Genetic Variation and Cluster Formation of the Ant *Azteca* in a Coffee
Agroecosystem

By

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ABSTRACT

The spatial distribution of organisms is an important concept in ecology and it has been recently recognized that large-scale spatial patterns may depend only on local-level interactions. The ant *Azteca instabilis* has been shown to have a spatially aggregated distribution despite a homogeneous environment in a coffee agroecosystems with evenly planted shade trees,. It has been argued that this self-organized pattern is the result of endogenous local forces that include a process of local expansion and density dependent control. In this study we sample ants from a 45-hectar plot within a coffee plantation and used behavioral assays of aggression, mitochondrial sequence data, and five microsatellites to elucidate processes contributing to this self-organized pattern.

High levels of aggression between groups and strong genetic divergence provide strong evidence for the presence of two evolutionary distinct units in this system. Low levels of aggression among nests within a cluster, lack of genetic differentiation, and significant isolation by distance all support a local nest expansion process through budding. Significant genetic differentiation between most clusters and lack of isolation by distance at the cluster level indicates that new cluster establishment could be happening via flying queens from other areas. Lack of differentiation between some clusters, however, suggest that large scale budding or short distance dispersal could also be responsible for the establishment of new clusters. This study confirms the existence of two evolutionary units co-inhabiting the 45-hectare plot. Furthermore, it confirms the formation of clusters through a colony budding process. However, the establishment of new colonies from which the cluster eventually emerge could be either through new

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flying queens from a regional pool, queens flying short distances or from long distance

bubbing.

INTRODUCTION

The spatial distribution of organisms is an important characteristic of a species and represents the population expression of individual behaviors (Taylor 1984). Early studies that examined the spatial distribution of mature colonies of ants found that colonies of the same or closely related species were overdispersed, in other words, that the distances among them were too nearly uniform to have been established at random (Elton 1932, Talbot 1943, 1954). Indeed, most studies reviewed by Hölldobler and Wilson (1990) on the spatial distribution of ant colonies reported overdispersion. They attribute this distribution to the important role of competition in determining the establishment and permanence of ant colonies. Additionally, factors such as predation and regularly spaced microhabitats can also result in overdispersion (Ryte and Case 1986, Deslippe and Savolainen 1995). More recent studies, however, have reported cases of aggregation among ant colonies (Rissing *et al.* 1986, Henderson and Jeanne 1992, Soares and Schroereder 2001, Vandermeer *et al.* 2008). Aggregated spatial distributions have been attributed to both exogenous and endogenous factors. Exogenous factors may include such outside forces as underlying environmental heterogeneity, which is then reflected in spatial patterns (Van de Koppet 2008). An aggregated pattern even in the absence of underlying habitat variation may also arise from endogenous factors such as intrinsic dynamic ecological forces operating at a local level (Vandermeer *et al.* 2008, Yitbarek *et al.* 2011). Such a pattern is considered a self-organized pattern (Pascal and Guichard 2005).

It has been recently recognized that large-scale spatial patterns may depend only on local-level interactions (Pascual *et al.* 2002, Couzin and Frank 2003). Self-organized

spatial patterns have been well described for sedentary organisms such as terrestrial plant communities (Klausmeier 1999, Reitkerk *et al.* 2008, Van de Koppel *et al.* 2008) and marine ecosystems (Wooten 2001, Van de Koppel *et al.* 2008). However, very few studies have documented spatial self-organization in animals (Maron and Harrison 1997, Vandermeer *et al.* 2008, Yitbarek *et al.* 2011). Ants, due to the sedentary nature of their colonies, represent ideal organisms to study spatial distributions and the possibility of a self-organized pattern. Indeed, of the three studies on terrestrial animal populations documenting spatial self-organization, two come from ant systems (Vandermeer *et al.* 2008, Yitbarek *et al.* 2011).

Multiple factors such as habitat availability, predation, competition and the manner in which new nests are formed, may govern the distribution of ant nests in a landscape. Intraspecific aggregation of nest distribution in ants may result from (i) specialization for a microhabitat, which itself is patchily distributed (an exogenous force), or (ii) colony fragmentation (or budding) through which several conspecific nests are formed close to the original nest (Herbers 1994). However, if colony fragmentation is occurring and there is no controlling mechanism to suppress the expansion of the ants, eventually, the colonies could fill all possible nesting sites. For an aggregated pattern to occur and be maintained over time, there must be a force preventing the continuous expansion of the species. Theoretical (Alonso and McKane 2002) and empirical (Vandermeer *et al.* 2008) studies have demonstrated that the combination of local expansion through colony fragmentation and density dependent suppression can generate a non-random spatial pattern similar to the Turing effect (Turing 1952) involving short-range activation and large-range inhibition (Murray 1989).

Vandermeer and colleagues (2008) argue that the spatially patchy distribution of nests of the arboreal ant *Azteca instabilis* in a southern Mexican coffee agroecosystem is the manifestation of a self-organized process. Farmers planted shade trees in the agroecosystem in such a manner as to be relatively uniformly distributed across the landscape (Fig. 1). Despite an apparently uniform environment from the perspective of potential nesting sites for the ant *A. instabilis* there is a distinct patchiness in the distribution of nests meaning ant nests form clusters of various sizes. Furthermore the frequency distribution of cluster size follow a power law distribution (Vandermeer *et al.* 2008). Importantly, Vandermeer and colleagues (2008) argue that this spatial pattern is maintained by internal self-re-enforcing dynamics where local expansion is counteracted by density dependent mortality. Several mechanisms leading to this pattern have been well studied. There is convincing evidence that density dependent control of *A. instabilis* nests is a result of dynamic population processes including attack by *Pseudacteon* phorid flies, coccinellid beetle predation on the scale insect, *Coccus viridis,* with which the ant has a mutualistic relationship, and attack on the scale insects by the fungal pathogen, *Lecanicillium lecanii* (Vandermeer *et al.* 2008, Liere and Perfecto 2010, Jackson *et al.* 2009, Hsieh and Perfecto 2012, Hsieh *et al.* 2012). Local expansion is presumed to occur through a budding process whereby new colonies are formed in close proximity to the original nest. As the local density of *A. instabilis* nests increases this sparks a density dependent attack response by the previously mentioned natural enemies, contributing to a distinctly patchy distribution of *A. instabilis* nests (Vandermeer *et al.* 2008). On the other hand, new clusters of nests are thought to arise from the establishment of colonies after

the flight of newly mated queens. Coupled with expansion via budding, this process should lead to clusters of closely related nests.

