Landscape genetics across the Andes mountains: Environmental variation drives genetic divergence in the leaf-cutting ant *Atta cephalotes*

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Abstract

Distinguishing among the mechanisms underlying the spatial distribution of genetic variation resulting from the environmental or physical barriers from those arising due to simple geographic distance is challenging in complex landscapes. The Andean uplift represents one of the most heterogeneous habitats where these questions remain unexplored since multiple mechanisms may interact, confounding their relative roles. We explore this broad question in the leaf-cutting ant *Atta cephalotes*, a species that is distributed across the Andes mountains, using nuclear microsatellite markers and *mtCOI* gene sequences. We investigate spatial genetic divergence across the western range of the northern Andes in Colombia by testing the relative role of alternative scenarios of population divergence, including isolation by geographic distance (IBD), climatic conditions (IBE), and the physical barriers presented by the Andes mountains (IBB). Our results reveal substantial genetic differentiation among *A. cephalotes* populations for both types of markers, but only nuclear divergence followed a hierarchical pattern with multiple models of genetic divergence imposed by the western range. Model selection showed that the IBD, IBE (temperature and precipitation), and IBB (Andes mountains) models, often proposed as individual drivers of genetic divergence, interact and explain up to 33% of the genetic divergence in *A. cephalotes*. The IBE model remained significant after accounting for IBD, suggesting that environmental factors play a more prominent role than with IBB. These factors, in combination with the idiosyncratic dispersal patterns of ants, appear to determine the hierarchical patterns of gene flow. This study enriches our understanding of the forces shaping population divergence in complex habitat landscapes.

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ABSTRACT

Distinguishing among the mechanisms underlying the spatial distribution of genetic variation resulting from the environmental or physical barriers from those arising due to simple geographic distance is challenging in complex landscapes. The Andean uplift represents one of the most heterogeneous habitats where these questions remain unexplored since multiple mechanisms may interact, confounding their relative roles. We explore this broad question in the leaf-cutting ant *Atta cephalotes* , a species that is distributed across the Andes mountains, using nuclear microsatellite markers and *mtCOI* gene sequences. We investigate spatial genetic divergence across the western range of the northern Andes in Colombia by testing the relative role of alternative scenarios of population divergence, including isolation by geographic distance (IBD), climatic conditions (IBE), and the physical barriers presented by the Andes mountains (IBB). Our results reveal substantial genetic differentiation among *A. cephalotes* populations for both types of markers, but only nuclear divergence followed a hierarchical pattern with multiple models of genetic divergence imposed by the western range. Model selection showed that the IBD, IBE (temperature and precipitation), and IBB (Andes mountains) models, often proposed as individual drivers of genetic divergence, interact and explain up to 33% of the genetic divergence in *A. cephalotes* . The IBE model remained significant after accounting for IBD, suggesting that environmental factors play a more prominent role than with IBB. These factors, in combination with the idiosyncratic dispersal patterns of ants, appear to determine the hierarchical patterns of gene flow. This study enriches our understanding of the forces shaping population divergence in complex habitat landscapes.

KEYWORDS

Andean uplift, western mountain range, spatial genetic structure, isolation by distance, isolation by barrier, model selection, isolation by environment.

INTRODUCTION

Population divergence can be determined by the spatial distribution of individuals, where geographic proximity modulates genetic similarity, leading to a pattern of isolation by distance – IBD (Lee & Mitchell-Olds, 2011; Shafer & Wolf, 2013; Slatkin, 1993; Wright, 1943). However, populations in complex landscapes are often exposed to environmental variation and physical barriers that can also contribute to genetic divergence (Manel & Holderegger, 2013; Manel, Schwartz, Luikart, & Taberlet, 2003; Shafer & Wolf, 2013). Populations could adapt to their local environment, maximizing the fitness of individuals under local conditions while decreasing the fitness of immigrants from alternative environments (Carro, Quintela, Ruiz, & Barreiro, 2019; Sobel, 2014; Wang & Bradburd, 2014; Wang, Glor, & Losos, 2013). This adaptive reduction in gene flow can produce a pattern of isolation by environment – IBE (Sexton, Hangartner, & Hoffmann, 2014; Shafer & Wolf, 2013; Wang & Bradburd, 2014). Alternatively, gene flow could be restricted by allopatric scenarios of genetic differentiation mediated by physical barriers in the landscape, generating patterns of isolation by barrier – IBB (De Queiroz, Torrente-Vilara, Quilodran, da Costa Doria, & Montoya-Burgos, 2017; Haffer, 2008; Rull, 2011; Turchetto-Zolet, Pinheiro, Salgueiro, & Palma-Silva, 2013). With increasing landscape complexity, gene flow is likely to be influenced by a combination of geographical and ecological factors in which these isolating mechanisms are not mutually exclusive (Crispo, Bentzen, Reznick, Kinnison, & Hendry, 2006; Edwards, Keogh, & Knowles, 2012; Noguerales, Cordero, & Ortego, 2016; Wang et al., 2013).

The Andean mountain ranges not only represent one of the most unexplored environments, but also offer a great complexity of landscapes, promoting the diversification of a wide range of taxa (Salgado-Roa et al., 2018). Across these mountains, restricted gene flow has been reported in multiple organisms, including birds (Cadena, Pedraza, & Brumfield, 2016), plants (Lagomarsino, Condamine, Antonelli, Mulch, & Davis, 2016; Luebert & Weigend, 2014; Pérez-Escobar et al., 2017), mammals (Antonelli et al., 2009; Hoorn et al., 2010), insects (Antonelli et al., 2009; De-Silva et al., 2017; Hoorn et al., 2010), and other arthropods (Salgado-Roa et al., 2018). However, organisms from contrasting populations in these habitats are often

exposed to a combination of distance, environmental, and physical barriers to dispersal, challenging the investigation of the relative roles of different isolating mechanisms (James, Coltman, Murray, Hamelin, & Sperling, 2011; Meirmans, 2015; Noguerales et al., 2016). It remains unclear how the Andean uplift has modulated patterns of gene flow and the evolution of several of the most ecologically important groups in the Neotropics, including social insects. For example, although the entire evolution of Neotropical ants occurs across the Andes (Mueller et al., 2017), the interplay between their population structure and environmental variation relative to the effect of these mountains on isolated populations remains largely unknown.

