Received: 1 November 2018 | Revised: 25 March 2020 | Accepted: 28 April 2020

DOI: 10.1111/mec.15468

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

The socially parasitic ant *Polyergus mexicanus* has host-associated genetic population structure and related neighbouring colonies

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Funding information
University of California, Santa Cruz Department of Ecology and Evolutionary Biology; Sigma Xi; University of California Natural Reserve System; University of California, Santa Cruz Services for Transfer And Re-entry Students (STARS)

Abstract

The genetic structure of populations can be both a cause and a consequence of ecological interactions. For parasites, genetic structure may be a consequence of preferences for host species or of mating behaviour. Conversely, genetic structure can influence where conspecific interactions among parasites lay on a spectrum from cooperation to conflict. We used microsatellite loci to characterize the genetic structure of a population of the socially parasitic dulotic (aka "slave-making") ant (*Polyergus mexicanus*), which is known for its host-specificity and conspecific aggression. First, we assessed whether the pattern of host species use by the parasite has influenced parasite population structure. We found that host species use was correlated with subpopulation structure, but this correlation was imperfect: some subpopulations used one host species nearly exclusively, while others used several. Second, we examined the viscosity of the parasite population by measuring the relatedness of pairs of neighbouring parasitic ant colonies at varying distances from each other. Although natural history observations of local dispersal by queens suggested the potential for viscosity, there was no strong correlation between relatedness and distance between colonies. However, 35% of colonies had a closely related neighbouring colony, indicating that kinship could potentially affect the nature of some interactions between colonies of this social parasite. Our findings confirm that ecological forces like host species selection can shape the genetic structure of parasite populations, and that such genetic structure has the potential to influence parasite-parasite interactions in social parasites via inclusive fitness.

KEYWORDS

dulosis, kin structure, microsatellites, population structure, population viscosity, social parasitism

1 | INTRODUCTION

Social parasites exploit the reproductive investment of their hosts for their own fitness benefit. Social parasitism occurs in a broad diversity of taxa, including birds, fish, and arthropods—particularly social insects (Davies, 2010; Field, 1992; Wisenden, 1999; Zink, 2003). Social parasitism has been particularly well studied in brood parasitic birds (Davies, 2010; Rothstein, 1990) but it is perhaps taken to even greater extremes among social insects (Aron, Passera, & Keller, 1999; Heinze & Keller, 2000; Hölldobler & Wilson, 1990). Most work on

social parasites has focused on host-parasite dynamics, with far less attention paid to interactions between the parasites themselves (but see Brooker & Brooker, 1989 for a notable exception for an avian brood parasite). This is somewhat surprising because it has long been recognized for endoparasites and diseases that parasite-parasite interactions are likely to have profound effects on how parasitic relationships evolve (Bull, 1994). For example, parasites may compete with each other for the same host, which can affect aspects of host-parasite interactions, such as the impact of the parasite on its hosts (e.g., virulence, Read & Taylor, 2001).

Interactions between social parasites should be influenced by their population structure. For example, parasites might compete among themselves for the same hosts, but such competition could involve relatives in viscous populations. Interactions among kin could potentially lead to a reduction in the intensity of competition due to kin selection (Hamilton, 1964), although in some contexts, kin competition might be expected to counterbalance any potential for kin selection (Gardner & West, 2004; Queller, 1994).

Conversely, population structure could reflect ecological interactions among parasites. For example, in some species of social parasites, individual parasites specialize on one of several possible host species, so ecological competition between parasites would be limited to parasites that share the same host species. Under some conditions, this type of host specialization can lead to the evolution of genetically distinct host races, which are thought to be important precursors to sympatric speciation (Buschinger, 1989; Gibbs et al., 2000; Marchetti, 1998; Torres, Tonione, Ramírez, Sapp, & Tsutsui, 2018; Via, 2001). The term "host race" is probably overused (Funk, 2012), given that the generally accepted definition requires documenting several criteria (Drès & Mallet, 2002) that are often very difficult to confirm. However, even for species where not all criteria apply, and hence where sympatric speciation is unlikely, the existence of host specialization and some population structure can have important consequences for parasite ecology and evolution. Specifically, the existence of genetic differentiation among parasite groups that use different hosts, despite some gene flow among them, is both consistent with host races and has ecological, social, and evolutionary implications by itself. Genetically differentiated groups of interacting conspecific parasites using different hosts may have different mating constraints, dispersal patterns, and social interactions than a panmictic population of the same parasites.

Eusocial insects are an ideal group for examining the genetics of social parasitism because they have complex societies and social parasitism is widespread (Bourke & Franks, 1995; Field, 1992; Heinze & Keller, 2000; Hölldobler & Wilson, 1990). For example, ants in the genus *Polyergus* are obligate social parasites that can have many conspecific interactions, so their population structure may be particularly relevant to understanding these interactions (Trager, 2013). These ants show a form of social parasitism called dulosis (often "slave-making", but see Herbers, 2007), which has evolved at least 10 times in ants (Beibl, Stuart, Heinze, & Foitzik, 2005; d'Ettorre & Heinze, 2001). Dulosis is invariably characterized by two essential features of the parasite's life cycle: (a) a newly-fertilized dulotic queen parasitizes an intact colony of a host ant species by killing the resident queen and usurping her workers; and (b) the dulotic queen's sterile daughters (dulotic workers) are reared to adulthood by the dead host queen's workers (host workers) and they then conduct raids on neighbouring host nests from which they steal brood (larvae and pupae) of the host ant species. The current generation of adult host ants raise the purloined brood to become the next generation of host workers (without a host queen, the parasitized colony cannot produce its own host eggs or larvae). As a result, all parasite nests contain mixed-species colonies that comprise a parasitic dulotic queen and her descendants, plus the stolen workers of numerous nearby host colonies, typically all from the same host species.

Polyergus are widely reported to be hostile towards conspecifics from neighbouring colonies (Bono, Gordon, Antolin, & Herbers, 2006; Mori, Grasso, Visicchio, & Le Moli, 2001; Topoff, Lamon, Goodloe, & Goldstein, 1984; Trager, 2013), and the strongest evidence of this is the occurrence of intraspecific raids, where the raided colony is destroyed (in contrast to the more common raids on pure host species colonies, where raided colonies typically survive) (Topoff et al., 1984). Similarly, Trager (2013) reports that when raids from neighbouring *P. breviceps* crossed paths, the interaction escalated into a two-day battle and culminated in the destruction of one of the two parasite colonies.

