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ENTOMOLOGY

Color polymorphism in an aphid is maintained by attending ants

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The study of polymorphisms is particularly informative for enhancing our understanding of phenotypic and genetic diversity. The persistence of polymorphism in a population is generally explained by balancing selection. Color polymorphisms that are often found in many insects and arthropods are prime examples of the maintenance of polymorphisms via balancing selection. In some aphids, color morphs are maintained through frequency-dependent predation by two predatory insects. However, the presence of color polymorphism in ant-attended aphids cannot be explained by traditional balancing selection because these aphids are free from predation. We examined the selective advantages of the existence of two color (red and green) morphs in the ant-attended aphid, *Macrosiphoniella yomogicola*, in fields. We measured the degree of ant attendance on aphid colonies with different proportions of color morphs. The results show that the ants strongly favor aphid colonies with intermediate proportions of the two color morphs. The relationship between the degree of ant attendance and the proportion of color morphs in the field is convex when aphid colony size and ant colony size are controlled. This function has a peak of approximately 65% of green morphs in a colony. This system represents the first case of a balancing polymorphism that is not maintained by opposing factors but by a symbiotic relationship.

INTRODUCTION

Polymorphisms are often found to persist in populations (*1–4*). The persistence of polymorphisms in populations may be explained by balancing selection. For genetic polymorphisms, two different nonmutually exclusive forms of balancing selection have been described: (i) negative frequency (or density) dependence of various morphs, and (ii) trade-off balance of two independent factors (*1–4*). For solely phenotypic polymorphisms (no genetic differences), adaptive coin flipping between different morphs is often the best solution for adapting to stochastic environments (*2*). In all cases, the balance between two different factors yields optimal polymorphisms (*1–4*).

In persistent genetic polymorphisms, a morph should become selectively advantageous when it is rare but disadvantageous when it is abundant (*4*). Negative frequency (or density)–dependent selection on various morphs is a prime example of this condition (*4–8*). Scale-eating cichlids are known to exhibit frequency-dependent fluctuations in the frequency of mouth types (*5*). A damselfly species is also known to show a similar fluctuation between the gynomorphs and andromorphs of females due to frequency-dependent male disturbances of oviposition by attempted mating (*6*). In some aphid species that are not attended by ants, color morphs are also maintained by negative frequency–dependent predation (*7*). There is also evidence that rare color morphs in male guppies are more attractive to females (*8*). Thus, negative frequency dependence is a well-known mechanism for the maintenance of genetic polymorphisms.

Another mechanism has also been reported to explain the maintenance of genetic polymorphisms: fitness equalization between two (or more) morphs by a selective trade-off between two independent factors under environmental heterogeneity. For example, color morphs in a web spider (*Nephila maculata*) are explained by the selective trade-off between foraging efficiency and abiotic factors (for deterring predation). This spider exhibits two color morphs: (i) normal type (stripes of green, black, white, and yellow) and (ii) black type (*9*). The colorful normal type is superior in attracting insect prey because the normal phenotype reflects more ultraviolet light than does the black phenotype. On the other hand, the black phenotype permits high tolerance to low temperatures because of its thermoregulatory advantages (black absorbs more light than does any other color). This phenotype is also considered a form of cryptic coloration. The balance between these opposing selective pressures would equalize the fitness of the two morphs (selectively neutral). However, if there were no negative frequency–dependent selection, this polymorphism would not be held by the effect of random genetic drift, causing a random walk leading to the eventual exclusion of one morph. Environmental heterogeneity is required to maintain the color polymorphism in this web spider because each morph would have an advantage over the other in microscale habitats distributed in patches across the entire habitat. Thus, each morph does not disappear from the whole habitat area because of random genetic drift.

Another mechanism that maintains a genetic polymorphism is overdominance between two alleles on a locus, where the heterozygote has the highest fitness. In this case, the two alleles and consequent phenotypic polymorphism are maintained. In conclusion, as explained above, there are three major mechanisms known for the maintenance of genetic polymorphisms.

