

Limited genetic differentiation of *Mycetomoellerius mikromelanos* in Parque Nacional Soberanía, Panama: Implications for queen dispersal

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Abstract

The coevolutionary relationship between fungus-growing ants (Formicidae: Attini: Attina) and their symbionts has been well studied in the Panamanian rain forests. To further understand the ecological context of these evolutionary relationships, we have examined the population-genetic structure of the fungus-growing ant species *Mycetomoellerius mikromelanos* Cardenas, Schultz, Adams 2021 in the Panama Canal Zone. We specifically investigated the presence of population structure, the significance of geographic features (i.e., creeks) limiting gene flow, and relatedness between ant colonies. To accomplish this, we genotyped 85 ant colonies from nine creeks across an approximately 30 km transect in Parque Nacional Soberanía, Panama, using double-digest restriction-site-associated DNA sequencing. We did not find distinct population structure using two genetic clustering methods; however, we did detect an effect of isolation by distance. Furthermore, related colonies were frequently detected on the same creek or neighboring creeks, and some at further geographic distances. Collectively, these findings demonstrate that new colonies tend to establish on natal creeks and occasionally on distant creeks following long-distance dispersal events. We discuss how population-genetic patterns reveal the natural history of *M. mikromelanos* in Parque Nacional Soberanía and how these results fit into the context of fungus-growing ant mutualisms.

Abstract in Spanish is available with online material.

KEYWORDS

dispersal, fungus-farming ants, fungus-growing ants, habitat preference, Panama Canal zone, Parque Nacional Soberanía, population genetics

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1 | INTRODUCTION

The Panamanian Isthmus has been an important location for both historical and contemporary movement of biota across the Americas (O'Dea et al., 2016). Its formation separated marine communities (Lessios, 2008), influenced oceanic currents and global climate (Haug et al., 2001; Lear et al., 2003), while also facilitating the interchange of terrestrial biota between the northern and southern continents (Bacon et al., 2013, 2015; Marshall et al., 1982; Winston et al., 2017). The construction of the Panama Canal in 1913 altered the surrounding forests while also creating Lake Gatun (Salgado et al., 2020) and Barro Colorado Island (BCI). In 1923, BCI became a nature reserve, and the Smithsonian Tropical Research Institute (STRI) established a biological research station shortly thereafter. Forest protections expanded in 1981 with the establishment of Parque Nacional Soberanía (Soberanía hereafter), which preserves two-thirds of the forested watershed east of the canal (Bagley & Johnson, 2014; Condit et al., 2001; Smythe, 1984). Soberanía offers access to pristine forests in its northern area and secondary forests to the south (Condit et al., 2011). The forests of the Panama Canal have been the focus of intense biological research for over 100 years (e.g., Dyar, 1914; Wheeler, 1925) with ants being an important focal taxon due to their conspicuousness and dominant ecological role in neotropical ecosystems (Hölldobler & Wilson, 1990; Nygaard et al., 2016; Schultz et al., 1998, 2002; Weber, 1940).

The fungus-growing ants (Hymenoptera: Formicidae: Attini: Attina; Ward et al., 2015) are diverse and abundant in Panama (Kaspari, Pickering, Longino, & Windsor, 2001; Kaspari, Pickering, & Windsor, 2001) and likely radiated from South America into North America due to ecological changes (Branstetter et al., 2017; Ješovnik et al., 2017; see also Barrera et al., 2021) and the success of their obligatory ant-fungal mutualisms (Mueller et al., 1998; Nygaard et al., 2016; Schultz & Brady, 2008). In addition, a broad network of complex symbioses evolved alongside the ant-fungal mutualism including social parasites (Adams et al., 2012), parasitoid wasps (Pérez-Ortega et al., 2010), fungal parasites (Currie et al., 1999, 2003), and bacterial mutualists (Sapountzis et al., 2019).

Studies on the population structure of fungus-growing ants can provide valuable context for understanding the factors driving their dispersal and ultimately their evolution with their symbiotic species networks (Caldera & Currie, 2012; Gerardo & Caldera, 2007; Hakala et al., 2019; Mikheyev et al., 2008; Mueller et al., 2017, 2018; Shik et al., 2021). This can provide a framework for future studies exploring the eco-evolutionary feedback between ants and their symbionts. Along with these symbiotic relationships, other factors should be considered that shape ant population structure, including historical land usage in Soberanía, metabolic limitations of dispersal by queens, ability to compete for resources, and abiotic factors (e.g., wind; Bruna et al., 2011; Helms & Godfrey, 2016; Kaspari, 2000; Suni & Gordon, 2010; Vogt et al., 2000). Studies of the ecological pressures that generate population structure can shed light on patterns of gene flow and isolation in natural populations and help our understanding of the potential for populations to adapt to

environmental change (Donoso et al., 2022; Kawecki & Ebert, 2004; Lenormand, 2002; Wang & Bradburd, 2014).

Habitat preferences and dispersal abilities are likely key synergistic factors that shape the genetic structure of fungus-growing ant *Mycetomoellerius mikromelanos* Cardenas et al., 2021. Drivers of dispersal (e.g., competition, parasite escape, and cost of inbreeding; Brown & Brown, 1992; Ronce, 2007) have not yet been identified; however, dispersal distances for this species are expected to be relatively short, ca. 0.1–1.0 km based on queen morphology (table 2 in Cardenas et al., 2021; see figure 3 in Helms, 2018), as has been shown in other ant species (Mikheyev, 2008; Rissing & Pollock, 1986; Sundström et al., 2005). *Mycetomoellerius mikromelanos* colonies (Figure S1) are typically found along creek embankments at high densities in Soberanía but are not abundant in the forest around the creek (see also supplemental extended data 10 in Shik et al., 2021; and Cardenas et al., 2021 for species identity). Consequently, creeks are likely important dispersal corridors for *M. mikromelanos* and thus important geographic features for gene flow. Despite the abundance of seemingly suitable micro-habitat (e.g., exposed vertical soil) along creeks, colony establishment can be fragmented. The heterogeneous distribution of ants may be due to intrinsic factors, environmentally driven extrinsic factors, or the presence of antagonistic symbionts (Combes, 2005; Doeschl-Wilson et al., 2012; Leihy & Chown, 2020; Lowe & McPeck, 2014; Ronce, 2007).