The leaf-cutting ant *A. cephalotes* is a major urban and agricultural pest in the Neotropics, colonizing a wide spectrum of environments (Della Lucia, Gandra, & Guedes, 2014; Fernández, Castro-Huertas, & Serna, 2015; Hölldobler & Wilson, 2011). In Colombia, its distribution overlaps with the maximum complexity of the Andean uplift, ranging from 0 to 2100 m.a.s.l. (Fernández et al., 2015), with a vertical thermal gradient of 0.6 °C/100 m (Hermelin, 2015). The northern section of these mountains in Colombia splits into three main branches: the western, central, and eastern ranges. Populations of*A. cephalotes* are separated by these mountains while simultaneously being exposed to complex combinations of topographical and environmental variation (Kattan, Franco, Rojas, & Morales, 2004; Pérez-Escobar et al., 2017; Salgado-Roa et al., 2018). Such conditions provide a tremendous climatic spectrum for local adaptation, with an interplay between population dynamics and species-specific dispersal patterns (Hakala, Seppä, & Helanterä, 2019). Evolution under such environmental heterogeneity could act to shape patterns of gene flow (De Queiroz et al., 2017; Lee & Mitchell-Olds, 2011; Noguerales et al., 2016; Wang et al., 2013), which often produces more complex scenarios than genetic divergence due to IBD alone (Slatkin, 1993; Wright, 1943). For example, we recently found that the eastern range of the Andes in Colombia plays a major role as a geographic barrier to historical gene flow, restricting the dispersion of *A. cephalotes* from north to south (Muñoz-Valencia, Vélez-Matínez, Montoya-Lerma, & Díaz, 2021). Although this initial study demonstrates the significant influence of the Andes on population divergence in the leaf-cutting ant at the phylogeographic scale, the role of local adaptation occurring at more regional scales across the Andes remains untested.

This study focuses on a finer and more complex geographic distribution scale of *A. cephalotes* : that of the western range of the Andean uplift in Colombia. We use a landscape genetic approach to investigate the role of geographic features and environmental variation in the definition of patterns of spatial genetic structure in *A. cephalotes* . Using nuclear (microsatellites) and mitochondrial (*mtCOI*) markers, we test the relative roles played by geographic distance, climate variation, and a major dispersal barrier (the western range) in modulating patterns of gene flow. As a monogynous (single-queen) species, *A. cephalotes* presumably undertakes long-distance nuptial flights that can potentially overcome isolating barriers (Cherrett, 1968; Helms, 2018; Moser, 1967). Our results demonstrate that gene flow is limited by a complex interaction of the three isolating mechanisms (IBD, IBE, and IBB) rather than IBD alone, while IBE appears to play a stronger role than IBB. Investigating the spatial genetic structure of a species in an exceptionally heterogeneous environment helps to elucidate the evolution and diversity of this ecologically dominant group of ants in the Neotropics.

MATERIALS AND METHODS

Sampling

Ant sampling was conducted in the Colombian Pacific and Andean regions, which are separated by the western mountain range of the Colombian Andes (Figure 1). The Pacific region is classified as a tropical rainforest with an extremely humid climate and an annual average temperature of 27 °C. The Andean region is further divided into two groups: Andean 1 (800 - 1050 m.a.s.l.) and Andean 2 (1300 - 2200 m.a.s.l.), with highly variable climatic conditions. The inner valleys in Andean 1 are climatically classified as tropical savanna and tend to be dry, with an annual temperature of 25 °C, while the range summits in Andean 2 are more humid, with a temperate climate, tropical monsoons, and an annual temperature of 21 °C (Supplementary table ST1) (Chen & Chen, 2013; Hernández-Camacho, 1992; Kattan et al., 2004; Köppen, 1884; Peel, Finlayson, & McMahon, 2007).

Environmental variation across the three regions was characterized by differences in temperature, humidity, and precipitation, measured as five-year averages of the annual temperature (°C), relative humidity (%), and precipitation (mm), respectively, for each location (IDEAM, 2019). In addition, a climate classification was represented using four categories (1 to 4) of different climatic conditions mediated by the tropical Andes. Variation in topography was estimated by elevation above sea level. A dummy variable was used to evaluate the Andean uplift as a major geographic barrier to gene flow. The code 0 was used for populations from the western side of the western range (Pacific region), and 1 for populations from the eastern side of these mountains (Supplementary table ST1).

Worker ants from nine to twenty nests in ten locations (total of 153 nests) were sampled in the period 2017-2018 (Table 1). The distance between nests in each location was at least 1.5 km, ensuring that the sampled nests were independent colonies. Three, two, and five locations were sampled from the Pacific, Andean 1, and Andean 2 regions, respectively (Table 1).

Molecular methods

DNA extraction and PCR amplification of microsatellite markers

Total DNA extraction was carried out for five workers from each nest (total 765 workers) using TNES lysis buffer (Tris 50mM, NaCl 0.4M, EDTA 100mM, SDS 0.5%), pH 7.5, and chloroform:isoamyl alcohol (24:1), following Wasko et al. (2003), with minor modifications as described by Muñoz-Valencia et al. (2020).

Thirteen microsatellite loci developed for *A. cephalotes*(Muñoz-Valencia et al., 2020) were used (Supplementary Table ST2). PCR reactions were carried out following Muñoz-Valencia et al. (2020) in a 10 µL volume containing 10 ng of DNA, 1 X Phusion Flash PCR Master Mix (Thermo Fisher Scientific), and 2 µM of each labeled primer. The thermal profile was: 98 °C for 1 min, followed by 34 cycles of 98 °C for 1 s, annealing temperature for 15 s, and 72 °C for 20 s, followed by a final extension step at 72 °C for 1 min. The fluorescent amplified fragments were visualized using an automated DNA sequencer ABI 3130 Genetic Analyzer (Applied Biosystems), and allele sizes were estimated using GeneMapper version 4.0 (Thermo Fisher Scientific).

Sequencing of the mtCOI gene

A 368 bp fragment of the mitochondrial cytochrome oxidase subunit I gene (*mtCOI*) was sequenced from one sample per nest, obtaining a total of 146 sequences after discarding failed amplifications and sequencing (GenBank accession numbers: MW245066 - MW245211). PCR amplification was carried out using the universal primers Ben and Jerry, following Kronauer et al. (2004) and Simon et al. (1994). PCR reactions were performed in a 10 µl volume, containing 10 ng of DNA, 1X GoTaq® Master Mix (PROMEGA), and 2 µM of each primer. The thermal cycling profile was 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. PCR products were confirmed by electrophoresis on 1 % agarose gels. All PCR products were sequenced by Psomagen, Maryland, USA.

Population genetics analyses

Genetic diversity of DNA microsatellites and the mtCOI gene

To test for null alleles, large allele dropout, and scoring errors, we used Microchecker version 2.2.3 (VanOosterhout, Hutchinson, Wills, & Shipley, 2004). Putative null alleles were detected in five loci (MAT2, MAT4, MAT10, MAT15, and MAT28) of five populations (DAG, GUA, DOV, QBY, and YOB; Table 2), which may have biased the estimates of population structure (Chapuis & Estoup, 2007). We accounted for this bias by comparing analyses after excluding these loci with those of the entire data set. Our main results and conclusions remained unchanged, however, regardless of the loci included in the analyses, and therefore only the results for the entire data set are presented here. Since *A. cephalotes* nests form family units and nestmates are closely related, only one individual per nest was used to evaluate the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD). Tests were performed for each locus (HWE) and each locus

pair (LD) for each population (Supplementary Table ST2) using GENEPOP (Rousset, 2008). A Bonferroni correction was applied with a global significance of α=0.05 for multiple comparisons.