In addition to the evidence of density dependence mortality caused by natural enemies of the ants or its mutualistic scale insect, the evidence presented by Vandermeer and colleagues (2008) of the self-organizing pattern in *A. instabilis* consisted of a cellular automata model that included simple rules of local expansion and density dependent suppression that generated a spatial pattern very similar to the observed pattern in nature. However, they presented no direct evidence that local aggregations of nests were generated through a budding process or that new clusters of colonies were formed by new queens coming from a regional pool. The goal of this study was to investigate the patterns of cluster formation of *A. instabilis* in the same shade coffee agroecosystem. We used behavioral and genetic data to determine i) whether local aggregation of nests is the result of colony budding, and ii) whether the establishment of new clusters is likely the result of founding by flying queens coming from a regional pool. If new nests are forming via budding to nearby trees we expect that nests within close proximity will have little genetic differentiation from one another and show low levels of behavioral aggression toward each other. If separate clusters are the result of extended budding we expect to see little genetic differentiation among clusters, and a pattern of genetic variation that is correlated with geographic distance. However, if separate clusters formed with the establishment of new queens flying into the system from other areas (i.e., from a regional pool) we will expect to see greater genetic differentiation between clusters than within cluster, and no spatial correlation.
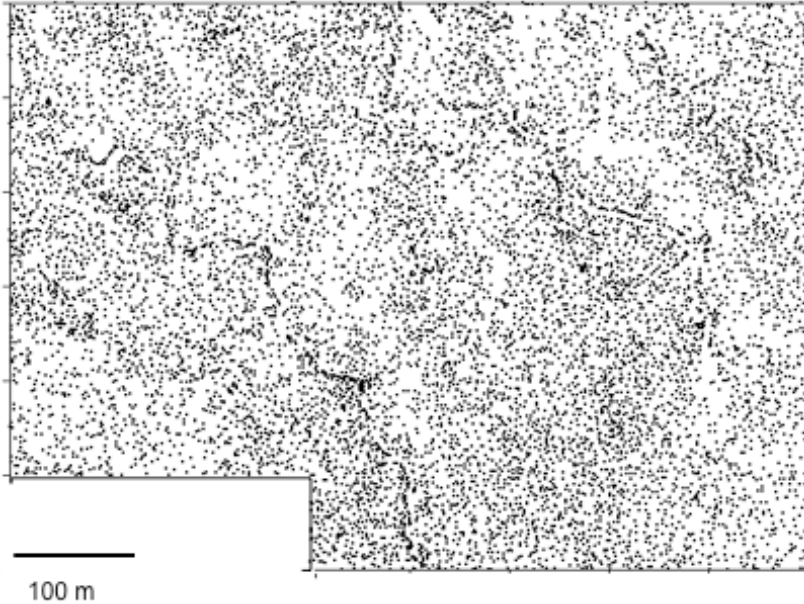
100 m

**Figure 1.  Map of shade trees in a 45-hectare plot in Finca Irlanda, a coffee plantation in southern Mexico.**

*Results from a previous study*

A previous attempt to elucidate the relationships among *Azteca* nests in a coffee plantation in Mexico found stark patterns in aggressive behaviors.  Behavioral assays of aggression revealed strong aggression among some *Azteca* nests and was nearly absent among other nests (Fig. 2, Taylor unpublished data).  Nests within the study segregated into two main groups characterized by low aggression within a 'group' and high aggression between 'groups.'  Each 'group' consisted of several clusters of nests that were distributed throughout the study plot.  Additionally, slight morphological differences were observed in workers representing the two groups, including differences in coloration of the head, thorax, and gaster.  Based on these differences we also wanted to examine the phylogenetic relationship between these two groups and the possibility that they may be distinct evolutionary units.
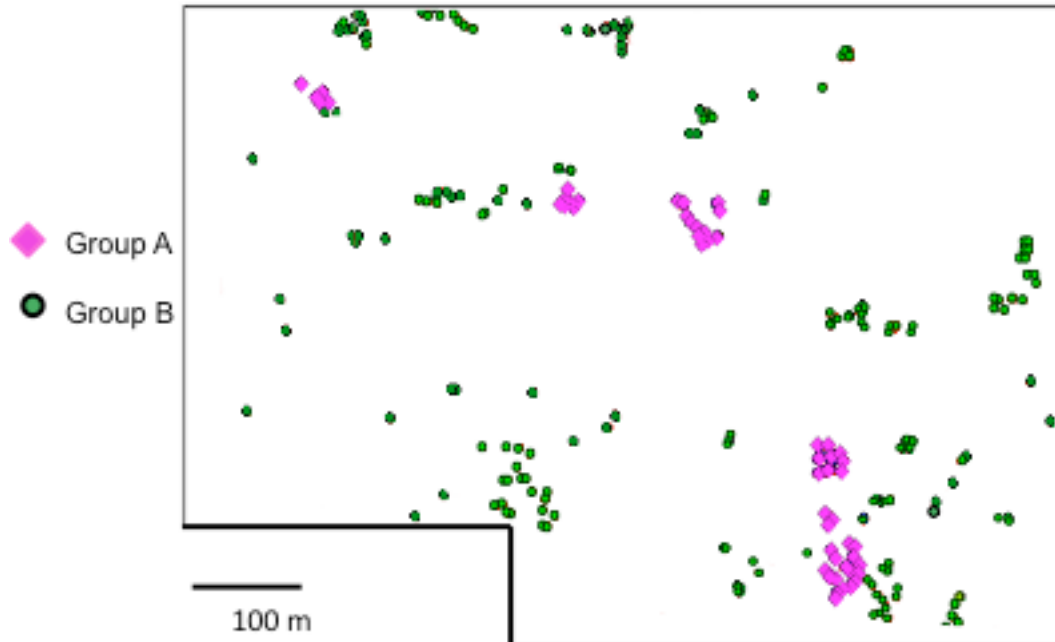
**Figure 1. Map of a 45-hectare plot in Finca Irlanda, coffee plantation in southern Mexico, delineating group A and group B based on previous assays of aggression. High levels of aggression occurred between groups A and B and low levels of aggression occurred within groups.**

METHODS

*Study site*

Our study site is a 45-hectare plot in a 300-ha shade coffee plantation in the Soconusco region of Chiapas, Mexico ($15^o$ 11' N, $92^o$ 20' W). Management of the plantation is characterized by a coffee understory grown under a diverse shade canopy comprised of approximately 90 tree species. The plantation is located 900-1150 m.a.s.l. and receives approximately 4500 mm of rain each year. Previously, all shade trees above 4.7 cm dbh were measured, tagged and mapped. A census of *A. instabilis* colonies is performed yearly by visiting each tree in the plot and recording the presence of the ant.