Many species of aphids exhibit color polymorphisms (*3*). Polymorphic coloration originates from the color of cells, hemolymph, or symbionts (*10*). Coloration in aphid species is known to have a genetic basis (*11*). Because color morphs are inherited through parthenogenetic reproduction, these morphs can be treated as a form of genetic polymorphism, even if coloration of morphs is controlled by symbionts. Few studies have investigated why these color polymorphisms are maintained in a population (*7, 12, 13*). A sympatric occurrence of a

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green and a red morph has been reported in the pea aphid, *Acyrthosiphon pisum*, which is not attended by ants. Previous studies have shown that frequency-dependent selection by two different predators on each morph maintains the color polymorphism in this species (*7*). A ladybug (*Coccinella septempunctata*) preys on the red morph preferentially, whereas a parasitoid wasp (*Aphidius ervi*) preys on the green morph (*7*). Thus, negative frequency–dependent selection acts on both morphs and maintains the coexistence of both morphs (*12, 13*).

Here, we investigated a color polymorphic mugwort aphid (*Macrosiphoniella yomogicola*; Fig. 1) that is attended by ants. Because ants attend this species, *M. yomogicola* should not experience strong predator-mediated selection. Thus, the coexistence of the two color morphs in this species cannot be explained by frequency-dependent predation. We should also note that the two color morphs cohabit sympatrically on a mugwort shoot (Fig. 1). This means that the balancing selection of two opposing selection pressures is not likely to explain this polymorphism, because the environmental heterogeneity that is required to maintain this type of polymorphism is unlikely to take place. Polymorphism by overdominance is also unlikely in this case, because the two color morphs reproduce parthenogenetically during the observed spring-summer seasons. Therefore, the former three hypotheses are not likely to explain the color polymorphism in *M. yomogicola*. Thus, we hypothesized that the color polymorphism in *M. yomogicola* is maintained by the attending ants because they prefer aphid colonies with a specific ratio of the two morphs. To test this hypothesis, we measured the preference of the ants to the aphid colonies with different proportions of the two morphs. We initially evaluated the effects of attending ants on the survival of the aphid colonies by experimental removal of the ants. We show that the attending ants are necessary for the persistence of the colony. We then

evaluated the preferences of ants for aphid colonies with different proportions of the two morphs.

RESULTS

The ant removal experiment showed that ant attendance is necessary for the survival of a colony (Fig. 2A). Most ant-attended colonies survived (seven of eight), whereas most colonies without ants became extinct (seven of eight) (Fig. 2A; Fisher's exact test, *P* = 0.010). Even in the survived colony with no ant attendance, only one aphid survived. Thus, ant attendance is necessary for the survival of aphid colonies in *M. yomogicola*. The total number of predators (larvae and adults of a ladybug, larvae of lacewing flies, and a parasitoid wasp) found on the mugwort shoots is marginally larger in the ant-removed treatments than in the ant-attended ones (*t* test, df = 12, *t* = 1.782, *P* = 0.087), suggesting that the ants are likely to remove these predators from the aphid colonies. Note that all the above predators were observed in other locations around the aphid colonies, and we never found any predator within the aphid colonies attended by the ants. This observation suggests that the ants completely removed predators from the aphid colonies.

The low survival rate of the aphid colonies with no ant attendance may stem from the effect of Tanglefoot (sticky insect repellent). However, the effect of Tanglefoot is likely negligible because the ratio of survived colonies is not significantly different between the control groups (*n* = 3) and the ant-attended groups (*n* = 8) (Fisher's exact test, *P* = 1).