Genetic diversity was characterized for each locus, region, and population as allele count (N_a), allelic richness (A_R), expected (H_e) and observed (H_o) heterozygosities, and the inbreeding coefficient (F_{IS}), using the software GenAlex v. 6.5 (Peakall and Smouse 2012) and FSTAT v. 2.9.3.2 (Goudet, 1995; 2001) (Table 2, Supplementary tables ST2, ST3). Tests for significant differences from zero in the diversity parameters were performed using the '*aov*'function in R (Chambers, Freeny, & Heiberger, 1992). We used the software Bottleneck (Piry et al. 1999) to estimate recent changes in population sizes by implementing the IAM and SSM mutation models and a Wilcoxon signed-rank test to evaluate statistical significances. We also determined whether the allele frequency distribution differed from the expected L-shape. Where necessary, significant values were adjusted for multiple comparisons through Bonferroni correction.

The genetic diversity of *mtCOI* data was estimated through the number of haplotypes (h), nucleotide diversity (π), and haplotype diversity (h_d), using DnaSP 5.19 (Librado & Rozas, 2009). Tajima's D and Fu's FS neutrality tests were performed to investigate signatures of recent population expansion (Ramos-Onsins & Rozas, 2002) when the null hypothesis of neutrality was rejected due to significant negative values ($P < 0.05$ for D, $P< 0.02$ for FS; Supplementary table ST3).

Spatial genetic patterns

We investigated spatial genetic patterns and restrictions to gene flow in *A. cephalotes* , using the software ARLEQUIN version 3.5.2.2 (Excoffier & Lischer, 2010) to perform alternative hierarchical Analyses of Molecular Variance (AMOVA). First, the populations were classified into two regions (Pacific and Andean), separated by the western range of the Colombian Andes, in order to study the effect of this range as a major geographic barrier to gene flow affecting the distribution of genetic variation in the data (isolation by barrier, IBB). Second, the populations were classified into three regions (Pacific, Andean 1, and Andean 2) defined by the climatic conditions mediated by the Andes mountains (isolation by environment, IBE; Supplementary table ST1). In the AMOVA, we estimated the associatedF_{ST} for microsatellites and the genetic distance-based Φ -statistic for *mtCOI* (Excoffier, Smouse, & Quattro, 1992; Meirmans, 2006; Meirmans & Hedrick, 2011), both globally and between all pairs of populations. The significance of the variance components and associated F_{ST} andΦ indices were calculated using 10,000 permutations.

We further investigated the structure of the *A. cephalotes*nuclear data using two clustering analyses. First, we used a model-based Bayesian clustering method implemented in the software STRUCTURE v. 2.3.4 (Pritchard, Stephens, & Donnelly, 2000), which estimates the number of genetic clusters (K) independent of spatial sampling. Analyses were performed using one individual per nest, with and without admixture, for correlated allele frequencies. A burn-in of 50,000 and 500,000 sampling generations were implemented for K ranging from 1 to 12, with 10 iterations for each value of K . Evanno's method (Evanno, Regnaut, & Goudet, 2005), implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2012), was used to estimate the optimal number of clusters from the STRUCTURE output (Supplementary figure SF1) and the results were visualized using CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015).

Second, discriminant analysis of principal components (DAPC) was performed using a principal component analysis (PCA) prior to the discriminant analysis (DA) (Jombart, Devillard, & Balloux, 2010). The DA partitions genetic variation, maximizing differences between clusters while minimizing within-cluster variation. We performed a DAPC analysis in the R package ADEGENET (Jombart et al., 2010) using one individual per nest for microsatellite data and a polymorphic nucleotide positions matrix for *mtCOI* . The function '*dapc* ' was used to estimate all available principal components (PCs), and to determine the optimal number of PCs used based on cumulative variance.

Redundancy analysis: disentangling patterns of isolation by distance, environment, and barrier

We implemented a set of redundancy analyses (RDA) to disentangle the relative contribution of alternative hypotheses for explaining the genetic differentiation in *A. cephalotes* : IBD, IBE, and IBB. RDA is a canonical

extension of PCA, in which the principal components are constrained to be linear combinations of a set of predictors (Bradburd, Ralph, & Coop, 2013; Legendre & Fortin, 2010; Meirmans, 2015). The goal of RDA here was to identify the best ordination model that describes genetic differentiation (James et al., 2011) in order to better understand how spatial heterogeneity across the Andes in the Colombian Pacific and Andean regions affects patterns of gene flow in*A. cephalotes* .

For the microsatellite data, a conventional RDA analysis was performed using population allele frequencies as a dependent matrix since the Euclidean distance estimates involved in the analysis are directly related to F_{ST} values (as long as the frequencies are not scaled) (De Queiroz et al., 2017; Noguerales et al., 2016). The*mtCOI* data were analyzed through a distance-based RDA, making direct use of a Φ_{ST}-based genetic distance matrix. In both cases, space (geographic distances matrix), environment (climate variables), and topography (barriers) were considered explanatory variables. These explanatory variables were grouped into three classes according to their resulting patterns of isolation: 1) IBD, with variables representing the geographic distance between populations (space); 2) IBE, with variables determining environmental differences between populations; and 3) IBB, based on the western range as a geographic barrier that splits populations in an allopatric manner. Since space was the only explanatory variable initially expressed as a distance matrix, it was transformed into a vector format (Oksanen et al., 2019) by PCA, using the '*pcnm'* function in the package VEGAN. Only the best explanatory PCNM components were retained. The significance of predictors was assessed using multivariate$F$ -statistics with 10000 permutations using the '*anova.cca* ' function included in the package VEGAN. All explanatory variables were scaled using the '*scale'* function in the package VEGAN. The allele frequencies used were not scaled in order to keep their inter-population relation with the Φ_{ST}.

Spatial explanatory variables were tested through an IBD analysis based on RDA, followed by sequential elimination of environmental variables, in which only variables with $| r | < 0.80$ were retained (Dormann et al. 2013; Supplementary figure SF2). The remaining variables were tested through a model selection approach, comparing all possible combinations or marginal tests to be finally included in the IBE analysis. Each marginal test was compared to the null model (intercept; AIC: 1.41). These combinations of environmental variables were used to identify the best model based on the Akaike information criteria (AIC). We selected the best model for significant predictors with the '*ordistep'* function, using the package VEGAN to prevent overfitting. IBB was tested using the classification of sampled populations represented by a dummy variable (see sampling). Once all three models of genetic isolation were defined and tested, both IBE and IBB were tested while controlling for spatial autocorrelation through a partial RDA (conditional test). Finally, the relative contribution of IBD, IBE, and IBB to the explanation of gene flow patterns in *A. cephalotes* was evaluated from the final complete model, including all mechanisms of genetic isolation, using a variation partitioning analysis with the function '*varpart* ' implemented in the package VEGAN. This analysis allowed us to disentangle total genetic variance according to the underlying mechanisms of isolation imposed by space, environment, and barriers, as well as their relative contributions when considered together.