Two different morphotypes of *Azteca* ants occur in the study site. These have been named as "group A" and "group B" (Fig 2).

*Aggression tests*

Nine nest clusters were chosen for aggression testing. Four clusters were from colonies previously identified as group A and five clusters represented group B. Clusters were identified from a census map of all living *Azteca* nests within the 45 hectare plot in 2011. Nests less than 20 m apart from each other were considered to be in the same cluster. The definition of cluster in this study is based on direct observations of groups of ants moving their nests 16 meters from where the original nest was located (Vandermeer, personal obs.). Within each cluster three nests were chosen for aggression (and genetic) studies. Between 20 and 100 individuals were collected per nest and kept in the laboratory with a cotton ball soaked in sugar water as a food source. Ants were not kept in the lab for more than 3 days. Pairwise aggression tests were conducted between the three nests in each cluster. Next, one representative nest was chosen from each cluster and pairwise fights were conducted between clusters. Finally, those same representative nests were used for pairwise tests between the two groups (A an B) in the farm.

Trials were conducted between individual workers (minors) in 40 mm diameter plastic cups. The sides of the plastic cups were painted with Insect-A-Slip (BioQuip) to prevent escapes, and the bottom of each cup was wiped with acetone between trials to remove alarm pheromones from the previous trial. Aggressive behaviors were scored for 3 minutes on the following aggression scale modified

from Suarez *et al.* (1999):  1 equals no aggression, 2 equals a less than two second

attack, 3 equals a greater than two second attack, and 4 equals an attack with

injurious behaviors (detaching segments from the body of the ant).  Five replicates

were conducted for each test.

*Genetic sampling*

Two hundred and twenty individuals were collected from *Azteca* nests

belonging to group A and 300 from group B.  Twenty workers (minors) per nest

were collected and stored in individual 0.65 ml tubes in 96% ethanol.  A total of 11

nests from four clusters were sampled for group A (three nests for three clusters

and two for one cluster).  For group B, 15 nests were sampled from five clusters

(three nests per cluster).  Workers were collected near nest entrances to ensure

nest fidelity.  Workers were collected using soft grip forceps that were flame

sterilized between collection of each individual.  DNA was extracted from 10 whole

ants per nest using a gentra Purgene protocol (Gentra Systems).  A 188 bp region of

COI was sequenced with primer sequences obtained from Ayala *et al.* 1996  to verify

taxonomic identity of the two morphotypes, (group A and group B).  Five

microsatellite loci (Az016, Az022, Az025, Az035, Az171) previously isolated from

*Azteca ulei* (DeBout *et al.* 2007) were genotyped for each individual.  Microsatellites

were multiplex PCR amplified using the Qiagen Type-it kit (Qiagen) following

manufacturer's protocols.  PCR conditions included an initial activation at 95 $^{\circ}$C for

5 min, followed by 28 cycles of denaturation at 95 $^{\circ}$C for 30s, annealing 46 $^{\circ}$C for

90s, extension 72 $^{\circ}$C for 30s, final extension 60 $^{\circ}$C for 30 min.  Molecular work was

conducted in the Genomic Diversity Laboratory at the University of Michigan. Genotyping and sequencing was done at the University of Michigan Core Facilities. Sequences were edited and aligned using Sequencher v 4.7 (GeneCodes). Microsatellite genotypes were scored using Genemarker v. 2.4 (SoftGenetics).

*Aggression analysis*

For each aggression trial the score for the highest level of aggression observed was averaged across replicates. We chose to base aggression scores on the highest level of aggression observed even though the number of aggressive interactions was recorded (Jaquiery *et al.* 2005). Ambiguity arose when some pairs demonstrated many level 2 attacks while other pairs had only one level 3 attack that lasted for the entire trial. The aggressive interactions, with both level 2 and level 3 attacks differed greatly in intensity to level 4 attacks. Level 4 attacks generally resulted in maiming (loss of extremities) or death of one or both ants while level 2 and 3 attacks never resulted in the loss of limbs or death. PASW was used to perform ANOVA on mean aggression scores within and between clusters.

*Phylogenetic tree*

Prior to analysis we determined an appropriate model of sequence evolution with jModel test (Posada 2008) With an HKY+I model we used Garli (version 2.0) to run maximum likelihood analysis with 100 bootstrap replications, with *A. longiceps* as an outgroup. Majority-consensus rule was used in PAUP v. 4.0b4a (Swofford, 2000) to find the best tree.

*Population genetic structure*

To examine genetic differentiation in our sample we calculated pairwise $R_{ST}$ values using GenAlEx 6.41 (Peakall and Smouse 2006). $R_{ST}$ comparisons were made at the cluster and nest level. At the nest level we expect to see very low levels of genetic differentiation if nests within a cluster are the result of budding, as new nearby nests would be forming from ants that are related. If nests within a cluster were genetically distinct from one another this would indicate that nests were originating from queens coming from other locations. At the cluster level it remains unclear what level of differentiation may exist. If clusters are the result of large-scale budding we expect low levels of differentiation due to common origin, and a signal of gradual reduction of genetic variation with geographic distance. The presence of genetically distinct clusters may indicate the establishment of new clusters by means other than continuous budding from a common nest (i.e., possibly from queens flying into the area and establishing new nests there).

An analysis of molecular variance (AMOVA) was used to determine the existence of genetic structure in our sample (GenAlEx 6.41). AMOVA is a hierarchical test that determines what level of data partitioning explains the variance in allele frequencies in our sample. We conducted a 3 tier AMOVA that partitioned data by individual, nest, and cluster.

