



RESEARCH ARTICLE - SOCIAL ARTRHOPODS

Clonal composition of colonies of a eusocial aphid, *Ceratovacuna japonica*

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Abstract

High degrees of relatedness among colony mates and kin recognition ability are important factors maintaining eusociality because kin-selection favors eusociality when donor and recipient of altruistic acts are related to each other. However, in eusocial aphids, clone mixing between different colonies occurs frequently, suggesting a lack of kin recognition. Studies investigating the clonal composition of eusocial aphid colonies have focused on the aphid generation on the primary host plant (gall generation) and showed that clone mixing occurs frequently among galls (= nest). To test whether clone mixing also occurs in open colonies of eusocial aphids on their secondary host plants (open-colony generation), we carried out an amplified fragment length polymorphism analysis to investigate clonal composition within colonies of the eusocial aphid *Ceratovacuna japonica*. The results showed that clone mixing occurred frequently in open-colony generation. They suggest that not only relatedness but also other factors (e.g., ecological background) are important for maintaining eusociality in eusocial aphids.

Introduction

Altruistic behavior, in which sterile individuals benefit their kin individuals, has evolved in eusocial insects of some taxonomic groups (Trivers & Hare, 1976; Hölldobler & Wilson, 1990; Pike & Foster, 2008). Eusocial aphids produce sterile individuals (so-called soldiers) that protect their colony mates from predators (Stern & Foster, 1996; Pike & Foster, 2008). Soldiers have morphological, behavioral and physiological traits that have specialized functions against predators (Kutsukake et al., 2004; Hattori & Itino, 2008; Hattori et al., 2013a, b). For example, soldiers of the eusocial aphid *Ceratovacuna japonica* (Homoptera, Hormaphidinae) have longer horns and forelegs than non-soldiers (Hattori & Itino, 2008). When they encounter a predator, they grasp it with their forelegs and then stick their frontal horns into the body of the predator to kill it or to delay its predation (Hattori et al., 2013a).

Kin selection theory predicts that such a reproductive division of labor is maintained by high genetic relatedness within a colony (Hamilton, 1964). In accordance with this

hypothesis, some hymenopteran insects can recognize non-kin individuals and prevent them from invading the colony (Hölldobler & Wilson, 1990). Such kin recognition may generally help maintain eusociality (Hamilton, 1964 but see Abbot, 2009), because an ability of kin recognition can keep high relatedness between donor and recipient in altruistic interactions (Gadagkar 1985). However, some eusocial aphids (e.g., *C. japonica*, *Ceratoglyphina bambusae*, and *Pseudoregma bambucicola*) cannot recognize kin individuals (Aoki et al., 1991; Carlin et al., 1994; Shibao, 1999; for a review see Pike & Foster, 2008).

Several eusocial aphids show a cyclic parthenogenesis which is composed of a single sexual generation and multiple parthenogenetic generations, and host alternation (i.e., seasonally migrating from the primary host to the secondary host vice versa) (Stern & Foster, 1996). In the primary host generation, aphid often construct gall (= nest) (Aoki & Kurosu, 2010). Several studies have shown that even within a eusocial aphid gall on the primary host (= nest), the degree of relatedness among colony mates is low (Abbot et al., 2001; Johnson et al., 2002; Wang et al., 2008). This low relatedness indicates that



clone mixing occurs due to the absence of kin discrimination and exclusion of non-kin individuals at the nest entrance. Therefore, we can hypothesize that clone mixing frequently occurs not only on primary hosts but also (even more frequently) on secondary hosts where free-living generation of aphids inhabits without a gall. In this study, to determine whether clone mixing occurs in colonies on secondary host plants, we surveyed the clonal composition of colonies of the eusocial aphid, *C. japonica*, by using amplified fragment length polymorphism (AFLP) analyses.

Material and Methods

The eusocial aphid

Ceratovacuna japonica Takahashi is a eusocial aphid that parthenogenetically produces sterile “pseudoscorpion-like” first-instar soldiers, which defend their colony on secondary host plants. This species has one primary host plant, *Styrax japonica* Sieb. et Zucc. (Ebenales, Styracaceae), and several secondary host plants (Poaceae species; *Sasa senanensis* Rehd. in central Japan) (Aoki & Kurosu, 1991, 2010). Adult individuals on secondary hosts produce alate individuals in the fall, which disperse to the primary host plants on which they overwinter. However, in central Japan where this study was conducted, *C. japonica* aphids are rarely observed on primary hosts. This suggests an overwinter on the secondary host *S. senanensis*. In fact, we observed apparently overwintering individuals (alone or with a few mates on a leaf) in the fall and winter season on *S. senanensis*. In this paper, we define an aphid colony as an aggregation of aphid individuals on a single leaf of a secondary host plant.