RESULTS

Genetic diversity

All microsatellite loci followed expectations from the HWE or LD tests. Most loci were highly variable, with the number of alleles ranging from 2 to 20 per locus (Table 2, Supplementary table ST2). The Pacific region showed the highest allelic richness, largely due to 17 private alleles, while the Andean 1 region had the lowest allelic richness, with four private alleles. Most genetic diversity parameters were significantly higher for the Pacific region, except for the $H_e$estimates (Figure 2A, Supplementary table ST4). An excess of heterozygotes was detected in the PPY population (IAM model: T= 3.40,$P < 0.01$; SSM model: T= 1.92, $P = 0.03$), while the allele frequencies differed from the L-shaped distribution. A putative bottleneck was supported by the low number of alleles per locus detected in this population (range: 1 to 6). Inbreeding coefficients did not differ significantly from zero, suggesting random mating in all populations (Table 2).

A 307 bp *mtCOI* gene fragment was successfully sequenced in 146 individuals. Ten nucleotide sites (3.3%) were polymorphic and defined 10 different haplotypes that differ in 1-3 positions (0.4% sequence divergence). The two most common haplotypes, Hap1 (frequency= 0.51) and Hap4 (frequency = 0.27), were found in almost all sampling locations. One haplotype was specific to the Pacific (Hap3), one to the Andean 1 (Hap6), and three to the Andean 2 (Hap8, Hap9, Hap10; Figure 2C, Supplementary Table ST5) regions. Overall, haplotype and nucleotide diversities were H_d= 66% (range: 0.0 – 76%) and π = 0.3% (range: 0.0 – 0.4%), respectively. In contrast to the microsatellite data, the Andean 1 region presented the highest diversity (Table 2, Figure 2B). However, the differences in mitochondrial diversity across regions were not significant (Figure 2B, Supplementary Table ST4).

Population structure for nuclear and mitochondrial data

Two regional models were used in the hierarchical AMOVA, following IBB and IBE scenarios (Table 3). For the microsatellite data, the results were similar under IBB and IBE scenarios, showing substantial differentiation among regions and populations within regions. Moreover, genetic differentiation was higher among regions when compared with that among populations, and was slightly higher under the IBE model when compared to the IBB model (Table 3).

Pairwise F_{ST} estimates between populations ranged from 0.01 to 0.28 (mean = 0.10) and were significant following Bonferroni corrections, except for the BVT-QBD comparison in the Pacific region (Figure 3A). Pairwise comparisons between regions under the IBE model were significant, showing the highest differentiation values for comparisons involving the Andean 1 region (Pacific-Andean 1: 0.04; Pacific-Andean 2: 0.02; and Andean 1-Andean 2: 0.10), consistent with the DAPC analysis (Figure 4A). However, regional comparisons under the IBB model were also significant (F_{ST} = 0.04).

Hierarchical AMOVA for *mtCOI* data indicated that populations within regions harbored 34% of the genetic variance, but no region effect was detected under the IBB and IBE models (Table 3). The pairwise Φ_{ST} values estimated among populations ranged from 0.00 to 0.86, and 56% of these comparisons were significant (Figure 3B). Moreover, DAPC clustering analysis also failed to group populations by regions (Supplementary figure SF3), supporting the AMOVA results.

Clustering analyses

Evidence for hierarchical population structure in microsatellite data was also found from the STRUCTURE runs. The ΔK *ad hoc*statistic by Evanno et al. (2005) indicated an optimal $K = 3$ [Pr(X / K : 2) = -5061.73] as the uppermost hierarchical level (Supplementary figure SF1). These three genetic clusters correspond to the individuals belonging to the three regions originally considered under the IBE model, except for the PPY population from the Andean 2 region. The first group (blue in Figure 4B) clustered most samples from the Pacific region. The second group (purple in Figure 4B) clustered most samples from the Andean 1 region, as well as samples from Andean 2 region (PPY). The third group (orange in Figure 4B) clustered most samples from Andean 2 region, apart from the PPY population. This pattern suggests a hierarchical north-south structure in the Andean region rather than a climate-related structure, as tested through the IBE-based AMOVA (Figure 4C), and suggests that IBD is influencing regional differentiation for the Andean regions rather than, or in addition to, IBE.

To further investigate the role of IBD in the genetic structure of*A. cephalotes* , we performed a new AMOVA by moving PPY from the Andean 2 to the Andean 1 region. However, the results were similar to those produced under the original IBE scenario (results not shown). We also ran another STRUCTURE analysis, this time only including samples from the Andean 1 region and the PPY population (southern Andean cluster). This analysis revealed two genetic groups (K = 2), in which PPY formed a sole cluster (Figure 4B).

The results for the IBE model were supported by the DAPC analysis, which clustered defined regions based on environmental variables. The first two principal components of the DAPC analysis explained 77.1% of the variance in allele frequencies (50 PCs retained; Figure 4A). In this case, the first principal component

clustered the Pacific and Andean regions, while the second principal component reflected the differentiation between Andean 1 and Andean 2 regions (Figure 4A).

RDA analysis – Alternative scenarios of genetic differentiation: IBD, IBE, and IBB

Significant IBD was detected from the RDA analysis for microsatellite data, in which the geographic distance expressed as the first axis of the PCNM analysis (PCNM1) contributed to the genetic structure of*A. cephalotes* , based on model selection. This suggests that distance is an important determinant of genetic structure in *A. cephalotes* (Figure 5). These results agree with those obtained from STRUCTURE, suggesting that genetic differentiation in the Andean regions can also be explained by an IBD pattern (north to south) and not exclusively by IBE across regions (Figure 4B). The IBD results followed different patterns between nuclear and mitochondrial markers since IBD was not detected from the *mtCOI* data.

Although alternative models used for regional differentiation suggested the stronger influence of IBE, based on its higher regional genetic differentiation when compared to IBB or IBD, all of the models were significant (Table 3). This was also observed from the RDA results, where two environmental variables (temperature and precipitation) and the barrier variable (Andes classification) were significantly associated with genetic divergence (Table 4, Figure 5). Moreover, a significant contribution of alternative models of genetic differentiation was only observed from the microsatellite data (Supplementary table ST6). The optimal model, including all mechanisms of genetic isolation as tested for IBD, IBE, and IBB, accumulated up to 33% of explained variation, which remained marginally significant after accounting for IBD in conditional tests (Figure 5).