To put our genetic data in a geographical context we conducted a Mantel test that tested for the relationship between geographic distance and genetic distance (GenAlEx 6.41). Genetic distance was calculated between clusters and geographic

distance was obtained from x, y coordinates within our study plot. Since there were two to three nests per cluster we found the geographical center between nests within a cluster and used this point as the cluster location. The null hypothesis of the Mantel test is that nests in close geographic proximity will be more genetically similar than nests further apart. Examining this relationship will give us insight as to whether queens that are establishing new nests are coming from nearby nests or from elsewhere. Short distance dispersal of queens or continuous large-scale budding would result in a positive correlation between genetic distance and geographic distance. If queens are coming from a regional pool we expect there to be no significant relationship between geographic and genetic distance.

## RESULTS

*Aggression*

Aggression levels between groups were consistently high (ave=3.64) and significantly higher than among individuals within the same group (Fig. 3). Additionally, level 4 aggression was generally only observed in envounters between individuals of group A *vs.* individuals of group B. These aggressive encounters often extended well past the 3-minute trial and often ended with no clear winner. In fact, these encounters mostly resulted in the dismemberment and death of both workers.
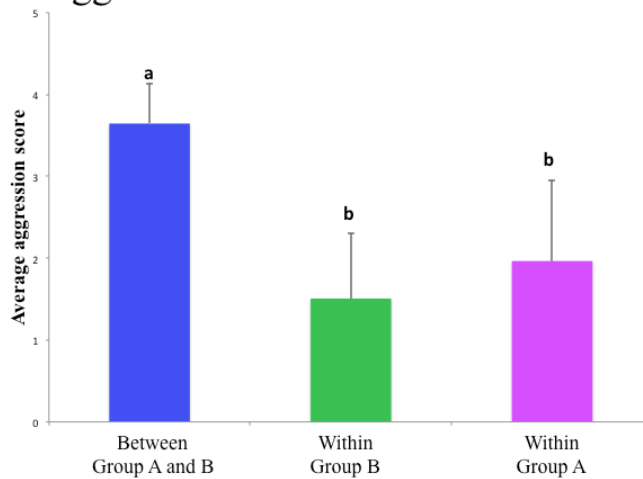
## Aggression Between and Within Groups

**Figure 3.** One way ANOVA of average aggression scores between groups A and B and within group B and within group A. Different letters above the error bars indicate significantly different values ($P=0.01$).

Aggression levels of trials between clusters were significantly higher than within clusters (Fig. 3, $P<0.001$). However, aggression levels were not uniformly higher across all inter-cluster trials. Clusters B1 and B4 showed little aggression with each other (ave=1.4). Cluster B2 also showed little aggression with cluster B3 (ave=1.2). Cluster B5 showed significantly higher levels of aggression ($P<0.001$) with cluster B1 and B4 (ave=2.4 and 2.6 respectively) than with B2 and B3 (ave=1 and 1.4 respectively).

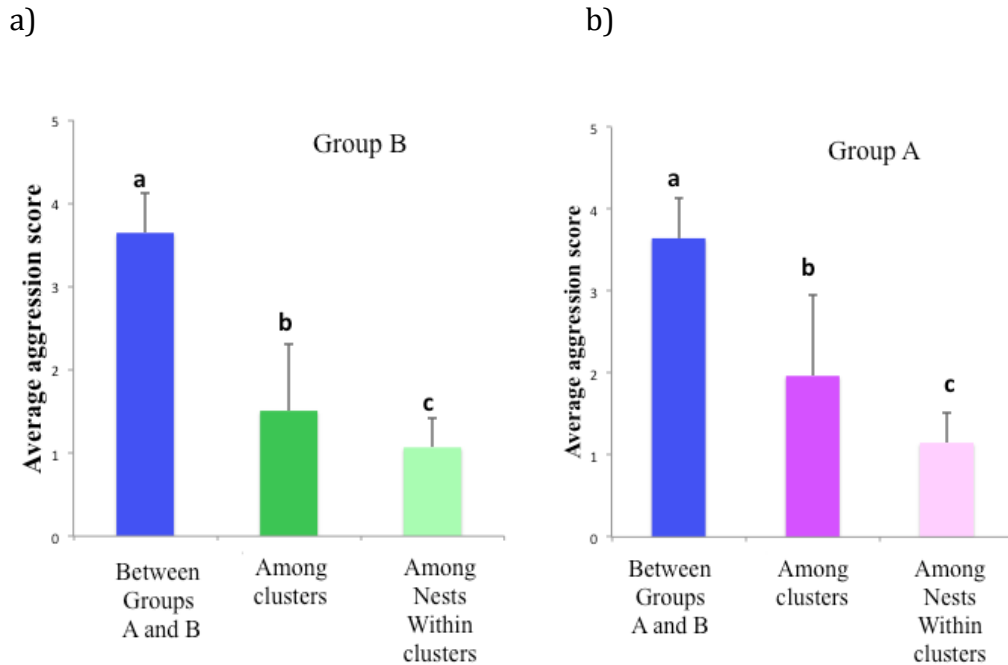a)                                          b)



**Figure 4. a) One-way ANOVA of average aggression scores between groups A and B, among clusters within group B and within group A, and among nests within clusters. Different letters above the error bars indicate significantly different values (*P*=0.01).**

Aggression was never observed between nest mates and very low levels of aggression were observed in pairwise tests between nests within a cluster (<20 m distance between nests). Level 3 aggression was only observed twice during within cluster trials and level 2 aggression was observed only once. Level 4 aggression was never observed during within cluster trials. The overwhelming majority of within cluster interactions resulted in no aggression.