The number of attending ants per aphid (APA) is highest when the green morph comprises 65% of the colony (Fig. 2B). The summary of the multiple regression analysis is presented in Table 1. The number of aphids was negatively correlated with APA (*P* = 0.0066). There was a marginally significant positive relationship between ant colony size and APA (*P* = 0.0617). Thus, we use a two-dimensional plane to control for the effects of both factors (ant colony size and aphid colony size) when examining the effect of the proportion of the green morph in an aphid colony (PG) on APA. In addition, the relatively low determinant coefficient (R^2 = 0.1173) shows that these two factors explain only 11.7% of the variance in APA. The residuals of APA from the plane were calculated, and we tested for equality in variance of the residuals using the Breusch-Pagan test. The results showed that the null hypothesis that the variance of the residuals is distributed equally was not rejected (*P* = 0.3166). Thus, the simple regression analysis of the residuals of APA on PG can be performed without statistical complications. Table 2 shows the results of the regression analyses of APA on PG. The linear regression was not significant (*P* = 0.8538), and the parameters of the two-dimensional regression ($Y = aX^2 + bX$) were not significant either (*P* = 0.1733 for *a* and *P* = 0.0592 for *b*). Thus, these two regressions did not adequately explain the distributions of the residuals of APA.

The smoothing spline regression was statistically significant (*P* = 0.0015), and AIC was −61.68 (see Table 2). In the three-dimensional regression ($Y = aX^3 + bX^2 + cX$), each coefficient was highly significant (*P* < 0.0001 for *a*, *b*, and *c*), and AIC was −64.835 (Table 2). The above results indicate that the three-dimensional regression provided the best model for explaining the distribution of the residuals of APA. The results of the fit are shown in Fig. 2B. The maximum value of the residual of APA is ca. 0.65 in PG. Thus, an intermediate ratio of both color morphs appears to attract ants maximally.
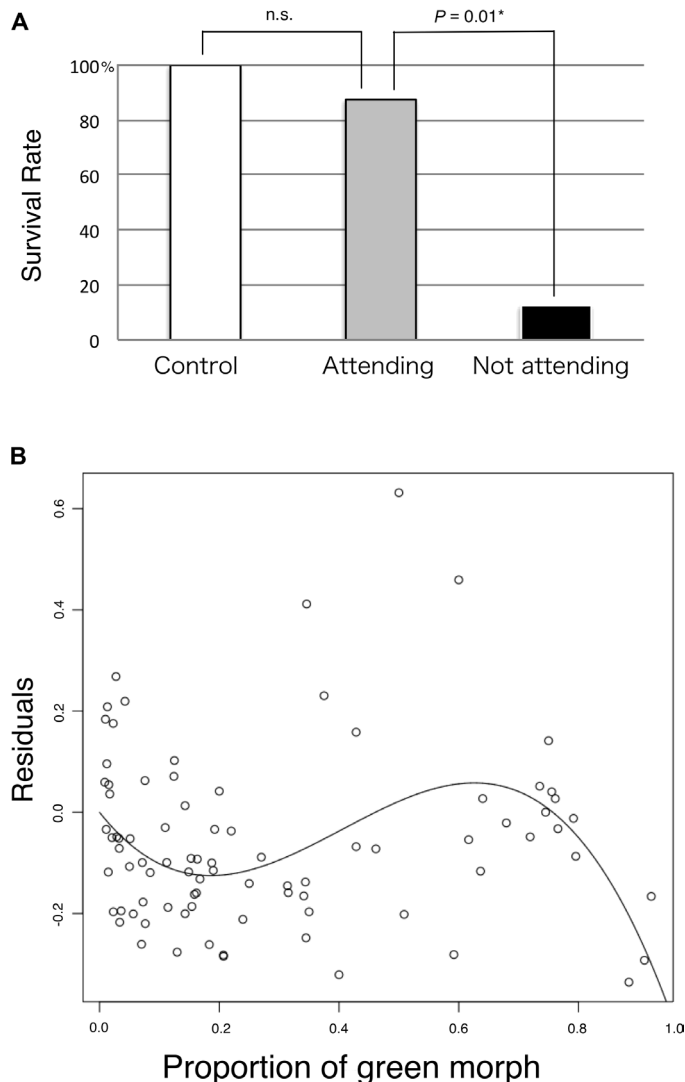

Fig. 1. Color polymorphism in *M. yomogicola* and attending ant, *L. japonicus*. Large black aphids and small green ones are green morphs, whereas large and small orange and brown ones are red morphs.