Other evidence suggests tolerance or at least less dramatic antagonism among conspecific *Polyergus* colonies. Some colonies do manage to persist much closer to each other than expected based on their typical raid distance, despite the fact that the mechanics of preraid host-nest searching by *Polyergus* scouts suggests such neighbouring colonies must be aware of each other's proximity (Bono et al., 2006). Bono et al. (2006) suggest that directional and temporal bias in raids from neighbouring nests may reflect a strategy of mutual avoidance among competing conspecific parasites. However, distinct host preferences—and potentially concomitant genetic differences—among neighbours may also influence the proximity of colonies and their levels of intraspecific antagonism. In sum, *Polyergus* colonies exhibit widely varying levels of hostility towards conspecifics, and it is unknown how the interacting forces of host specialization and genetic structure influence these interactions.

While definitive examples of host races are lacking for socially parasitic ants, at least some of the criteria that define host races have been documented for several dulotic species (Bono, Blatrix, Antolin, & Herbers, 2007; Goodloe & Sanwald, 1985; Goodloe, Sanwald, & Topoff, 1987; Schumann & Buschinger, 1994, 1995), and recent evidence suggests that they may occur in our study population as well (Torres et al., 2018). For all of these examples, a single ant species parasitizes multiple species of host ants, but individual colonies use only a single host species. This host fidelity has also been documented at our site (Torres et al., 2018) and is generally common among *Polyergus* (Trager, 2013).

Host fidelity alone, while not sufficient to prove the existence of host races, has important implications for dulotic ants. Parasites' perceptions of nestmates, kin, and conspecific competitors are all likely to be profoundly influenced by the cuticular hydrocarbons of their

host species of ant (Bos & d'Ettorre, 2012; Martin & Drijfhout, 2009; Topoff, 1990). Since parasites acquire both their chemical recognition templates and their own cuticular hydrocarbons from host nestmates, parasites are likely to both choose mates from, and to show competitive interactions with, other colonies that use the same host species. Thus, host preference alone may influence both genetic population structure and the competitive interactions of neighbouring parasites independently.

Relatedness is another factor that might affect parasite intraspecific interactions. Two behaviours that could lead to kin-structured populations often occur in *Polyergus* species. First, mating behaviour varies across the genus *Polyergus*, but observations at our site are consistent with the "female calling syndrome" (see pp. 144–146 of Hölldobler & Wilson, 1990): new queens forgo flying altogether and instead attract males via pheromones. After mating monogamously on the ground near their natal nests, females remove their wings and search on foot for host colonies to infiltrate. Consequently, mating and parasitic nest founding happen close to a new queen's natal nest. This should lead to relatedness among neighbouring colonies and possibly a pattern of average relatedness that decreases with the distance between nests. Second, conspecific colony destruction affects the spacing of parasitic nests and, if kinship influences the pattern of colony destruction, neighbouring colonies are likely to be closely related to each other.

In this study, we investigate patterns of population structure with respect to host use and relatedness in the dulotic ant *Polyergus mexicanus*. First, we test the idea that host preference influences genetic structure, spatial ecology, and intraspecific interactions. We quantify the population structure and relatedness of neighbouring colonies in a spatially contiguous range of *P. mexicanus* nests and contrast this population structure with host species use, nest location, and intraspecific interactions. Second, we quantify the potential for kin population structure in two ways: (a) we determine the relationship between the relatedness of pairs of colonies and their distance from each other; and (b) we determine the frequency with which colonies had any first order relatives as neighbours.

Our initial goal was to focus on kin structure in our population, but it became clear that the existence of host races could confound such an investigation. Specifically, ignoring genetic structure from host races could inflate apparent kin structure because colonies that share the same host species could have increased genetic similarity (and hence apparent relatedness) relative to the entire population, due to genetic isolation or selection from the history of host use and not from true relatedness. We therefore expanded the scope of the study to include an analysis of host races but note that we are not the first to examine host races in this species. Torres et al. (2018) previously examined the existence of host races occur in this same population, but with a much more restricted sample of colonies (18 colonies with mtDNA, 10 colonies with microsatellite DNA) than we used (82 colonies, seven of which were shared by both studies). Torres et al. (2018) found evidence of three distinct genetic subpopulations using the same biparentally inherited microsatellite markers we use here but found evidence of four subpopulations using mtDNA markers. By using the same microsatellite markers on a much larger sample of colonies (82), we can test whether the reported differences in number of genetic populations revealed by mtDNA and microsatellite markers is real (and perhaps a reflection of different inheritance patterns of the two markers) or is an artifact of small sample size and the potentially highly related individuals (nestmates) analysed. Specifically, evidence for three subpopulations would support the Torres et al. (2018) finding that maternal and paternal patterns of gene flow differ for this parasite and would correspond to the three known host species *Formica accreta*, *F. argentea*, *and F. subaenescens* (Schär et al., 2018). Conversely, evidence supporting four subpopulations would suggest that maternal and paternal gene flow follow the same pattern, and that population structure is influenced by some unknown factor, possibly a cryptic 4th host species.

2 | MATERIALS AND METHODS

2.1 | Study site and subjects

We studied a population of *Polyergus mexicanus* on the east slope of the northern Sierra Nevada Mountains, approximately 20 miles north of Lake Tahoe at the Sagehen Creek Field Station ("SCFS", a University of California Natural Reserve; 39.432181, −120.241263). Note that *P. mexicanus* at this site was previously identified as *P. umbratus*, characterized by a long and often convex mesonotum compared to *P. mexicanus*. Although *P. umbratus* was recently synonymized with *P. mexicanus* (Trager, 2013), recent genetic work indicates that *P. umbratus* is actually a distinct species, so the name may soon be resurrected (J. C. Trager, personal communication).

The site comprises a variety of habitats, but nests were typically found within 200 m of dirt roads in disturbed (mechanically thinned for fire control) mixed-conifer forest on the south-facing slope of the Sagehen Creek drainage basin. Nests were often found associated with downed tree trunks, stumps of harvested trees, or the root structure of common understory plants such as *Ceanothus prostratus* and *Wyethia mollis*.