*Phylogenetic relationship of groups A and B*

All workers identified as belonging to group A or group B based on phenotype (morphology and aggression) also separated into distinct clades in a phylogenetic tree (Fig. 5). Branching patterns between group A and B were

congruent with divergence seen between other known *Azteca* species in our tree, *A. longiceps, A. nigricans,* and *A. alfari.* There was strong branch support for the division of group B from other samples with a bootstrap value of 91. Support for the distinction of group A was lower with a bootstrap value of 61. Bootstrap values for branches within groups A and B were not strongly supported making branching patterns between nests within the group ambiguous.
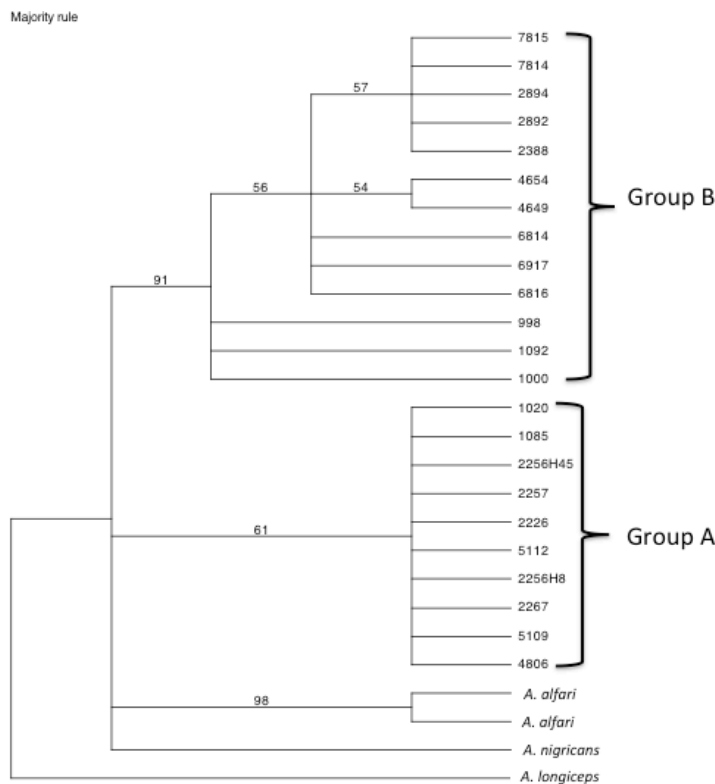


**Figure 5. Majority rule consensus tree showing phylogenetic relationships between group A and B and other *Azteca* species. Support from 100 bootstrap repeats are above branches.**

At the group level we saw significant differences in aggression between and within groups A and B and large genetic divergence between groups. For these

reasons we treated groups A and B as distinct evolutionary units and microsatellite data for each group was analyzed separately.

*Genetic differentiation*

Pairwise comparisons of $R_{ST}$ between clusters showed both significant and non-significant results (Fig. 6). In group B, cluster B1 and B3 are significantly genetically differentiated from all other clusters (*P*<0.05). Cluster B2, B4, and B5, however, show no significant genetic differentiation between them. This pattern was followed when pairwise comparisons were made at the nest level except three comparisons between cluster B1 and B3 were only marginally significant (Fig. 7b, *P*=0.06, 0.06, 0.07). Levels of genetic differentiation were not significantly different from 0 for pairwise comparisons between nests within the same cluster.
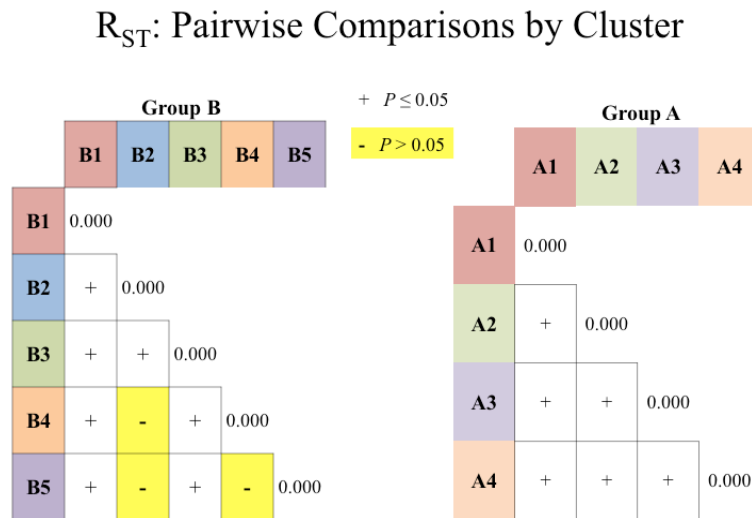


**Figure 5. Pairwise $R_{ST}$ comparisons at the cluster level. + indicates significant values (*P*<0.05); - indicates non-significant values (*P*>0.05). See text for detailed explanation.**

# $R_{ST}$: Group B Pairwise Comparisons by Nest

|       | B1 |     |     | B2 |     |     | B3 |      |     | B4 |     |     | B5 |     |     |
|-------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|       | i    | ii   | iii  | iv   | v    | vi   | vii  | viii | ix   | x    | xi   | xii  | xiii | xiv  | xv   |
| i     | 0.0000 | | | | | | | | | | | | | | |
| ii    | -    | 0.0000 | | | | | | | | | | | | | |
| iii   | -    | -    | 0.0000 | | | | | | | | | | | | |
| iv    | +    | +    | +    | 0.0000 | | | | | | | | | | | |
| v     | +    | +    | +    | -    | 0.0000 | | | | | | | | | | |
| vi    | +    | +    | +    | -    | -    | 0.0000 | | | | | | | | | |
| vii   | +    | -    | +    | +    | +    | +    | 0.0000 | | | | | | | | |
| viii  | +    | -    | +    | +    | +    | +    | -    | 0.0000 | | | | | | | |
| ix    | +    | +    | -    | +    | +    | +    | -    | -    | 0.0000 | | | | | | |
| x     | +    | +    | +    | -    | -    | -    | +    | +    | +    | 0.0000 | | | | | |
| xi    | +    | +    | +    | -    | -    | -    | +    | +    | +    | -    | 0.0000 | | | | |
| xii   | +    | +    | +    | -    | -    | -    | +    | +    | +    | -    | -    | 0.0000 | | | |
| xiii  | +    | +    | +    | -    | -    | -    | +    | +    | +    | -    | -    | -    | 0.0000 | | |
| xiv   | +    | +    | +    | -    | -    | -    | +    | +    | +    | -    | -    | -    | -    | 0.0000 | |
| xv    | +    | +    | +    | -    | -    | -    | +    | +    | +    | -    | -    | -    | -    | -    | 0.0000 |