Watanabe *et al. Sci. Adv.* 2016;2:e1600606   7 September 2016

2 of 5

Fig. 2. Results of experiment 1 (A) and experiment 2 (B). (A) Effect of ant attendance on the survival of aphid colonies. The ratio of survived aphid colonies. Aphid colonies were attended by ants (Attending), not attended by ants (Not attending), or attended by ants but experienced the effects of Tanglefoot (Control). n.s., not significant. **(B)** Effect of the proportion of the green morph in an aphid colony on the degree of ant attendance. The plots represent the residuals from the two-dimensional regression plane, with ant colony size and aphid colony size as independent variables. The superimposed function (solid line) is a three-dimensional function that was selected as the best fit model from the four candidate functions based on Akaike's information criteria (AIC) comparisons.

DISCUSSION

Our analyses showed that an intermediate proportion (~65%) of the green morph in an aphid colony is favored by the attending ant species, *Lasius japonicus*. *M. yomogicola* is an aphid that is attended obligately by ants. In the field, initial colonies (a few larval aphids with the stem mother) are always attended by ants. Our ant removal experiment showed that ant attendance is essential for aphid survival (Fig.

2A). Agawa and Kawata (*14*) have shown that more polymorphic colonies of *M. yomogicola* tend to decrease more slowly in populations. This trend may have occurred because of the difference in the degree of ant attendance. Our second result demonstrated that the number of ants per aphid has a peak at ~65% of the green morph in an aphid colony (Fig. 2B). This result suggests that these polymorphic colonies are protected more efficiently from predators by the attending ants than less polymorphic colonies. Thus, ant attendance may maintain the observed color polymorphism in *M. yomogicola*.

The present results suggest that attending ants select the optimal intermediate. This colony-level selection is a novel mechanism for balancing selection. The known traditional balancing selection includes frequency-dependent selection, balancing selection between opposing factors under heterogeneity, and overdominance. None of them fit in the current case.

There is another interesting point to consider. Although our results suggest that selection favors an intermediate ratio of the two morphs at the level of the entire colony, the two morphs are likely to compete with each other within a colony. The body color of aphids reflects their condition (*15*). Therefore, there may be a difference in ecological characters (such as increasing rate and quality of honeydew) between the red and green morphs in *M. yomogicola*. If this difference exists, an increasing rate of return per unit time may be higher in one morph than in the other because the former invests less energy into honeydew. If both morphs compete within a colony, one morph would soon become more common and most colonies would end up monomorphic. However, because the entire colony requires ant attendance for survival, a morph with a high reproductive rate producing presumably low-quality honeydew (red morph here) may obtain benefits from the existence of the other more attractive morph (green morph) and hence increased ant attendance.

If this is the case, individuals of the stronger morph under intermorphic competition may behave cooperatively with the other morph to secure their own survival. This would be a very interesting hypothesis for future studies.

Why the ant, *L. japonicus*, prefers an intermediate ratio of both morphs would be an interesting question to examine in detail. In previous studies, *Lasius niger* ants have tended to prey on aphid individuals that produce less honeydew within an aphid colony (*16*). Ants prefer to attend and guard aphid species that produce condensed honeydew and maintain a ratio of such aphids (*17–19*). *L. niger*, which is closely related to *L. japonicus* (previously, both have been classified as the same species), especially prefers trisaccharides in honeydew (*20*). On the other hand, ants tend to prey on aphid individuals that produce less honeydew (*16, 21*). Ants also attack and prey on aphid individuals from which ants have never collected honeydew within an aphid colony (*22*). How these factors interact in our system is important for establishing the reasons for the symbiotic relationships between the ants and the two color morphs of *M. yomogicola*. We would require detailed chemical analyses of honeydew contents in both morphs. In addition, how the ants control the morph ratio in an aphid colony to achieve the preferred ratio is an interesting question. More studies are needed to elucidate the proximate causes that achieve the observed symbiotic relationships among ants and aphids.