The elevation of study populations ranged from 1,930 to 2,125 m over a contiguous area of approximately 9 km^2. We estimate a density of *P. mexicanus* nests at 8.4 per 100 m^2, which is greater than any we are aware of elsewhere in the literature for any species of *Polyergus*. While *P. mexicanus* colonies at SCFS are only known to parasitize *F. accreta*, *F. argentea*, and *F. subaenescens*, there are approximately 20 species of *Formica* at our site, many of which are quite similar in habitat and appearance to the three most common host species.

2.2 | Field sampling methods and design

From 2008 to 2010, we searched for raids and nests on two 1-hectare study plots. To better characterize the genetic diversity of the population of *P. mexicanus*, we also searched a ~15 km^2 area for nests along

roads and trails throughout the reserve in 2010. In 2011, we conducted daily observations on four additional smaller (2,500 m^2) focal plots to provide more independent observations of unique pairs of interacting colonies for behavioural studies and broaden our genetic sample of parasite colonies for this study. All six plots were centered on a *P. mexicanus* nest and were chosen because of the high density of surrounding *P. mexicanus* nests in the area as revealed through preliminary pilot searches for nests. Our observations and collection of specimens were not limited to plot boundaries: when we detected raids and nests near but outside plot boundaries, we included them in this study.

We searched for raids at these plots daily for at least a month during the peak of the raiding season (typically during July) and used the conspicuous raids to locate the inconspicuous nests of both the mixed-species *P. mexicanus* colonies and the colonies of their host *Formica* species. We did not attempt to estimate the relative abundance of nests of the three known *Formica* species in this study because our study is focused on interactions among parasites and because *Formica* nests are cryptic unless being raided. We collected data on the scale, frequency, and nature of intraspecific parasite interactions by measuring the distance of raids, recording the relative locations of all parasite nests, and making special note of any raids that crossed any other active raids and intraspecific raids (i.e., raids from one parasite nest to another). We collected between 1–12 individuals from 82 nests for a total of 397 *P. mexicanus* female workers and six males for genomic DNA extraction. Live ants were frozen and preserved in 95% ethanol.

To assess host species identity, we collected host *Formica* workers either from a focal *P. mexicanus* colony or from a *Formica* nest raided by that focal *P. mexicanus* colony. Because it is well established that individual *P.* mexicanus colonies use a single host species, we often opted to collect *Formica* specimens and raiding *Polyergus* at the site of a raided nest to minimize disturbance to our long-term focal objects: the parasitic *Polyergus* colonies. For each parasite colony, we attempted to collect at least three host *Formica* workers, which we mounted according to museum standards for species identification. We used the dichotomous keys developed by Francœur (1973) as well as several characters known to be locally diagnostic for the different host species (P. S. Ward and C. W. Torres, personal communication) to determine the species identity of the *Formica* host workers. However, characters were sometimes ambiguous and *Formica* species within the *subaenescens*-group (as are the three host species at SCFS) are notoriously difficult to identify (Glasier, Acorn, Nielsen, & Proctor, 2013; Mackay & Mackay, 2002). To avoid making classification errors we assigned ambiguous individuals as "cf. *Formica subaenescens*" and "cf. *Formica argentea*". For all analyses, we checked to see if inclusion or exclusion of these ambiguous individuals changed the results. For analyses unaffected by inclusion of these individuals, we report the statistics that include these individuals assigned to their most likely species.

2.3 | Microsatellite protocol

We extracted genomic DNA from the collected *P. mexicanus* workers and six males using either Qiagen DNEasy Kits or Quick-gDNA MiniPrep Zymo kits according to the manufacturer's instructions. We amplified the DNA with PCR using six primers developed by Bono et al. (2007): Pol1, Pol2, Pol3, Pol4, Pol5, and Pol12. We modified these original primers to use an M-13 dye-tagging protocol (Schuelke, 2000). Each PCR was labelled with one of Applied Biosystems DS-33 Dyes (LIZ, 6-FAM, VIC, NED, PET).

The amplification process for all six loci differed only in annealing temperature. For all loci, extracted DNA was initially denatured at 95˚C for 5 min, then run through 36 cycles, each of which consisted of additional denaturing at 95˚C for 30 s, 30 s at one of two annealing temperatures, and 30 s of extension. After these 36 cycles, there was a final extension step of five minutes at 72˚C before samples were stored at 4˚C. The annealing temperature was 58˚C for Pol1, Pol4, and Pol12, and 53˚C for Pol 2, Pol 3, and Pol 5. Amplified DNA was preserved in HiDi Formamide and sent to the University of California at Berkeley Sequencing Facility for microsatellite fragment size analysis using Applied Biosystems 3730XL DNA Analysers and LIZ size standard. We determined peaks and bins of each locus on the resulting electropherograms using GENEIOUS version 6.0 (Kearse et al., 2012).

2.4 | Population structure analysis

To estimate population assignment of individuals, we used STRUCTURE (Pritchard, Stephens, & Donnelly, 2000) which uses Bayesian techniques to form clusters of individuals that best meet the assumptions of Hardy-Weinberg equilibrium and linkage disequilibrium. We tested hypotheses for K = 2–10 populations with 50 independent runs for each hypothesized K value (400 total runs). We used a burn-in length of 10,000 followed by 100,000 Markov Chain Monte Carlo (MCMC) repetitions. We set λ = 1 to assume that allele frequency could vary independently among subpopulations (a standard assumption when the K value is unknown) and did not use any prior population information (POPFLAG = 1). We used the online tool STRUCTURE HARVESTER (Earl & vonHoldt, 2012) to collate these results to determine which hypothesized K was most likely, using the Evanno, Regnaut, and Goudet (2005) method. We then used CLUMPP (Jakobsson & Rosenberg, 2007) to create a consensus data set that used the data from all the structure simulations of the best supported K values. Because our inclusion of nestmates potentially violates STRUCTURE's assumption that all samples are unrelated individuals, we repeated the above steps with only one individual from each colony to confirm that the best supported K, and the subpopulation assignments of individuals did not change. We analysed the goodness of fit between our estimated genetic subpopulations and host species groupings we described based on the morphology of host species for each *P. mexicanus* colony. Finally, we used the graphical software DISTRUCT (Rosenberg, 2004) to visually contrast these genetic populations with the three host species groupings we determined by examining host *Formica* workers.