$+\ \ P \leq 0.05$
$-\ \ P > 0.05$

# $R_{ST}$: Group A Pairwise Comparisons by Nest

|       | A1 |     | A2 |     |     | A3 |     |      | A4 |     |     |
|-------|------|------|------|------|------|------|------|------|------|------|------|
|       | i    | ii   | iii  | iv   | v    | vi   | vii  | viii | xiv  | x    | xi   |
| i     | 0.000 | | | | | | | | | | |
| ii    | -    | 0.000 | | | | | | | | | |
| iii   | +    | +    | 0.000 | | | | | | | | |
| iv    | +    | +    | -    | 0.000 | | | | | | | |
| v     | +    | +    | -    | -    | 0.000 | | | | | | |
| vi    | -    | +    | +    | +    | +    | 0.000 | | | | | |
| vii   | -    | -    | +    | +    | +    | -    | 0.000 | | | | |
| viii  | -    | +    | +    | +    | +    | +    | -    | 0.000 | | | |
| xiv   | +    | +    | -    | +    | +    | +    | +    | +    | 0.000 | | |
| x     | +    | +    | -    | +    | +    | +    | +    | +    | -    | 0.000 | |
| xi    | +    | +    | -    | -    | -    | +    | +    | +    | -    | -    | 0.000 |

$+\ \ P \leq 0.05$
$-\ \ P > 0.05$

**Figure 7. Within and between cluster pairwise $R_{ST}$ comparisons at the nest level for group B (a) and within and between cluster pairwise $R_{ST}$ comparisons at the nest level for group A (b). + indicates significant values ($P<0.05$); - indicates non-significant values ($P>0.05$)**

$R_{ST}$ results for group A show similar overall patterns as group B. At the cluster level we see all pairwise comparisons showing significant differentiation

(Fig. 6, *P*< 0.05).  At the nest level, however, there are several inter-cluster comparisons that show no significant levels of differentiation (Fig. 7b).  Again, all but one pairwise comparison between nests within the same cluster showed no significant differentiation (*P*>0.05).

A three-tier AMOVA based on $R_{ST}$ estimates is shown in table 1.  AMOVA only recovered significant population structure at the cluster level with the 26 % of the detected variation being apportioned among clusters ($R_{CLUSTER-TOTAL}$=0.415, *P*=0.01) and 0% among nests within clusters ($R_{NEST-CLUSTER}$=0.001, *P*=0.460) for group B.  For group A we see a similar pattern with cluster partitioning explaining 32 % of the variance in allele frequencies and nest level partitioning explaining 0 % of the variance.  This indicates that the unit of structure in our samples is at the cluster level for both group A and group B while within clusters there is no population genetic structure.

## Analysis of Molecular Variance:  AMOVA

| Source | Group B | | | Group A | | |
|---|---|---|---|---|---|---|
| | df | Est. Var. | % | df | Est. Var. | % |
| Among Clusters | 4 | 15533.58 | 26 | 3 | 1532.07 | 32 |
| Among Nests Within Clusters | 10 | 0.00 | 0 | 7 | 0.00 | 0 |

**Table 1.  Hierarchical Analysis of Molecular Variance (AMOVA) for groups A and B. Analyses performed among and within cluster levels.**

*Geographic distance versus genetic differentiation*

Examining the relationship between genetic distance and geographic distance at the nest level resulted in a significant positive correlation between genetic distance and geographic distance for both group B (correlation *r*=0.528, *P*=0.01) and group A (correlation *r*=0.135, P=0.02) (Fig. 8). At the cluster level no significant relationship was observed ( group B, correlation *r*=0.018, *P*=0.390; group A, correlation *r*=0.22, *P*=0.130) (Fig. 9).
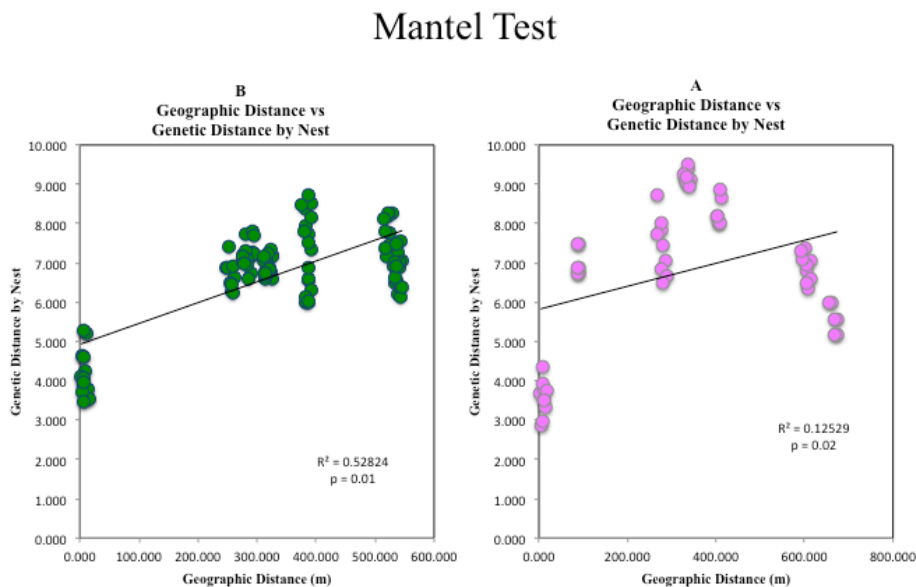


**Figure 8. Mantel test of genetic distance by nest versus geographic distance in groups A and B. A significant positive correlation is seen in both groups indicating isolation by distance.**