Previous studies have shown that ants recognize prey or nestmates by cuticular hydrocarbons (*23*). *Stomaphis* sp. produces cuticular hydrocarbons and thereby obviates predation by their host ants (*22*). If there are differences in the cuticular hydrocarbons produced

by the red and green morphs in *M. yomogicola*, the ants can discriminate between the color morphs. In addition, aphids invest in honeydew to induce ant attendance and thereby receive protection from predation (*17, 24*). This fact leads to the following prediction about proximate mechanisms: a morph's investment in honeydew would be negatively correlated with the proportion of that morph in the subpopulation (*25*). For example, a morph (probably the red) increasing in relative proportion may produce honeydew with a lower quality and vice versa. If there are certain advantages for the ants to rear the morph producing the low-quality honeydew, the present symbiotic relationships among the two morphs and the ants would be long-lasting. One such possibility is that the red morph may bear a higher ability to suppress the budding of mugworts' inflorescences than the green morph. When a mugwort buds inflorescences in autumn, the aphid colony on that mugwort becomes extinct without sexual reproduction via overwintering eggs. A few aphid colonies surviving on the mugworts that do not bud inflorescences reproduce sexually to produce overwintering eggs. Thus, if the red morph bears a high ability to suppress the inflorescence budding of mugworts, the ants gain indirectly when rearing the red morph to exploit its honeydew resources in the following year. Thus, the ants should control the ratio of color morphs to achieve optimality as determined by the cost-benefit ratio for the ants. This hypothesis could be further investigated in *M. yomogicola*.

Body colors in *A. pisum* are determined by carotenoid pigments (*26, 27*). The ability to produce body colors may be determined genetically or environmentally (*27*). In addition, the removal of internal symbionts in aphids has changed body color in *A. pisum* (*15*). In *Myzus persicae*, body colors are fixed for syngenesis generation (parthenogenetic reproduction) but are determined by one pair of allelomorphs for sexual generation (sexual reproduction) (*28*). Unfortunately, a

mechanism for the inheritance of body color in *M. yomogicola* is not known, although its body colors are parthenogenetically inherited. Investigations of the inheritance manner of body colors are important to elucidate the origin of color polymorphism in this aphid.

In summary, we detected an effect of the proportion of aphid color morphs on the degree of ant attendance—even when the effects of both aphid colony size and ant colony size were statistically removed. We found that there is an optimal proportion of the two morphs in a colony that maximizes the attractiveness to ants. Our results suggest that color polymorphism is maintained by the optimality of the intermediate combinations of the two morphs. This mechanism may be quite different from previously known mechanisms via balancing selection.

MATERIALS AND METHODS

Insects
M. yomogicola is an aphid distributed in Japan and inhabits a mugwort, *Artemisia montana*, in the Hokkaido area. This species shows a color polymorphism with several color morphs (*14*). Although there are several color morphs in a population in Shizuoka, Japan, only two morphs (red and green) are found at our field site in Hokkaido, Japan. Several ant species were observed to attend the aphid colonies in the study area: *L. japonicus*, *Formica japonica*, *Formica sanguinea*, *Pheidole fervida*, *Myrmica kotokui*, *Camponotus japonicus*, and *Lasius nipponensis*. Because most aphid colonies were attended by *L. japonicus*, we studied aphid colonies that were attended by this species only.