Variation partitioning for the full model, including all alternative models of genetic isolation, showed that IBD and IBE explained the highest proportion of genetic variation relative to IBB (Figure 5). Interaction effects between major mechanisms of genetic isolation were low for the triple interaction (IBD + IBE + IBB = 2%) and were not significant for paired interactions (Figure 5). The higher fraction of unexplained variation (residuals) might reflect the occurrence of genetic drift within populations, which is not associated with the explanatory variables.

DISCUSSION

The complex landscape across the Andean uplift is an important barrier, isolating populations and increasing genetic divergence on both sides of the mountains (Antonelli et al., 2009; Hoorn et al., 2010; Salgado-Roa et al., 2018). The western range of the Andes in Colombia is one of the most biodiverse ecosystems in the Neotropics (Kattan et al., 2004; Salgado-Roa et al., 2018) and is the most complex landscape within the distribution of the leaf-cutting ant *A. cephalotes* . By integrating hierarchical population structure with models of isolation by distance (IBD), environment (IBE), and barrier (IBB), we explored the role of the western mountain range in the distribution of genetic variation of *A. cephalotes* . Here, we demonstrated that the environmental heterogeneity imposed by the Andean uplift has highly influenced the population structure of *A. cephalotes* .

Genetic diversity

Our results indicate that the populations of *A. cephalotes* are highly variable at nuclear markers, with significant genetic differentiation at both region and population levels. In contrast, populations are highly differentiated for the *mtCOI* gene, but no region effect was detected with this marker. Ten *mtCOI* haplotypes were detected in 146 nests analyzed, showing low nucleotide and moderate haplotype diversities, with most populations dominated by the same haplotype (Hap1). As expected considering the common biogeographical history, similar *mtCOI* patterns have been detected in the leaf-cutting ant *A. colombica* (Helmkampf, Gadau, & Feldhaar, 2008), where six *mtCOI* haplotypes and relatively low levels of nucleotide diversity (0.1% population-wide) were detected across 20 colonies, with most specimens sharing the same haplotype. However, these results were obtained from a small sample size in a small area. Despite the

fact that several other genetic studies have been reported in Attini (*e.g., Acromyrmex*) (Cantagalli, Mangolin, & Ruvolo-Takasusuki, 2013; Diehl, Cavalli-molina, & Mellender de Araujo, 2002; Diehl, de Araújo, & Cavalli-Molina, 2001; Pinheiro dos Reis, Fernandes Salomão, de Oliveira Campos, & Garcia Tavares, 2014), the nature of the markers and sampling employed prevents us from drawing meaningful comparisons with these studies regarding diversity estimates.

Spatial differentiation at regional and local levels

We detected significant genetic differentiation across regions from the nuclear data, as evidenced from the AMOVA analyses and clustering methods, suggesting the potential role of the Andean landscape in restricting gene flow in *A. cephalotes* . We tested alternative scenarios causing regional differentiation, considering IBE as a model of divergence, as well as the geographic barrier imposed by the western range under an IBB model.

Regional differentiation from microsatellite data was higher under the IBE than the IBB model, suggesting the more prominent role of climate compared to the geographic barrier in restricting gene flow in *A. cephalotes* . On the other hand, the STRUCTURE results support a regional differentiation with mixed effects between IBE and IBD, evidencing a north-south spatial pattern for Andean populations rather than a regional climate model. However, this IBD pattern was likely due to a single population. PPY was initially classified within the Andean 2 region, but clustered mostly ($Q > 0.95$) with the geographically closer Andean 1 region. Nevertheless, this pattern occurs only in the Andean region, suggesting that population differentiation in this particular case results from dispersal distance rather than environmental conditions or a geographic barrier imposed by the western range. Medium to long dispersal distances have been reported for*A. cephalotes* , with queens flying up to 50 km during nuptial flights (Cherrett, 1968; Helms, 2018). This distance is at least one order of magnitude smaller than the inter-population distance used in this study, but it is also probably an underestimate since it was originally measured on a small island (Helms, 2018). Moreover, only a small number of individuals are required in order to homogenize population allele frequencies, and gene flow can also be conveyed stepwise. Although the genetic variance at the region level suggested IBE, all of the models of hierarchical differentiation were significant for the RDA analysis, suggesting the likelihood that all three leading causes of genetic isolation are contributing to the observed patterns of gene flow.

Significant nuclear differentiation at the population level suggests that other features besides proximity, environment, and major dispersal barriers act to restrict gene flow. The markedly increased variance in the PPY population is consistent with a recent population expansion signal. Whether the bottleneck signal is a product of a founder effect (Barton & Turelli, 2004; Matute, 2013; Parisod, Trippi, & Galland, 2005) or a recent change in effective population size cannot be determined with the current data. However, the proximity and clustering of PPY to Andean 1 populations rather than the Andean 2 region in the STRUCTURE results, suggests a recent founder effect or an admixture between the two Andean regions.

In contrast to the nuclear data, there was no regional differentiation in the *mtCOI* data, but populations were significantly differentiated within the regions in both the IBE and IBB models. Muñoz-Valencia et al. (2021) used *mtCOI* data to study the genetic structure of *A. cephalotes* from a larger geographic range covering most of the distribution of the species in South and Central America. These authors found significant genetic differentiation, both at the regional scale and among populations within major regions. Together, these results show that the eastern range of the Andes appears to be a major dispersal barrier driving regional differentiation, while the western range forms a relatively homogenous biogeographic area.

Disentangling the patterns of IBD, and IBE, IBB

Our RDA framework provides strong evidence for restricted gene flow in*A. cephalotes* across the western mountain range of the Colombian Andes by all of the mechanisms tested. Furthermore, we found that the explained genetic variation was maximized when all spatial and environmental variables associated with these mechanisms were included in the model. These multifactorial patterns are often expected in complex and heterogeneous environments (Sexton et al., 2014; Shafer & Wolf, 2013), such as in the Colombian

Andes (Kattan et al., 2004; Pérez-Escobar et al., 2017; Salgado-Roa et al., 2018). Regions classified according to climate variation and geographic barriers follow IBE but also include IBB, supporting the higher differentiation estimate (F_{CT}) obtained under the IBE model and the significance when both models are included in the RDA (IBE + IBB). Migration through low elevation passes in the western mountain range (Hernández-Camacho, 1992; Kattan et al., 2004) would allow gene flow between regions, explaining why such a major geographic barrier did not have a more pronounced effect. Spatial and environmental variables presumably associated with restriction to gene flow often suffer from spatial autocorrelation, challenging the differentiation of their underlying causes (Crispo et al., 2006; Edwards et al., 2012; Wang et al., 2013). Our RDA framework accounted for this issue by demonstrating strong IBE even after controlling for IBD; while IBB was not significant, even when ignoring IBD. Temperature and precipitation therefore appear to be the leading causes of IBE in *A. cephalotes* .