Our main objective is to investigate the genetic structure of *M. mikromelanos* to provide insight into patterns of dispersal and connectivity in Soberanía. With limited dispersal and micro-habitat preferences, creeks may act as corridors limiting the gene flow of *M. mikromelanos*. We hypothesize that populations are finely structured across Soberanía, and key geographic features, such as creeks and watersheds, drive overall population structure. From this hypothesis, we predict high relatedness between ant colonies found along the same creek compared with colonies from different creeks. To accomplish this, we use double-digest restriction-site-associated DNA sequencing data (ddRADseq; Peterson et al., 2012) to study the population-genetic structure of *M. mikromelanos* colonies across nine creeks over a 30km transect in the Panama Canal Zone. We answer the following questions: (a) Are there distinct genetic populations in the Panama Canal Zone? (b) Do populations conform to isolation by distance? (c) Do geographic features, such as creeks, limit fine-scale gene flow in these populations?

2 | METHODS

2.1 | Collection methods

To collect *M. mikromelanos*, we sampled worker ants from colonies along nine creeks across a ca. 30km-wide region in the Panama Canal Zone in May 2017 and June–July 2018 (Figure 1). Sampling efforts were focused in Soberanía near Gamboa (Table S1). We located nest entrances for each colony along creek embankments and then collected ants either by luring out workers from their underground

nest using polenta and oats as bait or by extracting ants from excavated nests. We collected 10–15 workers in 95% EtOH from multiple colonies from each creek totaling 132 colonies.

2.2 | Genomic data collection and bioinformatics

We chose one worker ant from each of the 132 colonies for our genomic data collection because the genotype of a single worker ant is a strong representation of the genotype of the colony (see also, Doums et al., 2002; Hakala et al., 2020; Janda et al., 2016; Ueda & Itino, 2017). This is because *M. mikromelanos* colonies have a single queen that mates once (i.e., monandry; Villesen et al., 2002). In monandrous haplodiploid reproductive systems, relatedness among offspring is high due to shared haploid genes among offspring.

We extracted genomic DNA from crushed worker ants using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. We quantified DNA and adjusted the concentration to 200ng of DNA for each sample. We modified the ddRADseq protocol from Peterson et al. (2012) for restriction-site-associated DNA sequencing using enzymes *Msp*I and *Spf*I-HF (New England Biolabs, Inc., Ipswich, Massachusetts, USA). For library preparation, we selected genomic fragments (250–600bp) using a Blue Pippin (Sage Sciences, Inc. Beverly, Massachusetts, USA), cleaned the genomic libraries, and amplified them according to standard Illumina multiplexed paired-end sequencing protocols. Our libraries were sequenced at Nationwide Children's Hospital in Columbus, Ohio on an Illumina HiSeq 4000 that produced 150bp paired-end reads.

We used a combination of bioinformatic software to process the genomic read data and generate two types of single nucleotide polymorphism (SNP) files, one that filtered out loci violating

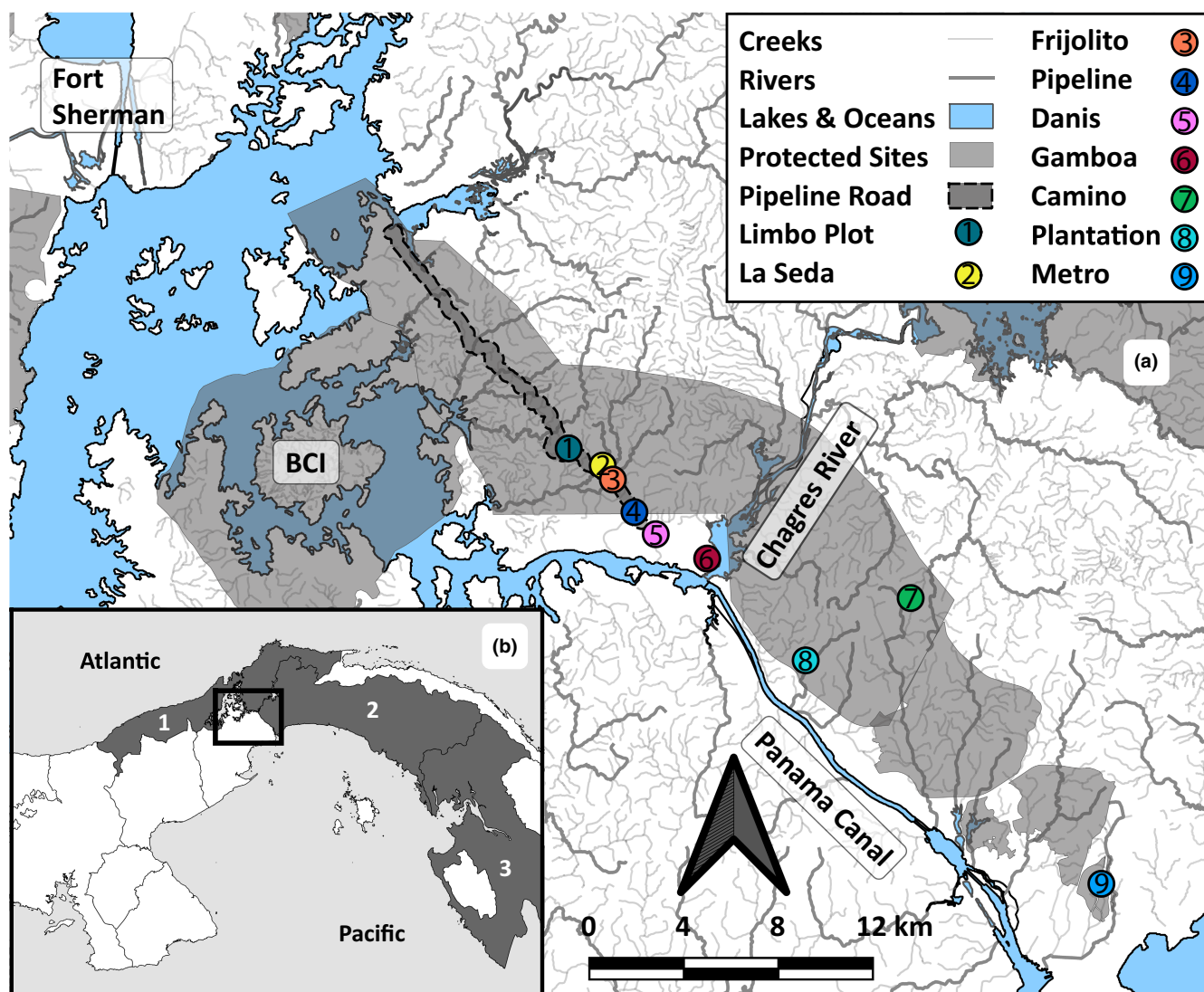


FIGURE 1 (a) Sampling localities for *M. mikromelanos* from creeks in the Canal Zone. Fort Sherman in the upper left, Barro Colorado Island (BCI) center left, Chagres River center, and the Panama Canal are labeled. Protected areas such as Soberanía National Park are indicated by dark gray shaded areas. (b) Inset shows Panama with the three provinces from east to west (1) Colón, (2) Panamá, and (3) Darién shaded in dark gray denoting where *M. mikromelanos* has been reported to occur (Cardenas et al., 2021).