Experiment 1
To examine the effect of ant attendance on the survival of aphid colonies, we selected three groups of eight, eight, and three mugwort shoots on which aphids colonized on 21 August 2015 at a field site at Hokkaido University, Sapporo, Hokkaido, Japan. On 21 August, the numbers of colonized aphids were counted for each colony. Ants were removed from the first eight colonies by painting Tanglefoot (sticky material) at the base of the mugwort shoots. Painting Tanglefoot prevents ants from moving up to the mugwort because of its stickiness, but most predators can reach the aphid colonies because they can fly. No Tanglefoot was applied to the eight colonies in the second group so that ants continued to attend the aphid colonies. To control for the effect of Tanglefoot application on aphid survival, Tanglefoot was painted at the surface of a leaf in the three colonies in the control group so that ants could continue to attend the aphid colonies. The numbers of colonized aphids were counted 9 days later. The number of predators found on each mugwort shoot was recorded three times during the experiment.

Table 1. The results of the multiple regressions of the two factors (aphid number is the number of aphids in a colony and ant head width is an index of ant colony size) on attending ants per aphid. Aphid number is negatively correlated with attending ants per aphid ($P = 0.00659$). Statistical significance is marginal for ant head width ($P = 0.06171$). $F = 5.449$; df = (2,82); $P = 0.006$; $R^2 = 0.1173$.

Factor	Estimate	SE	t	P
Intercept	−0.0209	0.119257	−0.175	0.8613
Aphid number	−0.00031	0.000112	−2.788	0.00659
Ant head width (∝ant colony size)	0.21410	0.113024	1.894	0.06171

Table 2. Regression coefficients and AIC values for the four regressed functions. Significance levels of the coefficients are indicated by asterisks (***$P < 0.001$).

Function type	Equation	a	b	c	P	AIC
Linear	aX	−0.0255	—	—	0.854	−50.931
Second dimension	$aX^2 + bX$	0.3304	−0.3312	—		−49.963
Third dimension	$aX^3 + bX^2 + cX$	−4.2858[***]	5.2189[***]	−1.4946[***]		−64.835
Smoothing spline	—	—	—	—	0.0015	−61.681

Experiment 2

Field collections and measurements. We collected a total of 85 *M. yomogicola* colonies from three sites and one site in Sapporo City in July 2012 and 2013, respectively. A colony on a mugwort shoot was collected and brought back to the laboratory in a vinyl bag with attending ants. To avoid multiple samplings from the same ant colony, we took a single shoot from the lumped shoots. The insects were frozen at −25°C in a freezer for measurements. For each shoot, the numbers of green morph, red morph, and attending ants were counted. The proportion of the green morph on the shoot was calculated using these data. The monomorphic colonies were removed from the analysis. For the rest of the colonies, APA was calculated.

Because the body size of ant workers became larger as colony size increased (*29*), we used the average head width of the attending ants as an index of ant colony size. The head of an ant was separated and fixed on a glass plate and was measured to 0.01 mm by a micrometer on a microscope (Macromax SCORP-ON Direct USB, SCORPION). The head widths of all the attending ants on a shoot were averaged. This value was used as the index of ant colony size on the shoot.

Statistical analyses

Three factors affect APA: the number of aphids on a shoot (aphid colony size), ant colony size, and the PG. Generally, the number of aphids is positively correlated with the number of attending ants. A large ant colony can mobilize more workers to an aphid colony than a small ant colony. We wanted to examine the effect of PG on APA without the effect of these other two variables. To remove the effects of both of these parameters, we first conducted a multivariate regression of the APA on both ant colony size and aphid colony size as independent variables. Second, the residuals of APA from the regression plane were calculated. Next, a simple regression of the residuals of APA on PG was conducted to examine the relationship between PG and APA.

We used four types of regression functions in the simple regression of APA on PG: linear function, two-dimensional function, three-dimensional function, and smoothing spline function. Each function was fitted on data, and AIC was calculated. The fitted function showing the lowest AIC was considered the regression that explained the data most effectively.

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