The significant effect of the environment on the genetic structure of*A. cephalotes* populations indicates the potential of this species to adapt to local environmental conditions. This may occur when processes such as sex-biased dispersal (Edelaar & Bolnick, 2012) or selection against migrants (Hendry, 2004; Weber, Bradburd, Stuart, Stutz, & Bolnick, 2016; Wright, 1943) decrease the rate of gene flow, as has been observed in several other species (De Queiroz et al., 2017; Lee & Mitchell-Olds, 2011; Wang et al., 2013). Moreover, geographic distance and barriers in between can restrict gene flow by reducing dispersal efficiency, which is more associated with local genetic drift (Clémencet, Viginier, & Doums, 2005; Cross, Naugle, Carlson, & Schwartz, 2016; De Queiroz et al., 2017; Noguerales et al., 2016; Smith et al., 2018), leading to combined patterns of IBD and IBB. These complex patterns have been detected, for example, in the Amazonian common sardine *Triportheus albus* (De Queiroz et al., 2017). However, disentangling the individual environmental drivers was not possible due to strong correlations across environmental variables (Crispo et al., 2006; Edwards et al., 2012; Wang et al., 2013). The underlying causes of genetic isolation of populations have been rarely explored in leaf-cutting ants (Branstetter et al., 2017). In *A. sexdens rubipilosa* , geographic separation of populations alone did not explain population divergence (Cantagalli et al., 2013), and significant IBD and biome fragmentation imposed physical barriers to gene flow in*A. robusta* (Pinheiro dos Reis et al., 2014). All of these results together strongly suggest that the isolating mechanisms studied are not mutually exclusive (Crispo et al., 2006; Edwards et al., 2012; Wang et al., 2013) and are important factors in the evolution of Neotropical species.

Agricultural activities have significantly contributed to the colonization processes of leaf-cutting ants (de Carvalho Cabral, 2015). Conversion of their natural habitat into cultivated lands may favor population growth and dispersal of leaf-cutting ants (Montoya-Lerma, Giraldo-Echeverri, Armbrecht, Farji-Brener, & Calle, 2012; Schowalter & Ring, 2017). Queens of *Atta laevigata* have been shown to prefer nesting in open areas rather than closed forests (Vasconcelos 1997). Our exceptional study population PPY is located in a large city (Popayán). It is possible that the city infrastructure and constant human interference, rather than natural dispersal associated with nuptial flights, have influenced its genetic structure. This pattern is more compatible with human-mediated dispersal (Zheng, Yang, Zeng, Vargo, & Xu, 2018), where environmental changes associated with human expansion appear to promote population growth in leaf-cutting ants (Montoya-Lerma et al., 2012; Siqueira et al., 2017). However, PPY is most likely an exception to a general large-scale migration process in*A. cephalotes* .

The Andean uplift appears to modulate the population structure of*A. cephalotes* in a more complex manner than previously thought. The eastern mountain range of the Andean uplift in Colombia plays a major role as a geographic barrier to historical gene flow, restricting the dispersion of *A. cephalotes* from north to south (Muñoz-Valencia et al., 2021). By exploring a finer scale across the western mountain range and incorporating neutral genetic markers with environmental variables, we clearly show that the observed genetic differentiation in *A. cephalotes* is mainly affected by the combined multifactorial effect of different isolating mechanisms, mediated by the landscape complexity. Together, these results suggest that studies directed at exploring historical gene flow across the Andes should be interpreted with caution, since the complexity and history of this landscape can dramatically influence results at different scales.

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DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study will be openly available in DataDryad after manuscript is accepted for publication.

AUTHORS' CONTRIBUTIONS

VMV participated in the conceptualization, ant sampling, data analysis, original draft, reviewing, and editing of the manuscript. JML participated in conceptualization, original draft, reviewing, and editing of the manuscript. PS participated in data analysis, reviewing, and editing of the manuscript. FD participated in conceptualization, data analysis, original draft, reviewing, and editing of the manuscript. All authors have read and approved the final version of the manuscript.

TABLES AND FIGURES

Table 1. Sampled populations of *A. cephalotes*. Locations with their regional classifications (Pacific, Andean 1, and Andean 2 regions from Colombia) and the number of nests are indicated for each population.

Code	Location	Region	No. of nests
BVT	Buenaventura	Pacific	9
QBD	Quibdó	Pacific	15
GUA	Guapi	Pacific	20
DAG	Dagua	Andean 1	10
CAL	Cali	Andean 1	20
PPY	Popayán	Andean 2	14
DOV	Dovio	Andean 2	19
QBY	Quimbaya	Andean 2	16
YOB	Yolombó	Andean 2	19
MNZ	Manizales	Andean 2	11

Table 2. Microsatellites and mitochondrial *mtCOI* marker variation for sampling locations of *A. cephalotes* from the Pacific, Andean 1, and Andean 2 regions of Colombia. N is the sample size (number of nests). N_a : mean number of alleles; AR : mean allelic richness; H_e : mean expected heterozygosity; F_{IS}: inbreeding coefficient; HWE : Hardy-Weinberg Equilibrium test; h : number of haplotypes; H_d : haplotype diversity; π: nucleotide diversity. (***) $P < 0.0001$. Location abbreviations are as presented in Table 1.

	Nuclear data	Nuclear data	Nuclear data	Nuclear data	Nuclear data	Mitochondrial data	Mitoch
	N	N_a	AR	H_e	F_{IS}	N	h
Pacific							
All pop n = 3	44	6.95	6.56	0.57	0.01	42	5
BVT	9	6.08	6.02	0.59	-0.06	9	2
QBD	15	7.46	6.97	0.58	0.00	13	4
GUA	20	7.31	6.68	0.55	0.04	20	4
Andean 1							
All pop n = 2	30	4.62	4.38	0.49	-0.01	29	6
DAG	10	3.92	3.85	0.46	-0.01	10	3
CAL	20	5.31	4.91	0.51	-0.01	19	4
Andean 2							
All pop n = 5	79	4.74	4.51	0.53	-0.01	75	6
PPY	14	3.69	3.58	0.54	-0.01	14	3
DOV	19	4.38	4.18	0.48	-0.05	19	3
QBY	16	4.77	4.58	0.52	0.02	15	2
YOB	19	6.38	5.83	0.61	-0.01	16	1
MNZ	11	4.46	4.37	0.50	0.02	11	3
Total n = 10	153	10.38	7.06	0.60	-0.01	146	10

Table 3. Hierarchical AMOVA for microsatellites and *mtCOI* markers. The analyses included two intermediate levels of variation following: 1) isolation by barrier (IBB), with populations separated by the western range (Pacific and Andean), and 2) isolation by environment (IBE), with populations classified into three regions following environmental conditions (Pacific, Andean 1, and Andean 2). The proportion of variation (%) and associated F_{ST} and Φ are given for each hierarchical level.