Hardy–Weinberg Equilibrium (HWE) and another without filtering based on HWE. We produced these different SNP files because haplodiploidy systems may not conform to observed genotype distribution under HWE expectations (Amorim & Pinto, 2022; Pearman et al., 2022; Waples, 2015). We used both the unfiltered and filtered dataset for calculating the summary statistics, but only used the filtered dataset for the rest of the downstream analyses related to estimating genetic structure and colony relatedness. To generate the datasets, we first used *FastQC* (v0.11.7; Andrews, 2018) to review the quality of the raw sequencing reads. We then used *trimmomatic* (v0.38; Bolger et al., 2014) to remove low-quality sequencing reads (Phred quality score < 25) using a sliding window and only including sequences that were > 100 bp in length. We then used *ipyrad* (v0.9.31; Eaton & Overcast, 2020) for SNP discovery (see Supporting information for *ipyrad* parameters). In *ipyrad*, we removed poor-quality sequences with less than one million raw reads and removed samples from Limbo Plot and La Seda that were overrepresented in the dataset compared with other sites. This reduced our sample size to 85 colonies from nine creeks (eight colonies per site on average; see Frantz et al., 2009; Schwartz & McKelvey, 2009; Sánchez-Montes et al., 2017). Next, we used the *R* (v3.6; RStudio Team, 2018; v1.2; R Core Team, 2019) package *radiator* (v1.5.5; Gosselin et al., 2020) with the interactive filtering function to remove loci and SNPs (see Supporting Information for all filtering parameters). The unfiltered dataset was generated in *radiator* prior to the step for HWE filtering. We did not use *radiator* to generate a dataset that filtered out loci that violated HWE because it requires a minimum number of samples to calculate HWE from each site (creek). Instead, we generated the HWE-filtered dataset using the *R* package *pegas* (v0.12; Paradis et al., 2010).

2.3 | Summary statistics

We quantified genetic diversity of our filtered and unfiltered datasets by calculating multiple summary statistics in the *R* package *hierfstat* (v0.04–30; Goudet, 2005), including observed (H_o) and expected heterozygosity (H_e), inbreeding coefficient (F_{is}), and pairwise fixation indices (F_{st}) with the *R* package *hierfstat*. Additionally, we calculated size-corrected F_{st} and G_{st} statistics based on recommendations by Meirmans and Hedrick (2011).

2.4 | Isolation by distance

We searched for patterns of isolation by distance (IBD) across all colonies using a Mantel test with the filtered dataset in *poppr* (v2.8.3; Kamvar et al., 2014, 2015). To calculate pairwise geographic distances, we determined the Mercator coordinates for all samples for each creek (see Table S1) with *geosphere* (v1.5–10; Hijmans, 2019). We performed a Mantel test with 1000 replicates with the previously calculated pairwise F_{st} values and a matrix of geographic distances between each creek.

2.5 | Population structure

We estimated population-genetic structure with two different approaches to account for the potential statistical challenges of estimating structure in the presence of IBD. First, we estimated population structure without accounting for IBD using *STRUCTURE* v2.3.4 in the *ipyrad* application programming interface (API) analysis tool kit (v0.9.31 Pritchard et al., 2000; Falush et al., 2007; Eaton & Overcast, 2020). For this analysis, we used an SNP dataset that required each locus to be represented in at least 50% of our colonies from every creek, and of those loci, at least 75% were globally shared across all colonies. We then ran *STRUCTURE* for clustering values ranging from $K = 2$ to $K = 12$ with 30 replicates and with 1×10^6 Monte Carlo Markov Chain (MCMC) steps following a burn-in period of 5×10^5 for each run. From this result, we used the Evanno method to determine the optimal K value and *CLUMPP* to calculate membership probabilities (Evanno et al., 2005; Jakobsson & Rosenberg, 2007).

We also estimated the population structure that accounts for IBD using *conStruct* (v1.0.4; Bradburd et al., 2018) with the filtered dataset. This program models the number of ancestral populations with an explicit assumption that geographic distance influences genetic differentiation. It is important to note that *conStruct* performs poorly with missing data, and preliminary analyses with SNP matrices containing missing data resulted in poor models. Because of this, we calculated the center of all samples from a creek using *geosphere* (v1.5–10; Hijmans, 2019) and then the allele frequency for all colonies from each creek using *adegenet* (v2.1; Jombart, 2008; Jombart & Ahmed, 2011). We then modeled clustering values ranging from $K = 1$ to $K = 4$ with 2000 MCMC iterations and five chains using both spatial and non-spatial models. We checked diagnostic trace plots to confirm convergence and mean probability distribution for each iteration. Additionally, we tested the models using *conStruct*'s cross-validation method with a 90% training proportion, 2000 MCMC iterations, and 10 replicates for clustering values ranging from $K = 1$ to $K = 4$. Lastly, we tested whether the difference between the spatial and non-spatial models cross-validation results was statistically significant using a paired t-test.

2.6 | Analysis of molecular variance

We investigated patterns of geographic partitioning of genetic variation among regions, watersheds, and creeks using a hierarchical analysis of molecular variance (AMOVA) with our filtered SNP dataset (1133 SNP) in *poppr* (v2.8.3; Kamvar et al., 2014, 2015). For this analysis, we defined strata at three levels: (a) colonies north or south of the Chagres River, (b) colonies located within watersheds, and (c) colonies within creeks. We compared genetic variances among (a) watersheds, (b) between colonies within a watershed, and (c) variation of all colonies. We placed particular emphasis on the Chagres River because it serves as a natural divide that separates creeks and watersheds along the transect (Figure 1 and Figure S2). To test the significance of these strata,

we used *ade4*'s *randtest* (v1.7–13; Chessel et al., 2004; Dray & Dufour, 2007; Dray et al., 2007; Bougeard & Dray, 2018) function with 1000 permutations. This allows for a significance test of genetic variation between colonies, between colonies within creeks, between creeks within the watersheds, between watersheds to the north and south of the Chagres River, and between colonies to the north and south of the Chagres.