Since males are haploid, we coded their genotypes as missing a second allele at each locus for the STRUCTURE analysis, as

recommended (Pritchard et al., 2000). After the STRUCTURE analysis, we removed 11 individuals (including all six males) that either had less than 50% of loci amplify or had otherwise incomplete or inconsistent data from subsequent analyses on relatedness. We also excluded nine other individuals that had a maximum subpopulation assignment of less than 80% from relatedness analyses, because relatedness calculations are only sensible when computed between members of the same subpopulation; however, including all individuals did not qualitatively change our results here. We also excluded these nine individuals from tests of the relationship between host species and subpopulation assignment, because these analyses required unambiguous assignment into one subpopulation.

We checked for null alleles, allelic dropout, and stutter in each of subpopulations we detected via the structure analysis using MICROCHECKER (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004) software that compares molecular data to Hardy Weinberg expectations and thus requires an estimate of population boundaries among samples. Because MICROCHECKER assumes that samples within a population are from unrelated individuals, we randomly chose only one individual per sampled colony for the MICROCHECKER data set. However, this random subsample excluded seven rare alleles that were present in the complete sample, so we also added six individuals from colonies that were already represented to ensure that these alleles were included in our analyses. In other words, we allowed a slight violation of the software's assumption regarding relatedness of samples in favor of including all alleles. We found evidence of null alleles for only one locus (Pol 1) in one of our estimated subpopulations (Population 3), and we used the "Brookfield 2" corrected allele frequency provided by MICROCHECKER for subsequent relatedness analysis (Brookfield, 1996).

2.5 | Comparing population structure to host species use

To assess the relationship between host *Formica* species use and genetic subpopulation, we first calculated a colony-level subpopulation probability of assignment score for each colony by taking the highest subpopulation probability score assigned by STRUCTURE to each nestmate and averaging them together. In all cases, nestmates had the highest probability of assignment to the same subpopulation, indicating that a colony average is biologically meaningful. We tested the dependence of host species use on a parasite's genetic subpopulation using a chi-squared contingency test.

2.6 | Relatedness analysis

We used KINGROUP (Konovalov, Manning, & Henshaw, 2004) to measure pairwise relatedness between all possible pairs of individuals within each subpopulation detected in our genetic structure analysis. Because kinship estimation relies on deviations from the assumption of panmixia, we restricted these calculations to all individual workers who were assigned to the same genetic subpopulations, as estimated from the STRUCTURE analysis. Within each subpopulation, we calculated the relatedness between all possible pairs of sampled *P. mexicanus* workers. We determined the relatedness between each pair of colonies by averaging all possible pairwise relatedness values of each nestmate from the first colony with each nestmate from the second colony.

We calculated the Pearson product-moment correlation for the pairwise relatedness of colonies and the distance between nests for all pairs of colonies, which included nest pairs as far as 4 km away from each other, a distance we considered reasonable for the dispersal of winged males. To focus on the effects of female dispersal (which is on foot) and intercolony interactions such as conspecific raids, we repeated this analysis with only colony pairs with nests that were less than twice the maximum observed distance of host raids, 155 m.

To assess the potential for kinship to influence interactions among colonies with neighbouring nests, we calculated the proportion of colonies that had at least one highly related neighbour within the maximum observed raiding distance (77.5 m) of a focal nest. We considered a relatedness value >0.375 as highly related; this is the expected relatedness between workers of one colony and workers of a new queen arising from that colony. We present these results both as a proportion of all 82 colonies observed, and a proportion of the 44 colonies that have at least one neighbour.

3 | RESULTS

3.1 | Population structure

We found the strongest evidence for three subpopulations (Delta K = 849 for K = 3, Table 1). The hypothesis of four subpopulations was poorly supported by the genetic structure analysis (Table 1) and did not align well with patterns of host species use (Figure 1c). The three well-supported subpopulations were very distinct, with most individuals very clearly assigned to one of the three populations (Figure 1a). Evidence for gene flow between subpopulations was minimal: we detected only 12 individuals out of 397 sampled whose membership coefficients were less than 0.8 for all three clusters, and three of these were individuals with poor microsatellite data (see annotations in Figure 1a).

The number of genetic subpopulations estimated by our population structure analysis (K = 3) equaled the number of known host *Formica* species parasitized by *P. mexicanus* at SCFS. Genetic subpopulations of *P. mexicanus* colonies were correlated with the host *Formica* species they used (Contingency test, χ^2 = 57.1, df = 4, p < .001), but this relationship was not perfect. Subpopulation 3 in particular showed little evidence of host specialization compared to the other two subpopulations. While colonies in Subpopulations

1 and 2 predominantly used *F. argentea* and *F. subaenescens* hosts, respectively, Subpopulation 3 used all three hosts with moderate frequency (Table 2).

TABLE 1 Evanno method statistics for each hypothesized number of subpopulations (*K*)

K	Reps	Mean Ln*P*(*K*)	*SD* of Ln*P*(*K*)	Ln′(*K*)	|Ln″(*K*)|	Delta *K*
2	50	−7317	81.51	–	–	–
3	50	−6550	0.67	767	568	849.31
4	50	−6351	78.96	198	67	0.86
5	50	−6221	125.11	130	16	0.13
6	50	−6107	93.60	114	5	0.06
7	50	−5988	43.82	119	35	0.79
8	50	−5903	90.30	85	23	0.25
9	50	−5842	97.08	62	7	0.08
10	50	−5773	99.37	69	–	–

Note: Ln*P*(*K*) is the log-probability of *K*. *SD* is standard deviation. *L*′(*K*) is the rate of change of the likelihood distribution. |*L*″(*K*)| is the absolute value of the second order rate of change of the likelihood distribution. Delta *K* is calculated as |*L*″(*K*)|/*SD* of Ln*P*(*K*).