Model				Microsate
	Source of variation	**Source of variation**	**df** F_{ST}	**df** F_{ST}

Model				Microsate
1) IBB: Pacific populations and Andean populations	Regions	Regions	1	1
	Pops (regions)	Pops (regions)	8	8
	Within pops	Within pops	296	296
	Total	Total	305	305
2) IBE: Pacific, Andean 1, and Andean 2 populations	Regions	Regions	2	2
	Pops (regions)	Pops (regions)	7	7
	Within pops	Within pops	296	296
	Total	Total	305	305

Table 4. Results of model selection as tested through the RDA analysis under IBD, IBE, and IBB scenarios of genetic divergence.

MODEL	**F**	P	**AIC**
One model			
IBD model	1.17	0.30	1.33
IBD model selected (PCNM1)	2.71	0.01*	0.49
IBE model (T + H + P + E + C)	1.46	0.07	1.65
IBE model selected (T + P)	1.81	0.03*	1.23
IBB model (Andes)	1.31	0.22	1.89
Two models combined			
IBE-IBB (T + P + Andes)	1.69	0.03*	1.28
IBE-IBD (T + P + PCNM1)	2.16	[?] 0.01*	0.09
IBB-IBD (Andes + PCNM1)	2.17	[?] 0.01*	0.58
Three models combined			
Whole model (IBD + IBE + IBB)	2.17	[?] 0.01*	-0.66
Conditioned models by IBD			
IBE model (T + P) ~ PCNM1	1.65	0.04*	0.09
IBB model (Andes) ~ PCNM1	1.48	0.13	0.58
Whole model (IBD + IBE + IBB) ~ PCNM1	1.74	0.02*	-0.66

T: Temperature; H: Humidity; P: Precipitation; E: Elevation; C: Climate. (*) Indicates significant value, P [?] 0.01. AIC null model: 1.41.

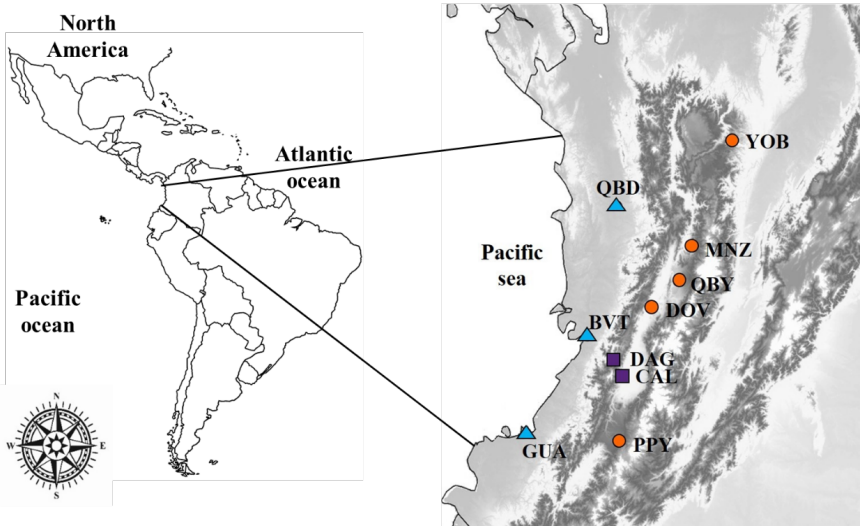

Figure 1. Study area and sampling locations of *Atta cephalotes* in Colombia. Different symbols and colors indicate sampling locations following regional classification: Blue triangles for sampling locations in the Pacific region (BVT, QBD, and GUA), violet squares for the Andean 1 region (DAG and CAL), and orange circles for the Andean 2 region (PPY, DOV, QBY, YOB, MNZ) (Table 1, and Supplementary table ST1).

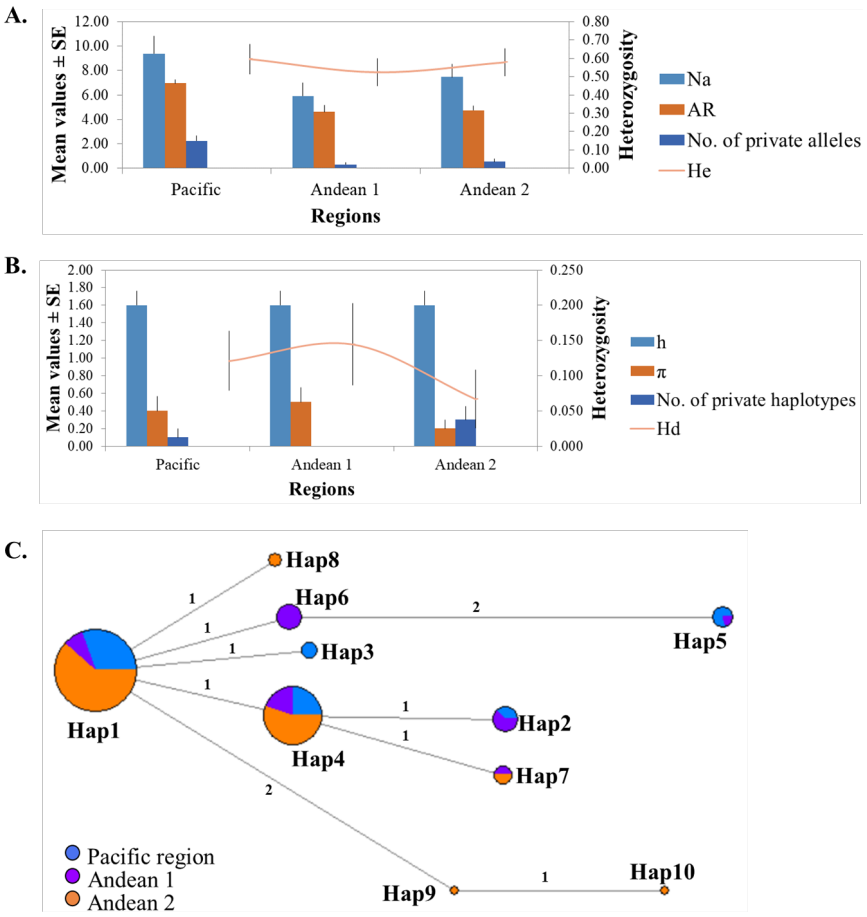

Figure 2. Patterns of genetic diversity and haplotype network for *A. cephalotes* **sampled in three different regions of Colombia**. **A.** Genetic diversity parameters for microsatellite markers (N_a: mean number of different alleles; A_R: mean allelic richness; H_E: mean expected heterozygosity). **B.** Genetic diversity parameters for the *mtCOI* gene (h: number of haplotypes; π: nucleotide diversity; H_d: haplotype diversity). **C.**Haplotype network based on 146 sequences of the *A. cephalotesmtCOI* gene. Numbers on the connecting lines denote the number of mutations.