Given our hypothesis that creeks and watersheds influence gene flow, we also performed two additional AMOVAs without hierarchy, with one considering only watersheds and the other only creeks. These were performed because the hierarchical analysis does not explicitly compare only creeks to each other, and watersheds are interconnected creeks. Lastly, to reduce the risk of Type I error from multiple tests (11 total comparisons), only measurements with a Bonferroni corrected p -value ($p < .0045$) were considered significant. As an additional note, AMOVA calculates ϕ statistics (ϕ_{st}), which are comparable to fixation indices allowing for an additional measure of population structure (see Excoffier et al., 1992; Michalakis & Excoffier, 1996).

2.7 | COLONY analysis

We estimated contemporary relationships of all colonies (i.e., sibship reconstruction; see Blouin, 2003; Jones et al., 2010) to better understand creek fidelity. We inferred relatedness using the filtered dataset in the program COLONY (v2.0.6.5; Jones & Wang, 2010). COLONY assumes individuals are from a large and randomly mating population and sorts out individuals belonging to the offspring category into the most probable full-sib groups. We used COLONY solely for inferring relatedness between colonies because it can accurately estimate relatedness in haplodiploid reproductive systems without including genotypes of the parents. Consequently, we disregarded the interpretation of the pedigree results. With all colonies as offspring, we ran COLONY with five “long runs” and a full-likelihood analysis as advised by Jones and Wang (2010). Allele frequencies were updated throughout each run, and we considered inbreeding absent based on the summary statistics. In COLONY, we specified that females and males are monogamous. Monogamy was previously determined in *M. mikromelanos* gynes (Villesen et al., 2002) and the current consensus for ants is that males are as well (Hölldobler & Wilson, 1990; but see: Baer & Boomsma, 2004; Shik et al., 2012). We interpreted the full-sibling dyad output, because the other dyads (e.g., half-sibling) are not calculated without samples categorized as parents.

3 | RESULTS

3.1 | Sequencing and filtering

High throughput sequencing of the ddRADseq fragments resulted in 490.7 million reads. After demultiplexing and thinning samples, there were approximately 211.7 million raw reads for 85 colonies

(averaging 2.5 million reads each) from nine sampling sites (creeks). Locus discovery and genotype calling with *ipyrad* resulted in 6447 loci in total, averaging 4165 SNPs (s.d. 622) per colony, and 16,409 total SNPs. During filtering in *radiator*, 11 colonies were removed leaving 74 colonies ($N = 74$). The unfiltered dataset retained 1805 loci, 2482 unlinked SNPs. After filtering for HWE, the filtered dataset retained 937 loci and 1113 unlinked SNPs from 74 colonies.

3.2 | Summary statistics

The observed heterozygosity from the unfiltered dataset was much lower than the expected heterozygosity for colonies from each site (Table 1a). The unfiltered dataset also showed high proportions of F_{is} , indicating high levels of inbreeding for each creek. Nei's F_{st} was high (0.2802), which indicated moderate levels of population differentiation between colonies (Table 1b; see also Figure S3a). However, the fixation indices calculated using Hedrick's G'_{st} (0.1268), which considers the number of potential populations, were much lower than Nei's F_{st} (G'_{st} 0.1268, F_{st} 0.2802; Table 1b). This is likely due to an insufficient number of heterozygous loci observed, as shown in the inbreeding coefficient ($F_{is} = 0.4084$; Table 1b). The filtered data show that the heterozygosity observed was much closer to heterozygosity expected with a small excess of heterozygous loci ($F_{is} = -0.0664$; Table 5b), while both fixation indices are comparable and low (G'_{st} 0.1101, F_{st} 0.1176; Table 5c), suggesting weak population differentiation (See also Figure S3b). Pairwise Nei's F_{st} calculations between nine creeks ranged from 0.0052 to 0.1954 (Table 1c).

3.3 | Isolation by distance

Plotting the pairwise F_{st} values as a function of geographic distance shows a positive correlation (Figure 2). Moreover, the Mantel test of 74 colonies of *M. mikromelanos* from across ca. 30 km of the Panama Canal Zone shows some effect of IBD ($r = 0.6555$, $p = .0369$). Both of these results indicate there is an effect of IBD on colonies sampled.

3.4 | Population structure

For our STRUCTURE analysis, we obtained a 74×624 SNP matrix representing 74 colonies and 624 SNPs that contains 4.32% missing values. The STRUCTURE analysis suggests an optimal $K = 6$ based on delta K (Table 2, 7.49). However, the estimated log probability of the mean was highest at $K = 10$ (Table 2, -10240.2) and the lowest estimated log probability of the standard deviation indicated $K = 5$ (Table 2; 205.82). Sample assignment probability plots do not result in clear population clusters (see Cullingham et al., 2020), and the most conservative representation of our STRUCTURE analysis is $K = 2$ or $K = 1$ (Figure 3a; for all K values see Figure S4).

Estimating allele frequencies for each creek resulted in a 9×1113 SNP matrix representing all nine creeks with no missing

TABLE 1 (a) Percentage of loci in Hardy–Weinberg equilibrium (HWE), heterozygosity observed (H_o), heterozygosity expected (H_s), and inbreeding coefficient (F_{is}) of creeks where $N = 74$ *M. mikromelanos* colonies were collected.