3.2 | Relatedness between parasite colonies and among individuals within colonies

The global average relatedness of all non-nestmates was close to zero ($R \pm SD = 0.03 \pm 0.27$; $n = 26{,}410$ unique pairs of non-nestmate workers), indicating that most colonies are not closely related to each other. However, 75 colonies pairs (out of 1,363 possible combinations of colonies) had an average relatedness equal to or greater than 0.375; the expected relatedness between workers of one colony and workers of a new queen arising from that colony (Figure 2a). Eleven of the 130 (8.5%) unique pairs of colonies with nests within 155 m of each other had a relatedness value of 0.375 or higher (Figure 2b).

We found that 44 colonies of the 82 colonies in our genetic sample had at least one neighbour from their subpopulation within the maximum raiding distance (77.5 m). Of these 44 colonies, 16 (35.4%) had a neighbouring colony whose average relatedness was 0.375 or greater.

There was a weak but significant negative correlation between relatedness of colonies and the distance of their nests from each other over local distances (up to 155 m; Table 3). However, there was no such correlation in an analysis that included pairwise comparisons at all distances (Table 3). Separate analyses of each subpopulation

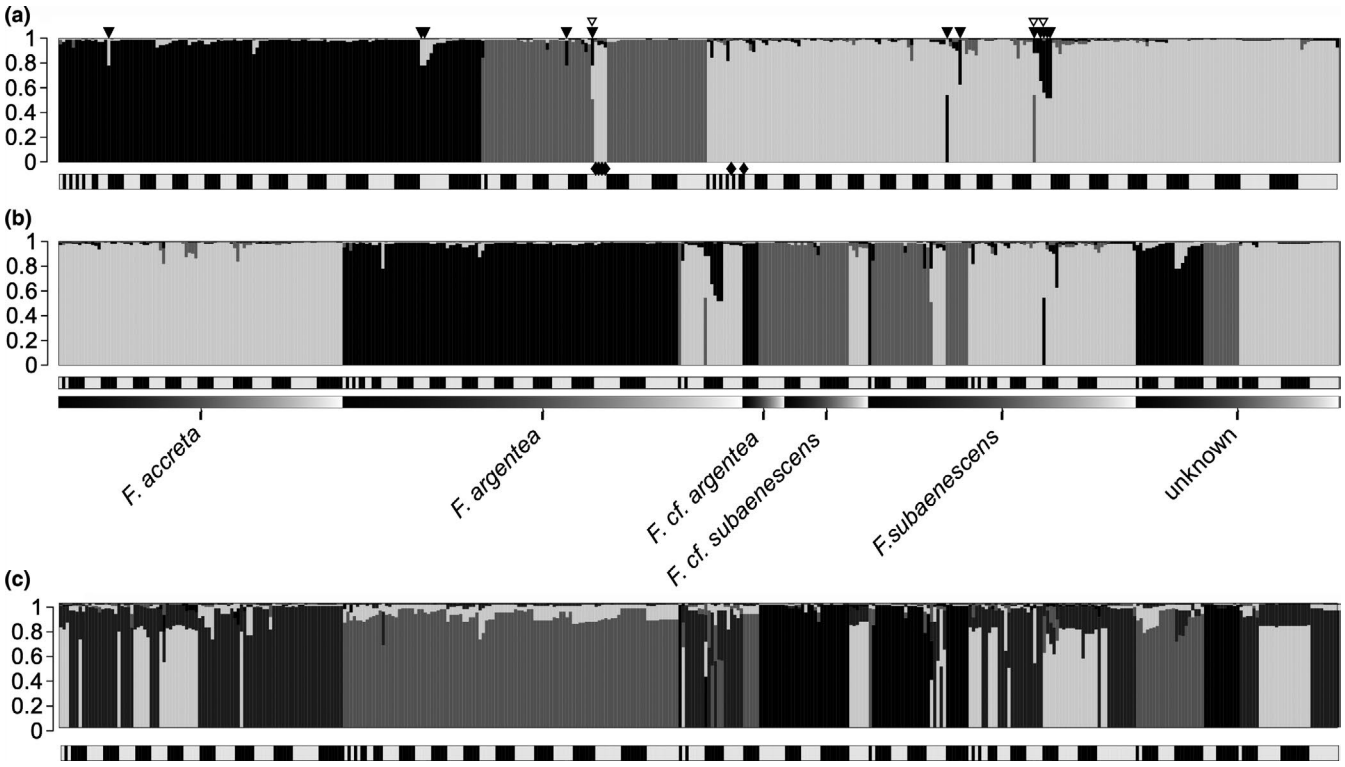

FIGURE 1 Structure plots for *Polyergus mexicanusstudy population*. Plots compare best supported number of genetic subpopulations, *K* = 3 (a, b) to *K* = 4 (c). Shading of main bars represents subpopulation (for *K* = 3: black is Subpopulation 1, dark grey is Subpopulation 2, light grey is Subpopulation 3. *K* = 4 populations were not named). The alternating black and white bar along the bottom indicates colony membership: a contiguous shading indicates that individuals belong to the same colony. (a) Individuals and colonies sorted by subpopulation assignment. The 18 individuals that were removed from subsequent analyses are noted with three symbols: along the top, solid triangles (▾) identify 12 individuals whose highest membership coefficient was <0.8, and hollow triangles (▽) indicate three individuals that had <50% scorable microsatellite loci. Along the bottom, six males are indicated by diamond (♦) symbols. (b) Individuals and colonies sorted by host species use. Gradient shaded bars along bottom group colonies that use the indicated *Formica* host species. (c) Individuals and colonies arranged as in (b), but with *K* = 4

revealed that only Subpopulation 1 had a significantly negative correlation between distance and relatedness, and that this correlation existed even when all distances were considered (Table 3).

Within colonies, relatedness was high: the global average pairwise relatedness of nestmates was close to the 0.75 value expected for full sibling haplodiploid sisters ($R \pm SD = 0.71 \pm 0.24$, $n = 939$ unique pairs of nestmates).

3.3 | Parasite intraspecific interactions

We observed 778 raids from the parasite colonies involved in this study. We combined these observations with those from other observations at this site (i.e., colonies with no genetic samples) for a total of 862 observed raids with the maximum raid distance of 77.5 (as used in above analyses), and an average raid distance of 19.2 m ($SD = 13.6$). Nests of parasitic colonies were frequently closer to each other than these raiding distances, and there was no apparent clustering by subpopulation or host use (Figure 3).