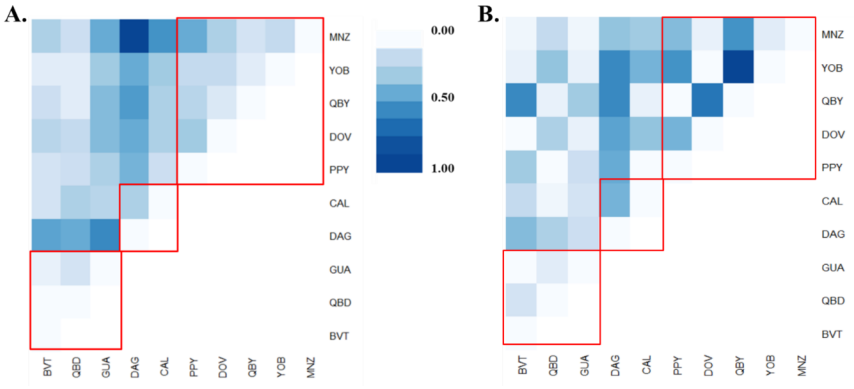

Figure 3. Heatmap for pairwise F_{ST} comparisons of *Atta cephalotes* populations. **A.** F_{ST} comparisons based on nuclear microsatellites and, **B.** Φ_{ST} comparisons based on *mtCOI* . Red squares denote intra-region comparisons.

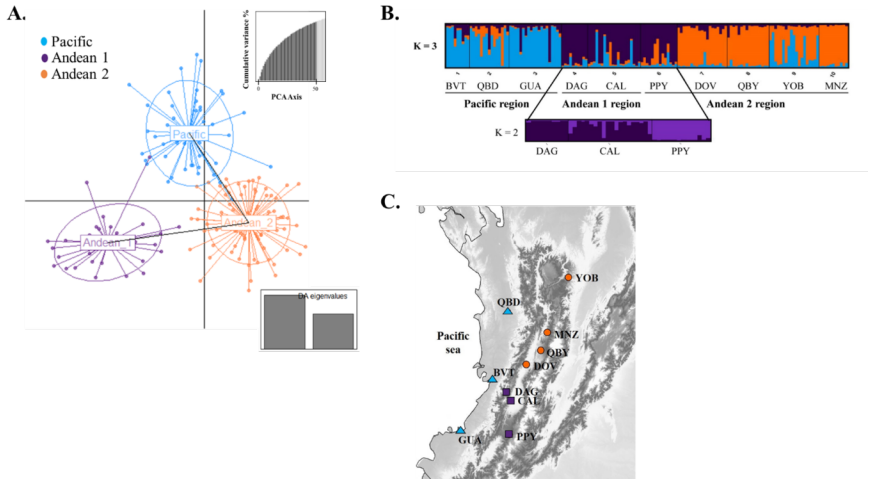

Figure 4. Population structure of *Atta cephalotes* as obtained from the STRUCTURE and DAPC analyses. **A.**Discriminant analysis of principal components (DAPC) scatterplot between samples. The key describes the colors attributed to each region and inertia ellipses describe the general distribution of points. Eigenvalues for each PC axis are shown (PC1, vertical; PC2, horizontal). The number of PCA axes retained in each DAPC analysis is shown in the bottom-right inset (gray bars). **B.** Multilocus genotype clustering of *A. cephalotes* populations, using STRUCTURE and following Evanno et al. (2005). Each region is characterized by color and each location is represented by a vertical bar. Locations are organized by region, starting with Pacific (BVT – GUA) followed by Andean 1 (DAG and CAL), and Andean 2 (PPY – MNZ). A different simulation was run for the southern cluster. **C.** Map for sampling localities according to the STRUCTURE clustering: Pacific (BVT, QBD, and GUA in blue), Andean south (DAG, CAL, and PPY in violet), and Andean north (DOV, QBY, YOB, and MNZ in orange).

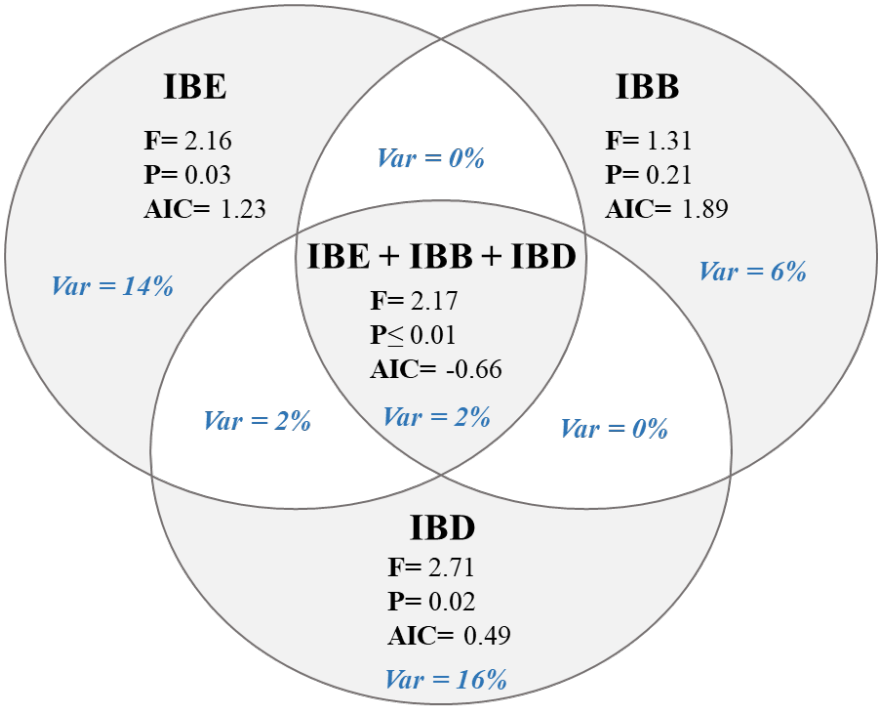

Figure 5. Variation partitioning and model comparison of isolating mechanisms driving nuclear genetic differentiation (F_{ST}) in *Atta cephalotes*. Variation partitioning analysis based on RDA results of the full model (IBE + IBB + IBD) into environmental (IBE: temperature and precipitation), barrier (IBB: Andes mountains), and spatial (IBD: geographic distance) components. Each circle represents the variation explained for each mechanism, and their overlap is the fraction of shared variation. Model selection and significance are indicated by the AIC and *P*-values. The IBE model remained significant after controlling for IBD (Conditioned model: F: 1.66, *P*: 0.04, AIC: 0.09), while IBB was not significant when accounting for IBD (Conditioned model: F: 1.49,*P*: 0.11, AIC: 0.56).