# Mantel Test

B

**Geographic Distance vs Genetic Distance by Cluster**



$R^2 = 0.01803$
$p = 0.390$

A

**Geographic Distance vs Genetic Distance by Cluster**
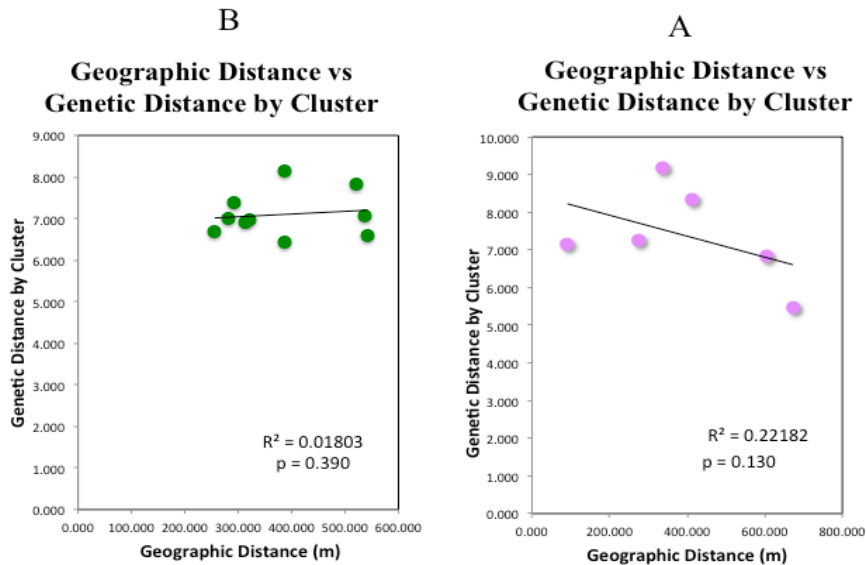


$R^2 = 0.22182$
$p = 0.130$

**Figure 9. Mantel test of cluster level genetic distance versus geographic distance for both groups A and B. There is no significant correlation in either group.**

## DISCUSSION

*Phylogenetic relationships between group A and group B*

Our data supports the presence of two distinct evolutionary units in our study. Levels of aggression between group A and group B were consistently high and reached intensities not seen in encounters between workers of the same group. This intensity of aggression was also seen when workers from group A or group B encountered other species of *Azteca* collected from near our study site including *A. alfari* and *A. nigricans*. Similar patterns in aggression have been found in other studies where intra-specific aggression was minimal but interspecific aggression was high during encounters with closely related species (Fournier *et al.* 2008,

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Holzer *et al.* 2006).  Additionally in our phylogenetic tree we see a clear division between workers from groups A and B with strong branch support.  The length of our sequenced fragment and lack of variation precluded finer scale definition between workers within group A or B, however, despite this lack of strong phylogenetic signal we still found divergence between groups A and B.  Aggression and genetic data were taken from individuals living within meters of each other and thus results do not reflect genetic drift or loss of recognition due to large geographic separations, and indicates that selective forces may be responsible for these differences.

*Cluster formation through budding*

Our data supports cluster formation through a process of new nests budding from an original nest.  Lack of significant allelic differentiation based on $R_{ST}$ values was seen consistently between nests within a cluster.  In contrast, significant differentiation was seen between several clusters in both groups.  Additionally, results from an AMOVA based on $R_{ST}$ values shows that 0% of the variance in allelic differentiation is attributed to within cluster differences, which indicates a lack of population structure at the nest level.  Finally, results of the Mantel test comparing genetic distance versus geographic distance showed a positive and significant correlation among nest comparisons in both groups (A and B).  While the correlation for group A is low there is a positive trend and except for two points representing genetically similar nests.  The correlation is clearly driven by nests that are less than 20 m apart, which is our definition of a cluster. Once the data are analyzed as clusters this correlation is absent (see below). Nests considered within

the same cluster are more genetically similar to each other than to nests that are over 100 m apart, or in this case in different clusters, which matches the predictions based on a budding hypothesis. Beye *et al.* (1998) found a similar pattern where increased genetic distance correlated with geographic distance in *Formica pratensis,* which they also attributed to a budding dispersal strategy.

Aggression between nests within a cluster was significantly lower than between clusters and only escalated to biting three times out of 50 trials. These results indicate a high level of tolerance between individuals among nests within the same cluster, which is consistent with behavioral predictions from a budding hypothesis. This lack of aggression could indicate a lack of non-nestmate recognition, however, our aggression assays were not sensitive enough to address this possibility. Holzer *et al.* (2006) measured behaviors such as prolonged antennation, which they interpreted as non-nestmate recognition. Aggression levels seen in intra-cluster trials in our study are similar to those seen between nests in the same supercolony or in unicolonial species (Fournier *et al.* 2009, Holzer *et al.*2006, Suarez *et al.* 1999).

Budding may be a strategy adopted by species when the probability of colony founding by one or a small group of queens is low. Budding is thought to increase the probability of successful colony founding since queens are accompanied by workers and brood when establishing a new nest (Buczkowski and Bennett 2009). In particular, budding is thought to occur when nests reach a sizeable population such that the parental nest and budded nest are both likely to survive (Fernandez-Escudero *et al.* 2001). Foitzik *et al.* (2010) found that long-distance dispersal and

independent colony foundation is rarely successful.  The presence of phorid fly

parasites and other predators may make establishment of new nests by a small

group of queens unlikely to be successful although we have not directly measured

nest establishment success rates.

*Inter-cluster genetic differentiation*

Genetic data examining inter-cluster relationships show two possible modes

of dispersal.  Significant genetic differentiation between clusters was seen for all

clusters sampled in group A.  Two clusters in group B were found to be significantly

genetically differentiated from all other group B clusters, while 3 clusters showed no

genetic differentiation from each other.  The presence of strong differentiation

between clusters is not surprising given the findings of structure at the cluster level.

A hierarchical AMOVA revealed structure at the cluster level for both groups.  This

indicates that rather than a panmictic population there is definite population

structure among clusters within the same group.  Finally, a Mantel test comparing

population genetic distances between clusters shows no correlation with

geographic distance.  While at the nest level intra-cluster nest comparisons were

driving a positive correlation between genetic distance and geographic distance, this

relationship disappears at the cluster level.  The lack of evidence for isolation by

distance and the significant genetic differentiation among clusters indicates that

cluster initiation may be happening as the result of queens flying from other areas

and establishing new nests and clusters.

There is also evidence for another possible dispersal method involved in

cluster establishment in our study.  The lack of genetic differentiation between three

clusters in group B that are spatially aligned indicates that some form of long

distance budding or short distance stepping stone mode of dispersal by neighboring

flying queens may be occurring.  Nonetheless, due to the low number of

microsatellite markers used in this study, it is also possible that lack of

differentiation is simply an artifact; however, we did see strong levels of

differentiation between other clusters.  Further sampling and genotyping of a larger

set of markers would be needed to more deeply address the origin of and levels of

differentiation among clusters throughout this Mexican agroecosystem.