Among these raids, 11 were intraspecific raids that occurred between seven unique pairs of colonies (i.e., some intraspecific raids happened repeatedly between the same pair of colonies). We had both genetic data and host species data for both colonies in four of the seven unique pairs of colonies. In all cases, the interacting colonies shared host species. In one of these four cases, the raided and raiding colonies belonged to different subpopulations (Subpopulation 1 raided Subpopulation 3). For the three other cases involving colonies of the same subpopulation, relatedness values ranged from −0.23 to 0.39.

Six pairs of raids crossed each other from five unique pairs of colonies. There were no signs of aggression or antagonism for any of these crossing raids, in stark contrast to all intraspecific raids we observed. We had complete genetic data and host species data for one pair, and host species data for three pairs. One pair was from the same subpopulation (Subpopulation 1) and had a colony-level relatedness value of 0.181. The three other pairs were from different subpopulations and all but one of these used different host species. The raids that crossed but used the same host species (*F. subaenescens*) were from Subpopulations 2 and 3.

TABLE 2 The relationship between host species use and parasite genetic subpopulation

	Genetic subpopulation			
Host species	**1**	**2**	**3**	**Totals**
Formica accreta	–	–	17	17
Formica argentea	23	2	3	28
Formica subaenescens	1	8	13	22
Totals	24	10	33	67

Note: Frequencies of observed *Polyergus* colonies that occur in each genetic subpopulation contrasted with frequencies that use each host *Formica* species.

4 | DISCUSSION

The relationship between genetic structure, parasite host use, population viscosity, and intraspecific interactions among these parasites is more complex than predicted. We found strong evidence that host species use restricts gene flow, and that genetic subpopulations are associated with the use of specific host species. However, the genetic subpopulations we detected do not perfectly match parasite host use. At a local level, the relatedness patterns we observe between pairs of colonies do not indicate high viscosity: neighbouring colonies are often not closely related, nor necessarily from the same subpopulation, nor using the same species of host (Figure 3). However, while most neighbouring colonies of the same subpopulation are not close relatives, colonies that are both closely related and spatially close enough to interact with each other occur regularly, and this could affect the nature of interactions between some colonies. The relationship between distance and relatedness between pairs of colonies within raiding distance of each other was weaker than expected from our observations of the mating behaviour of *P. mexicanus* queens and intraspecific raiding behaviour of *P. mexicanus* workers at SCFS. The interactions we observed between pairs of parasite colonies—raids on each other and raids from two different colonies that crossed—were extremely infrequent in comparison to normal raiding behaviour. While the rareness of these observations prohibits formal analysis of their relationship to other factors, it seems clear that conspecific raids only occur between colonies that use the same host species, and that no clear pattern exists between

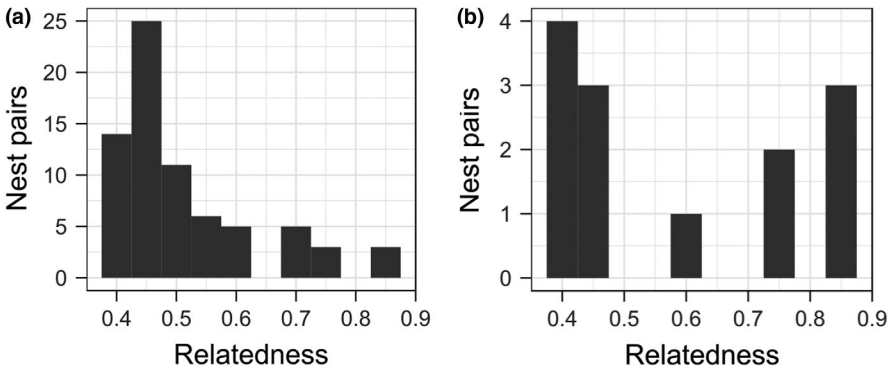

FIGURE 2 Histograms of average relatedness of pairs of colonies for highly related colonies at two spatial scales. Only colony pairs with average $R > 0.375$ are included. (a) All possible pairs of colonies at our study site, irrespective of distance between their nests. (b) Pairs of colonies with nests <155 m from each other

TABLE 3 Correlations between relatedness and distance for different subpopulations at two spatial scales

Distances	Genetic subpop	Sample size	*df*	*p*	*t*	Correlation
All	All	1,782	1,780	.06	−1.88	−0.04
<155 m	All	134	132	.04	−2.08	−0.18
All	1	627	625	<.01	−5.02	−0.20
All	2	104	102	.12	1.59	0.16
All	3	1,051	1,049	.38	0.87	0.03
<155 m	1	48	46	.02	−2.47	−0.34
<155 m	2	12	10	.22	−1.30	−0.38
<155 m	3	74	72	.76	−0.30	−0.04

Note: Subpop, subpopulation. Statistics shown are Pearson product moment correlations for relatedness versus distance for all unique pairs of colonies.