a									
Creek	N	HWE1	H_o1	H_s1	$F_{is}1$	HWE2	H_o2	H_s2	$F_{is}2$
Limbo Plot	15	0.8307	0.0587	0.1351	0.5657	0.9947	0.0555	0.0529	−0.0474
La Seda	11	0.9089	0.0435	0.0962	0.5474	0.9947	0.0434	0.0485	0.1034
Frijolito	13	0.8025	0.0606	0.1538	0.6061	0.9929	0.0495	0.0533	0.0703
Pipeline	4	0.9705	0.1376	0.1556	0.1159	0.9981	0.1000	0.0867	−0.1527
Dani's	5	0.9778	0.0714	0.1677	0.5746	0.9982	0.0421	0.0436	0.0355
Gamboa	8	0.9399	0.0808	0.1146	0.2953	0.9867	0.0636	0.0556	−0.1453
Camino	6	0.9632	0.0608	0.0847	0.2815	0.9982	0.0672	0.0622	−0.0807
Plantation	8	0.8658	0.0684	0.1472	0.5353	0.9885	0.0720	0.0699	−0.0294
Metro	4	0.9867	0.1178	0.1392	0.1534	0.9920	0.1137	0.0989	−0.1501
b									
		H_o		H_s		Nei's F_{st}		Hedricks G'_{st}	F_{is}
unfiltered		0.0770		0.1313		0.2802		0.1268	0.4084
Filtered		0.0674		0.0632		0.1101		0.1176	−0.0664
c									
	La Seda	Frijolito	Pipeline	Dani's	Gamboa	Camino	Planation	Metro	
Limbo Plot N = 15	0.0601 1.6	0.1007 2.2	0.1552 3.8	0.0941 5.1	0.0749 7.5	0.0911 15.8	0.1740 13.5	0.1954 29.2	
La Seda N = 11	-	0.0537 0.7	0.1074 2.4	0.0794 3.7	0.0727 5.9	0.1064 14.2	0.1108 11.9	0.1750 27.6	
Frijolito N = 13	-	-	0.0625 1.6	0.0137 2.9	0.0529 5.2	0.0540 13.5	0.0893 11.2	0.1329 26.9	
Pipeline N = 4	-	-	-	0.0825 13.0	0.1164 3.6	0.0940 12.2	0.0697 9.6	0.0091 25.3	
Dani's N = 5	-	-	-	-	0.1155 2.4	0.0986 11.1	0.0991 8.3	0.1521 24.0	
Gamboa N = 8	-	-	-	-	-	0.0764 8.7	0.1410 6.0	0.1521 21.7	
Camino N = 6	-	-	-	-	-	-	0.1098 5.1	0.1457 14.6	
Plantation N = 8	-	-	-	-	-	-	-	0.1516 15.7	
Metro N = 4	-	-	-	-	-	-	-	-	

Note: Creeks are ordered north to south. The unfiltered dataset is presented by HWE1, H_o1 , and H_s1 and the filtered data (HWE2, $H_o2...$).

(b) Summary statistics of unfiltered *M. mikromelanos* SNP data and filtered data. (c) Pairwise F_{st} calculation from the filtered data of the nine creeks sampled; distances in km are included in bold.

data. Our *conStruct* analysis revealed that the optimal K is 1. The diagnostic posterior probability trace plots all converge on a similar stationary mean probability distribution. Except for the spatial chain three for $K = 1$ (See Appendix S1), which has a bimodal distribution indicating it did not converge, all trace plots were acceptable. Layer contributions (Bradburd et al., 2018) show that layers above $K = 1$ contribute little to the overall covariance in these models (Figure 3b). The cross-validation analysis reveals that the optimal K value from the spatial analysis is either $K = 2$ or 3.

However, neither K 's mean predictive value is significantly different (Figure 3c,d; confidence interval ranges $K2$: −0.59 to −0.42 and $K3$: −0.55 to −0.38; the paired t-test $K2$: $t = -0.28446$, $df = 9$, $p = .6088$ $K3$: $t = -0.34728$, $df = 9$, $p = .6318$; see also Supporting Information Paired t-test). Examining the *conStruct* admixture plots (Figure 3e) for $K = 2$ shows that Pipeline Road, Plantation, and Parque Metropolitano have different ancestral proportions, and $K = 3$ and 4 follow a similar trend while Camino and Limbo Plot also show some difference, albeit small.

3.5 | Analysis of molecular variance

The AMOVA findings for the hierarchical stratification of geographic scales of 74 *M. mikromelanos* colonies show that a significantly large amount of genetic variation and population differentiation comes from all colonies (86.63%, $\phi = 0.1336$, $p = .0009$, Table 3a), indicating a largely panmictic population. The ϕ statistic from all colonies indicates some population differentiation, but not high enough for population structure. A statistically significant yet small amount of variation exists between creeks from Limbo Plot, Frijolito, and La Seda that share a watershed (Table S2). However, this only accounts for a small proportion of total variation and small amount of population differentiation (6.97%, $\phi = 0.0739$, $p = .0009$, Table 3a). Other

small and nonsignificant amounts of variation came from all other hierarchical stratifications.

The AMOVA results of creeks and watersheds also support a broadly panmictic population. A significant majority of genetic variation comes from all colonies (Watershed AMOVA: 86.05%, $\phi = 0.1395$, $p = .0009$, Table 3b; Creek AMOVA: 87.63%, $\phi = 0.1237$, $p = .0009$, Table 3c). The ϕ statistic of both indicates some population differentiation from colonies, but not enough for population structure, as expected from a panmictic population. The genetic variation and differentiation between watersheds are significant, but the genetic variation explained is low (10.28%, $\phi = 0.1028$, $p = .0009$; Table 3b). Similarly for creeks, genetic variation and differentiation are significant but with low genetic variation (11.35%, $\phi = 0.1395$, $p = .0009$; Table 3c). Both AMOVA findings between watersheds and between creeks show nonsignificant genetic variation coming from colonies on watersheds and creeks, respectively.

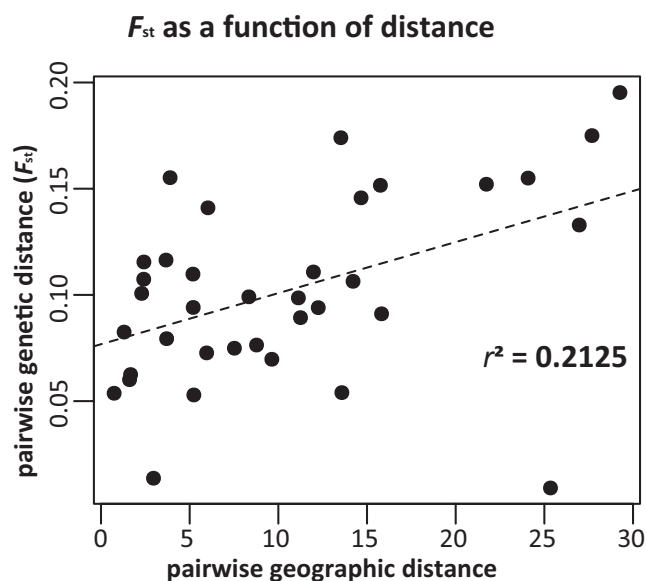


FIGURE 2 Plot of pairwise genetic distance (F_{st}) of nine creeks as a function of pairwise geographic distance in km ($r = 0.6555$, $p = .0369$) for 74 colonies from the Canal Zone. Dashed line is the linear model, $r^2 = 0.2125$.