Field sampling

Field sampling was conducted in an aphid population parasitizing a *S. senanensis* patch (about 10 m × 5 m in size) at the edge of a deciduous coniferous forest (dominated by *Larix kaempferi* [Pinales, Pinaceae]) at the foot of Mt. Norikura, Nagano, central Japan (1600 m above sea level; 36°8′16.64N, 137°36′24.5E). In August 2012, we randomly selected five

aphid colonies, each consisting of 200–1000 aphid individuals, and randomly collected 10 soldiers and 10 adults from each colony. We preserved the collected specimens in 100% ethanol before AFLP analyses.

Genetic analyses

We extracted DNA from the ethanol-preserved aphids by using a “salting-out” protocol (Sunnucks & Hales, 1996). We performed AFLP analysis according to the method of Vos et al. (1995) with some modifications. Genomic DNA was digested with the restriction enzymes *EcoRI* and *MseI* at 37°C for 1.5 h. Double-stranded adaptors were then ligated to the ends of the digested DNA fragments at 20°C, overnight. We performed pre-selective amplification performed for 20 cycles, using a primer pair with one additional nucleotide on each restriction enzyme (*MseI*-C/*EcoRI*-A) with the following cycle profile: a 30-s DNA denaturing step at 94°C, a 1-min annealing step at 56°C, and a 1-min extension step at 72°C. We performed selective amplification performed for 30 cycles with the following cycle profile: a 30-s denaturing step at 94°C, a 20-s annealing step, and a 2-min extension step at 72°C. The annealing temperature of the first cycle was 66°C, then for each of the next 10 cycles, it was reduced by 1°C each cycle, and finally it was maintained at 56°C for the remaining 19 cycles. Selective polymerase chain reaction (PCR) amplifications were then conducted with a fluorescence-labeled primer combination: *EcoRI*-ACA (FAM)/*MseI*-CCG. A Dice TP600 PCR Thermal Cycler (Takara Bio, Shiga, Japan) was used with the AFLP Amplification Core Mix (Applied Biosystems, Foster City, CA, USA) for both the pre-selective and selective amplifications. AFLP fragments were detected with an ABI Prism 3130 automated sequencer (Applied Biosystems) and Gene Mapper software v.4.0 (Applied Biosystems). In this analysis, we selected 8 AFLP bands and obtained fragment data from 57 of the 100 collected aphid individuals. We did not include the fragment data from the remaining 43 individuals in the following analyses because the quality of the data from these specimens was low.

To observe the clonal composition of the aphid colonies, we analyzed the fragment data of each aphid individual by conducting a principle coordinate analysis (PCoA) (Gower, 1966) with R Version 2.13.0 software (R Development Core Team, 2011). In this analysis, we considered specimens with identical PCoA scores to be clones.

Table 1. Clonal composition of the five *C. japonica* colonies. Lowercase letters indicate identical clones. S, soldier; N, non-soldier adult.

Colony No.	Clone (number of individuals by caste*)
1	a (1S, 2N), c (2S), f (1N), g (1N), j (1N), m (1S), n (2N), o (1S)
2	b (1N), c (2N), d (1N), I (1S), j (1S), n (2S, 1N), o (3S, 4N)
3	b (1S), c (1S), I (1N), n (N1), o (3N)
4	c (1S, 1N), e (1N), f (1N), g (1N), k (1N), n (1S), o (2S, 2N)
5	d (2S), h (1N), j (5N), o (2N)

*S: Soldiers, N: non-soldier adults

Results

We found 15 clones (designated by the letters a–o) among 57 individuals from five colonies of *C. japonica* (Table 1, Fig 1). The number of clones per colony ranged from four to seven, and many clones occurred in several different colonies. For example, the ‘a’ clone was found in only colony 1, and the ‘o’ clone was found in all five colonies (Table 1). Each clone may consist of (1) only soldiers (first-instar larvae) (‘i’ and ‘m’

clones), (2) only non-soldier adults ('f', 'g' and 'h' clones), or (3) both soldiers and non-soldier adults ('a', 'b', 'd', 'e', 'j', 'l', 'n' and 'o' clones) (Table 1). Furthermore, PCoA showed that frequency of clone mixing did not relate to genetic similarity among clones (Table 1, Fig 1). For example, in colony 1, distant clones (e.g., 'a' and 'j') coexisted.

