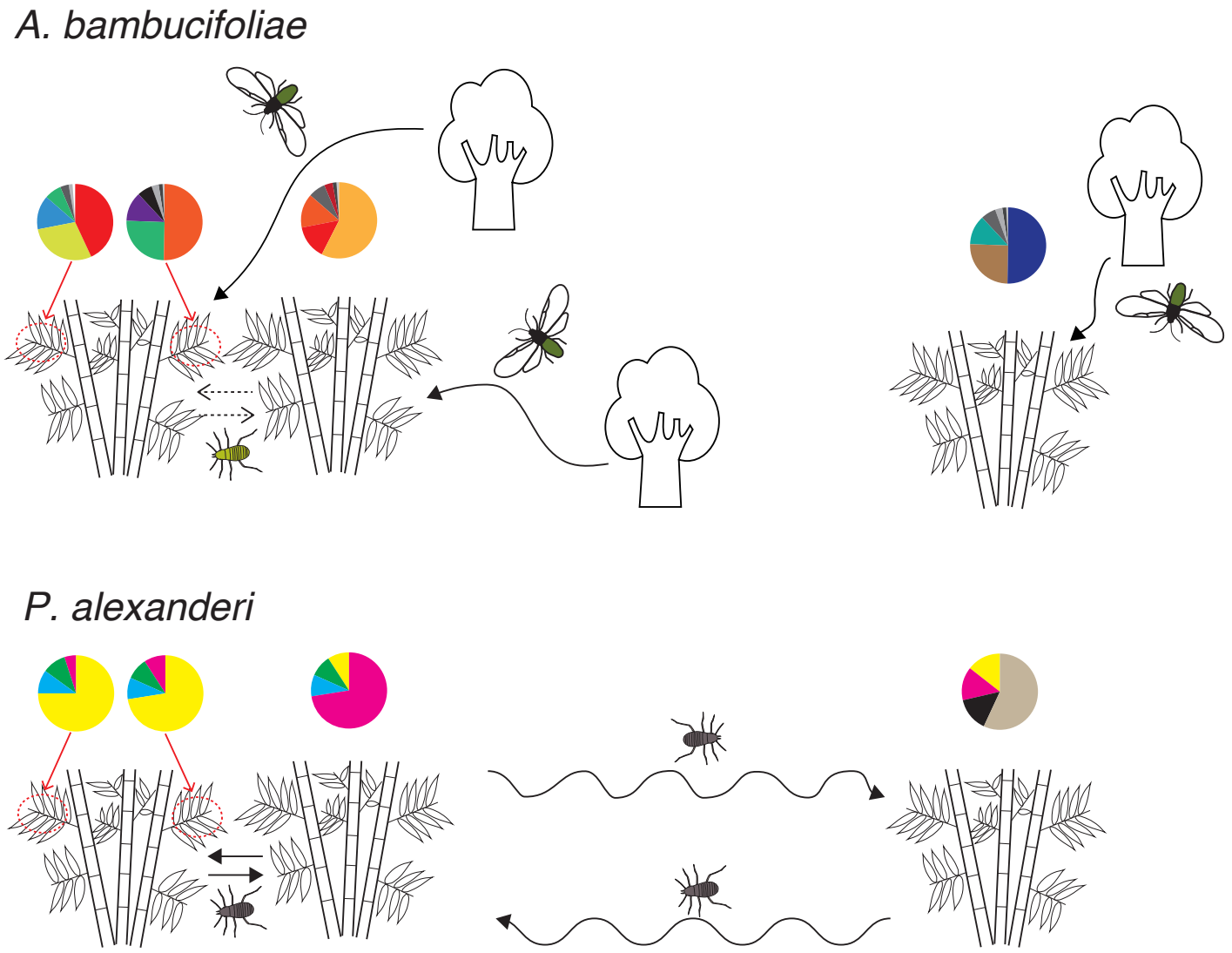



Figure 5.