3.6 | COLONY analysis

The COLONY analysis shows that a total of 51 of the 74 *M. mikromelanos* colonies have a high probability of relatedness. There are 93 relationships identified between colonies with a probability greater than 50%, indicating that nearly two-thirds of the colonies sampled have some degree of relatedness. Of the relationships identified, only two have probabilities less than 75% (2 of 93) and 64 have 100% probability (64 of 93; Figure 4). Over half of the relationships identified occur from colonies on the same creeks (56 of 93), but some of the identified relationships occurred between different creeks (37 of 93). For example, colonies sampled on Limbo Plot have a high probability of relatedness to colonies from Rio La Seda (ca. 1 km distant), Dani's Creek (ca. 5 km distant), and Gamboa (ca. 7 km distant; see Figure 4). Dani's Creek also has a high probability of relatedness to Rio Frijolito (ca. 3 km distant) and Rio La Seda (ca. 4 km distant). These creeks share watersheds or are on

TABLE 2 STRUCTURE analysis run using the *iPyRAD* method with filtered data and 30 replicates for K 2:12 presented as a table.

K	lnPK	lnPPK	deltaK	estLnProbMean	estLnProbStdev
2	0	0	0	-14710.2	213.67
3	1758.05	625.43	1.96	-12952.1	318.89
4	1132.62	713.61	2.53	-11819.5	281.50
5	419.01	12.43	0.06	-11400.5	205.82
6	406.57	1673.41	7.49	-10993.9	223.31
7	-1266.83	1543.45	0.26	-12260.7	5872.91
8	276.62	1161.05	0.21	-11984.1	5515.92
9	1437.67	1131.43	2.88	-10546.4	392.18
10	306.24	2769.22	6.58	-10240.2	420.87
11	-2462.98	2862.47	0.39	-12703.2	7268.27
12	399.49	0	0	-12303.7	5241.57

Note: The most likely deltaK, estimated log probable mean, and standard deviation based on our data are in bold.

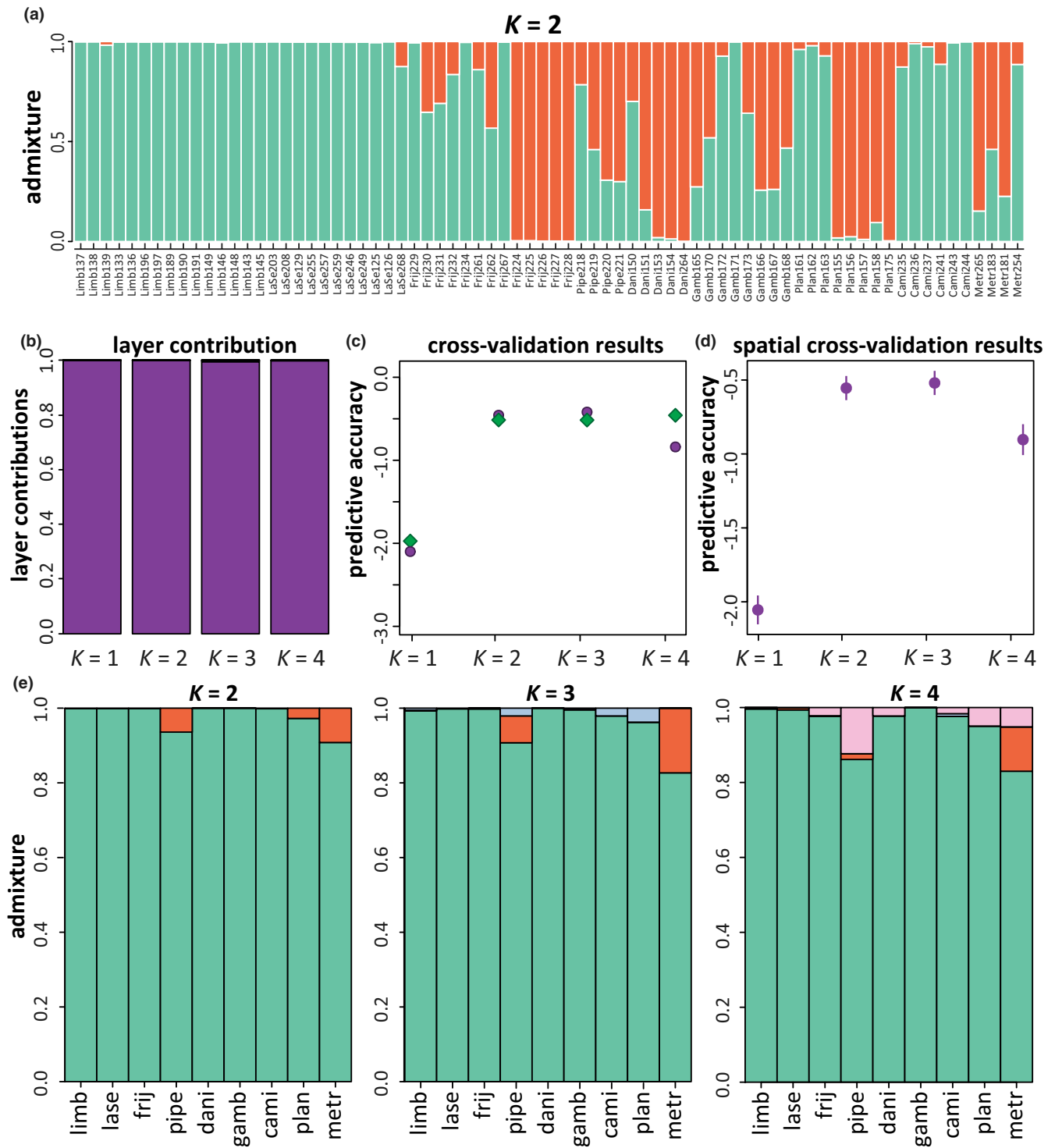


FIGURE 3 Results of clustering methods with *STRUCTURE* and *conStruct* using the filtered dataset of 74 *M. mikromelanos* samples from the Canal Zone. (a) The most probable *K*-value as determined by the magnitude of the calculated delta*K* from the *STRUCTURE* analysis. (b) Layer contributions calculated in *conStruct* from the allele frequencies of the nine creeks sampled. (c) Mean predictive accuracy of the spatial (purple circle) and non-spatial (green diamond) analysis in *conStruct* for *K* = 1:4. (d) Mean predictive accuracy of the spatial-cross-validation results with confidence intervals for the spatial model from the *conStruct* analysis. (e) The resulting spatial admixture plots for *K* = 2:4 using *conStruct*.

watersheds that are close in proximity. However, some colonies were found to be related to several colonies on separate watersheds (Dani's Creek, Gamboa, and Parque Natural Metropolitano).