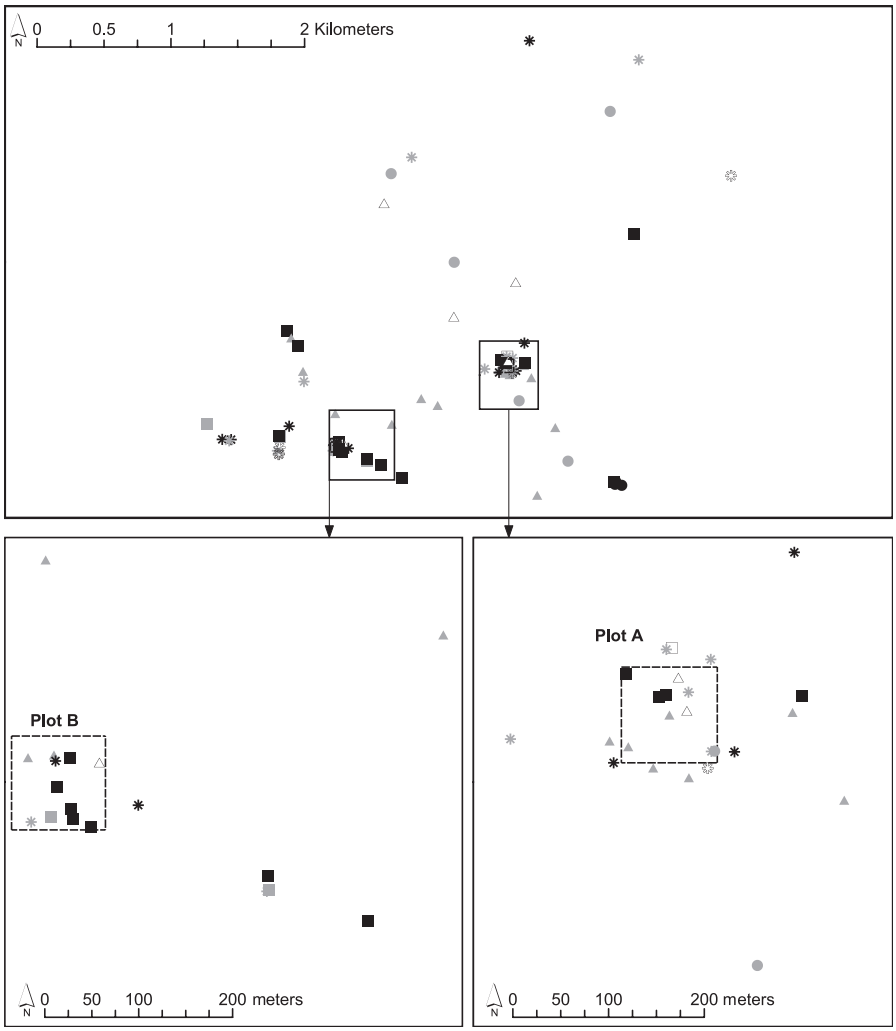

FIGURE 3 Distribution of *Polyergus mexicanus* nests in the study area. Map showing the locations of nests located in and around the borders of the two most intensively studied one ha plots established in 2008, which are represented by dashed lines and shown in detail in the two lower panels. Shape indicates host species: *F. accreta* = circles (*n* = 8), *F. argentea* = squares (*n* = 22), *F. subaenescens* = triangles (*n* = 25), and unidentified host species = asterisks (*n* = 27). The shading of each marker indicates if the colony was assigned to Subpopulation 1 (black; *n* = 30), Subpopulation 2 (white; *n* = 13), or Subpopulation 3 (grey; *n* = 39)

either type of intraspecific raid interaction and genetic subpopulation or relatedness.

When a parasitic species uses several different host species, co-evolution between host and parasite can, under some conditions, lead to the evolution of distinct genetic "host races" (Gibbs et al., 2000; McCoy, Boulinier, Tirard, & Michalakis, 2001). Jaenike (1981) outlined the criteria for distinguishing between simple differences in current host preferences and true genetic host races that have the potential to play a role in sympatric speciation of the parasites. These criteria include (a) sympatric subsets of a parasitic species that use different host species; (b) statistically significant genetic differences among the parasites using different hosts; (c) the genetic differences must extend beyond loci that affect host preference; and (d) the genetic differences must not be entirely the result of natural selection on the current generation. More recently, Drès and Mallet (2002) expanded these criteria to include the following: (a) greater genetic differences among host

races in sympatry than between individuals using the same host in allopatry; (b) parasite mate choice that is correlated with host choice; and (c) gene flow between races in sympatry.

While these criteria are necessary to fully demonstrate that an observed host-parasite association represents a step on the path to sympatric speciation, the difficulty in documenting all six criteria has made true examples of host races rare (Fitzpatrick, Fordyce, & Gavrilets, 2008; Via, 2001). Evidence for host races does exist for some parasite groups (e.g., ectoparasites [McCoy et al., 2001]) but similarly complete evidence in insect social parasites has rarely been studied (but see Fanelli et al., 2005), and not previously reported. Though not sufficient to prove host race formation, cases of clear genetic differentiation among parasites using different hosts have implications for the ecology and evolution of host-parasite interactions, even when there are mechanisms in place that prevent speciation. One example is the avian brood parasite, *Cuculus canorus*, in which speciation is prevented by cross-host mating by males, and genes responsible for host-specialization reside on the female sex chromosome (Gibbs et al., 2000; Marchetti, 1998).

In this context, the recent other work at SCFS showing a clear relationship between parasite genetic structure and host species use is noteworthy, especially since it also documents greater genetic similarity between allopatric populations using the same host than between sympatric populations using different hosts (Torres et al., 2018). Our results corroborate parts of this finding, while adding some complications. First, we confirm that there are three clearly defined genetic subpopulations at SCFS. This clear pattern is supported with 397 ants from 82 distinct colonies and suggests that the difference Torres et al. (2018) found between biparental markers (three subpopulations) and maternally inherited markers (four distinct clades) represents a real difference in inheritance patterns, which suggests differing maternal and paternal gene flow, or differing rates of evolution between the two markers. Indeed, our results indicate that the hypothesis of four genetic subpopulations is a poor fit with both our biparental microsatellite data (Table 1) and our host use data (Figure 1c). However, our results do not indicate that genetic structure maps onto host use as cleanly as suggested by Torres et al. (2018). This could be because the broader sample of parasite colonies here better captures true variable relationships between host use and genetics in this parasite population, suggesting that host species use is only one factor influencing genetic structure among other unknown factors. Alternatively, our results could indicate that the morphological indicators we used to determine host species are unreliable. Indeed, these *Formica* are infamously difficult to correctly identify (Glasier et al., 2013; Mackay & Mackay, 2002), and cryptic species and hybrids are common in the genus (Beresford et al., 2017; Bernasconi, Cherix, Seifert, & Pamilo, 2010; Seifert, 2009; Seifert, Kulmuni, & Pamilo, 2010). A modern phylogenetic analysis of host *Formica* species could help resolve this issue and help identify any currently cryptic species.

The genetic evidence, however, does not suggest that the inconsistent match between genetic subpopulations and host species use is caused by the existence of cryptic *Formica* host species, nor hybridization among the closely related *Formica* host species. While hybridization could explain the existence of the ambiguous host species we identified as *F.* cf. *argentea* and *F.* cf. *subaenescens*, and the use of putative *F. subaenescens* hosts by two distinct *Polyergus* subpopulations, we found exceedingly little evidence of gene flow between parasite subpopulations in the patterns of their genetic structure, in contrast to what we would expect if hosts were hybridizing. If cryptic host species occurred commonly, we would expect to find more subpopulations than known host species, but in fact we found strong support for three parasite subpopulations, the same as the number of known host species.