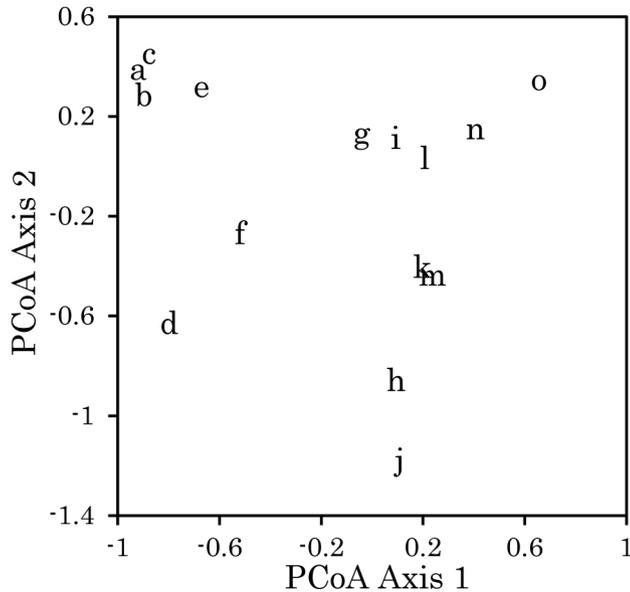


Fig 1. Two-dimensional principal coordinate analysis based on the result of AFLP. PCoA Axis 1 and PCoA Axis 2 explained 26% and 21% of the total variance, respectively. Lower cases indicate clones (c.f., Table 1). Distance between lower cases shows a difference of fragment pattern in the result of AFLP.

Discussion

Despite the limited samplings, our AFLP analysis results clearly showed that *C. japonica* colonies on secondary host plants are composed of multiple clones (Table 1, Fig 1). Such clone mixing in eusocial aphid colonies has been previously reported in gall-forming social aphids (Abbot et al., 2001; Johnson et al., 2002; Wang et al., 2008), but has not yet been reported in free-living social aphids such as *C. japonica*. The presence of multiple clones within a single colony indicates that aphid individuals often migrate from their natal colony to other colonies as was described for non-social aphid species (Dixon, 1998; Loxdale et al., 2011). Moreover, Carlin et al. (1994) showed that reproductive individuals and soldiers failed to exclude other colony members significantly more than their colony members when they met their colony members and other colony members. From this observation, they concluded that *C. japonica* aphids do not discriminate kin. Taken together, these findings suggest that colonies of this species accept non-kin intruders.

In eusocial aphids, gall on the primary host may have an important function as a physical wall to prevent intruding conspecific cheaters to some extent. In fact, some social aphids repair their galls when damaged; *P. spyrothecae* induces the host plant to create complementary regrowth in the gall

to fill a hole (Pike & Foster, 2004). Such behavior may be selected against predators and conspecific cheating intruders. On the other hand, surprisingly, *C. japonica* in the secondary host, cannot discriminate kin. Without kin recognition in the free-living, secondary host generation, the aphids may suffer intensive invasion by non-kin cheaters.

Kin selection theory predicts that a high degree of relatedness among individuals within a colony is a mechanism for the maintenance of sociality (Hamilton, 1964). In fact, in the gall-forming social aphid *Pemphigus obesinymphae* (Homoptera, Pemphigidae), which does not have kin-recognition ability, intruders to non-kin colonies behave and develop selfishly; they rarely attack predators and are more than three times as likely to be reproductive as individuals that remain in their natal galls (Abbot et al., 2001). Theoretically, acceptance of intrusions by unrelated selfish individuals should gradually increase dominance of non-kin offspring, which may eventually break the sociality of this aphid species. However the eusociality of *C. japonica* is maintained despite the occurrence of clonal mixing as shown in this study. This fact implies the maintenance of eusociality in this species is not explained by relatedness only. For example, ecological background such as predation pressure may be important for the maintenance of eusociality in aphids because predation may select for colonies composed of only altruistic clones even if relatedness between clones is low. To understand how some individuals come to migrate to and to invade into other colonies and the precise mechanism that allows the maintenance of eusociality in *C. japonica* despite clonal mixing, future studies should observe the migration and competition of clone-identified individuals.

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