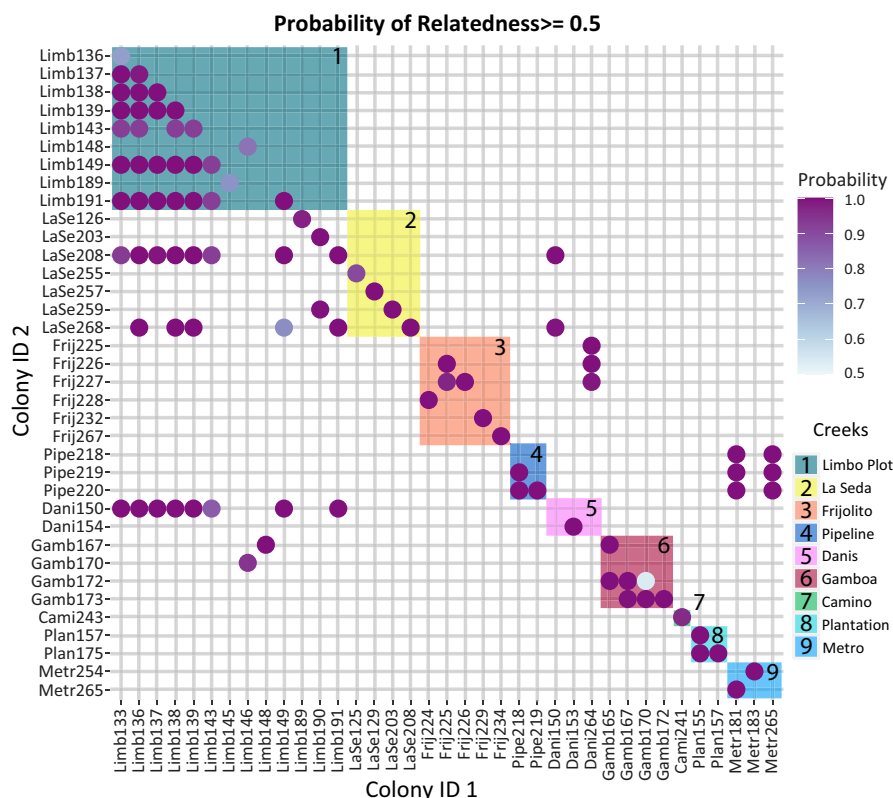
Lastly, colonies from Parque Natural Metropolitano are also related to multiple colonies on a different watershed, Pipeline Road, nearly 25km away.

TABLE 3 Results of the AMOVA of the filtered data with Bonferroni correction for multiple tests ($p < .0045$).

a	Hierarchical comparison of var.	df	σ	% var	ϕ	Alter	p
	N vs S of Chagres River	1	0.6376	0.0075	0.0075	Greater	.2247
	Watersheds within N vs S	5	4.1942	0.0494	0.0498	Greater	.2157
	Creeks within watersheds	2	5.9134	0.0697	0.0739	Greater	.0009
	Colonies within creeks	65	0.8502	0.0100	0.0114	Greater	.2717
	All Colonies	74	73.2453	0.8663	0.1336	Less	.0009
	Total	147	84.8409	1.0000			
b	Comparison of Watershed Var.	df	σ	% Var	ϕ	Alter	p
	Between watersheds	6	8.7515	0.1028	0.1028	Greater	.0009
	Colonies within watersheds	67	3.1269	0.0373	0.0410	Greater	.0460
	All colonies	74	73.2453	0.8605	0.1395	Less	.0009
	Total	147	85.1238	1.0000			
c	Comparison of Creek Variation	df	σ	% Var	ϕ	Alter	p
	Between creeks	8	9.4888	0.1135	0.1135	Greater	.0009
	Colonies within creeks	65	0.8502	0.0101	0.0115	Greater	.2877
	All colonies	74	73.245	0.8763	0.1237	Less	.0009
	Total	147	85.1238				

Note: (a) Hierarchical AMOVA for colonies, (b) an AMOVA of comparisons between watersheds of *M. mikromelanos*, and (c) an AMOVA comparison between creeks in the Canal Zone. Significant p -values are in bold.

FIGURE 4 Probability of relatedness between 51 of the 74 *M. mikromelanos* colonies sampled. Colored squares behind points represent creeks, are ordered from north to south, and are color coded to match Figure 1. Relatedness is presented in a pairwise dyad (pairs of colonies; see Blouin, 2003) as determined by the program COLONY. Circles indicate a sibling relationship greater than 50% with a gradient reflective of the probability of relatedness.



4 | DISCUSSION

Our investigation of the genetic variation of *M. mikromelanos* colonies within Soberanía revealed no fine-scale population structure

and limited support for our hypothesis that creeks and watersheds generate overall population structure. Consistent with the moderate pattern of IBD between ant colonies, we found higher probabilities of relatedness and low F_{st} values between ant colonies on the same

creeks or in watersheds. However, some closely related ant colonies were identified between distant creeks, suggesting occasional long-range dispersal by reproductive castes (queens or males). While geographic features such as creeks are biologically important due to the abundance of micro-habitat (i.e., vertical exposed soil), they do not generate a significant amount of genetic differentiation between *M. mikromelanos* colonies in Soberanía. Other factors (e.g., flight ecology, resource limitation, and abiotic features) should be considered, but our results still provide important groundwork. Future studies on intrinsic factors (such as metabolic limitations and sex-biased dispersal of reproductive castes) and extrinsic factors (e.g., habitat heterogeneity, resource availability, and human-driven deforestation) may better explain the genetic structure of ants in Soberanía. Additional work on this species and other fungus-growing ant systems, considering the eco-evolutionary feedback with other members of the symbiotic species network, would also be a fruitful scientific pursuit.