*Inter-cluster aggression*
Inter-cluster aggression was significantly less than aggression between

group A and group B and significantly greater than intra-cluster aggression.  Inter-

cluster aggression was generally peaceful but did escalate to biting and occasionally

prolonged biting.  *Azteca* ants are very aggressive ants and will flex their gasters

upon little to no obvious perturbation.  *Azteca* workers in both group A and group B

were observed flexing their gasters in the fighting rings even when no other ants

were present.  Therefore, we did not include this behavior in our aggression assays.

Additionally, it is difficult to determine the biological significance between multiple

level 2 attacks (<2 sec) and one level 3 attack (>2 sec).  However, even if level 2 and

3 attacks are lumped as one category there is still less aggression within clusters

than between clusters.  There is a major distinction between level 3 and 4 attacks as

level 3 attacks never resulted in dismemberment or death of either worker.  Inter

cluster aggression within groups never reached level 4 intensity.  Additionally, our

trials were conducted on a neutral field, which may elicit fewer agonistic responses

than those when foreign individuals attempt to enter a nest (Breed and Bennett 1987; Martin *et al.* 2009). This means it is possible we may have even underestimated aggression, particularly between clusters.

Inter-cluster aggression was variable and clusters with little genetic differentiation did not necessarily display less aggression. Although in some cases aggression clearly has a strong genetic component (Giraud 2002), aggression can be linked to a combination of factors (Liang and Silverman 2000). Drescher *et al.* (2007) found that aggression generally increased as relatedness decreased, but with some notable exceptions, which indicated that genetic and environmental factors could be contributing to aggression levels. It is possible that environmental factors such as differing diets are playing a significant role in aggression (Ichinose *et al.* 2009; Buczkowski *et al.* 2005; Silverman and Liang 2001).

*Unicoloniality/polydomy*

In the strictest sense polydomy in ants refers only to a colony that inhabits multiple nests that maintain social contact (DeBout *et al.* 2007). Low levels of aggression and genetic differentiation found at the intra-cluster levels indicate it is feasible that workers could move freely between nests, but short of tracking workers we cannot tell the extent to which nests within a cluster interact once budding has occurred. Thus this species may or may not fit a strict definition of polydomy. However, it is possible that polydomy is simply a transient state in this species where nests bud but social contact is eventually lost.

There is a significant body of literature examining unicolonialism or the existence of supercolonies, particularly in invasive ant species. DeBout *et al.* (2007) define unicoloniality as entire populations functioning as a socially interacting polydomous colony, which is characterized by low levels of genetic differentiation and aggression. Pedersen *et al.* (2006) state that unicolonial populations are made of one or more supercolonies. Supercolonies have no behavioral boundaries and encompass a large number of colonies over a geographic area expansive enough that distant colonies have no direct interaction. Some studies claim a species is uniclonal when workers from disparate regions show little aggression between each other (Fournier *et al.* 2009) even when significant levels of genetic variation are found (Holzer *et al.* 2006). However despite significant genetic differentiation, Holzer *et al.* (2006) also presented data that showed indiscriminate behavior toward non-nest brood indicating a lack of recognition. Supercolonies can be used to explain the existence of high levels of aggression and significant genetic differentiation in unicolonial populations as seen in the Argentine ant *Linepithema humile* (Suarez *et al.* 1999).

Our data shows significant and relatively strong genetic differentiation between clusters and relatively high levels of aggression indicating that *Azteca* is not acting as a unicolonial population. Low levels of genetic differentiation between three clusters in group B that span 538 m fit the supercolony predictions of genetic patterns. However, aggression observed between these clusters including prolonged biting is higher than reported for intra-supercolony or unicolonial aggression in other studies (Drescher *et al.* 2007, Abbott *et al.* 2007; Holzer *et al.*

2006; Pedersen *et al.* 2006, Giraud *et al.* 2002, Suarez *et al.* 1999). Suarez *et al.* (1999) and Giraud *et al.* (2002) found that intra-supercolony aggression in *Linepithema humile* never escalated beyond touch or avoid behavior. Additionally, there are also no clear signs that nests within or between clusters maintain social contact. There are no definite trails connecting nests and short of tracking individuals it would be difficult to determine exchange of workers between nests.

*Significance of 2 Groups*

The ecological significance of the presence of two species of *Azteca* in this system (where previously it was believed there was only one) remains unknown. From our data we can see evidence for budding strategies in both groups indicating similarity in local expansion processes. All clusters for group A were genetically distinct and this was also true for 2 of 5 clusters in group B. However, we saw no differentiation in three clusters separated by up to 500 m in group B, indicating there could be differences in mechanisms leading to new cluster initiation. Caution should be taken, however, when speculating on landscape scale differences between groups A and B from our study, since we have very coarse resolution in our study with only 4 clusters analyzed from group A and 5 clusters from group B. Additionally, with only five microsatellite markers it is possible we just do not have enough power to see differentiation between clusters B2, B4, and B5 in group B. There are however, stark differences in cluster abundance of group A and group B in our study plot. We have found only a total of 4 clusters of group A in the 45 hectare plot, whereas there are tens of clusters of group B, indicating that group B is numerically dominant. We do not have any direct observations concerning

competition between the two groups over food or nesting resources. Additionally, we do not know if density dependent controls are acting upon group A and group B in ecologically similar manners. However, phorid flies appear to be ubiquitous throughout the farm indicating that at least the potential for phorid attack exists regardless of whether a cluster is group A or group B. Our results here place new interesting hypothesis to be investigated in future studies.

CONCLUSION

In conclusion, we find strong evidence that the origin of clusters is the product of a process of budding, which supports the hypothesis by Vandermeer and colleagues (2008) concerning the local expansion process crucial to the self-organized pattern observed in *Azteca*. The presence of non-genetically differentiated clusters in geographically disparate locations implicate that a stepping stone model, or jump dispersal could be happening. This could be attained through a long distance budding process or via short distance dispersal of winged queens. However, the presence of strong genetic differentiation between other clusters and no correlation between genetic distance and geographic distance at the cluster level implicate that cluster establishment via queens flying from other areas may also be occurring. Additionally, it is apparent that there are two distinct evolutionary units existing sympatrically in our study site. From our data it appears that similar local expansion processes are operating in both groups. The ecological equivalence of these two units is unknown and is an important topic of exploration concerning the spatial distribution of these organisms and their role in the ecosystem service of biocontrol.

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