For ants, dispersal is a critical natural history trait prefacing colony foundation. We find indirect evidence that *M. mikromelanos* establishes nests on natal creeks, neighboring creeks, and within watersheds. This is supported by evidence of IBD and elevated inbreeding at the creek level. Our results of IBD and panmixia are comparable to larger and stronger flying fungus-growing ant species (Helmkamp et al., 2008; Mikheyev et al., 2008). While dispersal is limited in many ant species, some species have reported dispersal distances of 30 km (Helms, 2018; Rissing & Pollock, 1986; Ross et al., 1997; Seppä & Pamilo, 1995; Sundström et al., 2005; Suni & Gordon, 2010).

The lack of population structure in *M. mikromelanos* may be due to gene flow from occasional long-distance dispersal, just as theory predicts that only a few migrants prevent population differentiation (Slatkin, 1987). Gene flow in fungus-growing ants depends on (1) male and female reproductive dispersal, (2) success in colony and garden establishment, and (3) colony growth levels that result in the dispersal of new reproductives. Flight capability is correlated with reproductive morphology and nest founding strategy. Queens that forage during nest establishment (non-claustral founding, like *M. mikromelanos*) may be capable of flying longer and farther due to fewer morphological and metabolic limitations (Hakala et al., 2019; Helms, 2018). However, queen morphology of *M. mikromelanos* suggests short flight distances (in ants flight distances increase with body size, Helms, 2018), yet we still found evidence of occasional long-distance flight. Wind could also be an influencing factor, as is the case in some ant species (Helms, 2018; Vogt et al., 2000). Prevailing winds are strongest near Gamboa in the wet season when *M. mikromelanos*' reproductive are mating and dispersing (Cardenas et al., 2021; Cheng & Georgakakos, 2011). Successful nest establishment, leading to healthy garden growth, and the production of male and female reproductives also influence population-level gene flow. The ants and the mutualistic fungus-garden must thrive in the micro-habitat where a queen starts her nest for the colony to reproduce. Detrimental outcomes when dispersing beyond suitable habitat may lead to the nesting patterns that we observe in Soberanía (i.e., aggregations of young and old colonies on the creek banks and few in nearby forested habitat). Future work on

flight ecology, sex-biased dispersal, flight times, and altitude (see Doums et al., 2002; Helms, 2018; Johansson et al., 2018; Matthews et al., 2021; Sanllorente et al., 2015; Shik et al., 2012) will further elucidate gene flow between colonies in the Canal region.

The lack of population structure in *M. mikromelanos* is also a strong basis for predicting the population dynamics of their mutualists. (Currie et al., 1999; Wallace et al., 2014). For example, the population structure of the symbiotic fungal cultivars should reflect the ant's population structure because they are vertically transmitted by dispersing queens (Mikheyev et al., 2010; but see also Adams et al., 2000; Green et al., 2002; Mikheyev et al., 2008). However, the successful founding of ant colonies does not solely rely on the fungus symbiosis but also on the ecological conditions they have dispersed into. The presence of garden pathogens and host ant parasites may also decimate a colony or limit the production of reproductive castes (Adams et al., 2012; Currie et al., 1999; Pérez-Ortega et al., 2010; Wallace et al., 2014).

The rain forests of the Panamanian isthmus have a gradient of wet and less disturbed forests near the Atlantic coast (e.g., Fort Sherman, Figure 1) to drier and more disturbed forests near the Pacific (Condit et al., 2001; Curtis et al., 2021; Griscom & Ashton, 2011). We only found *M. mikromelanos* in the southern forests of Soberanía (Figure 1). Surveys in wetter forest types like Fort Sherman in the north, and even BCI (Figure S5), were unsuccessful. The damming of the Chagres River in 1913 and the building of the Panama Canal created large-scale perturbations that altered surrounding forests due to flooding and deforestation, resulting in the potential for subpopulation isolation. Barro Colorado Island has a mix of wet and drier forest types that likely cause micro-habitat features that influence ant colony success (Condit et al., 2011; Curtis et al., 2021). While we could not find *M. mikromelanos* along numerous creeks on the island, colonies have occasionally been reported (Donoso, personal communication). We argue that it is reasonable to expect that ants are capable of dispersing to BCI from the mainland because of the short distance (ca. 0.5 km) between them and because of our inferences to occasional long-range dispersal (Figures 1 and 4). However, we surmise through our field observations that *M. mikromelanos* prefers more disturbed and drier habitats, like what is found in southern Soberanía, as opposed to the less disturbed habitats found in BCI and the northern wet forests. Further studies into the habitat preferences of *M. mikromelanos* will help explain the heterogeneous distribution of colonies and potentially reveal drivers of long-distance ant dispersal. Comparative ecological studies in northern and southern forest types outside of well-studied sites like BCI in Panama would be useful to address this question (see Castilla et al., 2016).

5 | CONCLUSION

We have found that colonies of *M. mikromelanos* in the Panama Canal Zone belong to a nearly panmictic population with evidence of creek fidelity and genetic structuring at a relatively small scale

(30 km). We described the genetic variation between colonies using over 1100 neutral SNPs. We found that many colonies on the same or neighboring creeks were closely related, while a few colonies had a high probability of relatedness across our entire sampling range. This suggests that reproductive individuals tend to remain close to their natal creeks, yet occasional long-range dispersal does occur. This work currently stands as the first examination of the population genetics of *M. mikromelanos*. Broader sampling and interpretations of the landscape processes in Panama and throughout Central America would clarify the population dynamics of this species and their symbiotes while providing new insights for other creek-dwelling species.

AUTHOR CONTRIBUTION

CRC, AJM, ASC, and RMMA conceptualized the study, contributed to the methodology, validated the study, and contributed to the writing—original draft and writing—review and editing. CRC, AJM, and RMMA curated the data, investigated the study, and supervised the study. CRC and AJM contributed to the formal analysis and software, and visualized the study. CRC and RMMA acquired the funding and contributed to the project administration and resources.

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CONFLICT OF INTEREST

The corresponding authors declare, on behalf of all authors, there are no conflicts of interests that may raise the question of bias in the work presented.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.0k6djhb3g> (Cardenas et al., 2022).

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