Assuming our determination of host species identity is accurate, the observed relationship between genetic subpopulation and host *Formica* species suggests a more complex pattern of host use than the one-to-one host-species-to-parasite subpopulation pattern predicted by the host-race hypothesis. Genetic subpopulations 1 and 2 provide evidence reasonably consistent with the existence of *P. mexicanus* host races specializing on *F. argentea* and *F. subaenescens*, respectively. Despite this apparent host specialization by Subpopulation 2 on *F. subaenescens*, a parasite colony using *F. subaenescens* was more likely to belong to Subpopulation 3 than Subpopulation 2. Subpopulation 3's relatively even use of all three host species is evidence against potential host race formation in that subpopulation (Figure 1b and Table 2).

One interpretation of this pattern is that that Subpopulation 3 may be more generalist than the other two subpopulations and thus able to use *F. accreta*, *F. subaenescens*, and to a lesser extent, *F. argentea* as hosts. Host specificity may be caused by limited parasite dispersal or host-specific adaptation (Timms & Read, 1999). Given the general proximity of nests of parasite colonies using the different three host species at our site, and the intimate levels of deceptive chemical signaling by the parasites that must occur for dulosis to be successful (d'Ettorre & Heinze, 2001), host-specific adaptation seems a more likely driver of host specificity. Because host specificity has coevolutionary consequences for host resistance and parasite virulence (Little, Watt, & Ebert, 2006), determining the extent of host specificity in different parasite subpopulations, as well as its causes and consequences, is a valuable direction for future work.

Despite the diversity of hosts used by Subpopulation 3, one pattern does suggest its genetic structure is connected to host specialization: it is the only subpopulation we observed using *F. accreta* hosts (Table 2). This suggests that Subpopulation 3 may have coevolved to use *F. accreta* hosts historically, but still has the capacity to use the other two hosts when *F. accreta* hosts are limiting. This hypothesis is supported by the fact that *F. accreta* is the rarest host we observed, yet Subpopulation 3 is the most abundant parasite population: Subpopulation 3 parasites may prefer *F. accreta*, but can make the "best of a bad job" with *F. argentea* or *F. subaenescens* colonies when they are abundant while *F. accreta* colonies are rare. Future work that better documents the abundances and locations of free-living host *Formica* nests and that measures fitness proxies of parasite colonies as a function of the interaction between genetic

subpopulation and host species will be an important next step in testing this idea.

The mating behaviour of eusocial organisms has strong effects on their dispersal, reproductive output, social evolution, and ecology (Liautard & Keller, 2001; Vitikainen, Haag-Liautard, & Sundström, 2015). At SCFS, the mating behaviour of new *P. mexicanus* queens is characterized by short dispersal distances because queens do not fly, but attract flying males ("female calling syndrome"; Hölldobler & Wilson, 1990), and then mate monogamously with a single male. We expected that such limited dispersal by queens would lead to population viscosity, where individuals in spatial proximity would tend to be closely related. In addition to mating patterns, raids among parasites on each other's nests could also structure populations if the raids were biased towards unrelated individuals: local removal of unrelated individuals should further contribute to relatedness of neighbouring parasite colonies. While we did find that relatedness between pairs of colonies did decrease with distance over the probable range of *P. mexicanus* queen dispersal, the correlation was weak. Strangely, this pattern seems driven by entirely by Subpopulation 1, and the relationship persisted when all distances were considered for this subpopulation (Table 2). We have no a priori reason to suspect that one subpopulation would have different dispersal or mating behaviour than the others, even if we assume that subpopulations represent host races. Subpopulation 1 did have had a tighter correlation with one host species (*F. argentea*) than the other two subpopulations. Future work on host quality and the ecological and behavioural differences among parasites that use different hosts may illuminate why these subpopulations have differing relatedness patterns across the landscape.

Many ants have viscous populations, due to limited dispersal (Chapuisat, Goudet, & Keller, 1997; Pamilo, Gertsch, Thorén, & Seppä, 1997; Sundström, Seppä, & Pamilo, 2005). While viscous populations were once thought to promote kin-selected altruism, it is now appreciated that this altruism may be counteracted by the increased kin competition that low dispersal engenders (Griffin & West, 2002; Queller, 1994). While we find weak evidence supporting population viscosity overall, we found that enough colonies have a closely related neighbour to make kinship potentially relevant to some of the intraspecific interactions between neighbouring parasite colonies.

Some of these parasitic ants' most striking intraspecific interactions—raids on each other's nests and raids from adjacent nests that cross without aggression—are also among the most infrequent. We have too few observations on these interactions to permit statistical analysis, but the initial patterns are not consistent with predictions we might make based on kin selection or subpopulation structure, though host species seems relevant. All intraspecific raids occurred between colonies using the same host species, as predicted by the known host fidelity exhibited by *Polyergus* colonies at this site. Specifically, these intraspecific raids may be adaptive because raiders obtain additional brood of their desired host species, and because they remove or harm a neighbouring competitor for that brood. In contrast to the host species status of colonies involved in intraspecific raids, relatedness values between such pairs of colonies varied from colonies that were less related than the population average to colonies that appeared to be kin. This variation in relatedness values suggests that kinship is not an important determinant of parasite raids on other parasite colonies and that local competition is sufficient to overcome kin-selected altruism for these parasites, as many models of kin selection under limited dispersal indicate (Griffin & West, 2002; West, Pen, & Griffin, 2002).

In contrast to what has been reported for other sites (Trager, 2013), none of the six pairs of raids we observed crossing each other exhibited any discernible hostile interactions. Although we do not have sufficient relatedness or subpopulation data to correlate with these passive crossing raids, they are striking events that strongly suggest some form of intraspecific tolerance. It is likely that crossing raids are capable of hostility but choose peace, because two lines of evidence suggest *Polyergus* raids do indeed have the capacity to opportunistically attack each other when their raids cross in other contexts. First, Trager (2013) reports similar crossing raids quickly devolving into intraspecific hostility on par with intraspecific raids. Unfortunately, we do not know the host-species or relatedness context in which this conspecific interaction occurred, but it would be informative to know how many host species were common at that site. Second, as Topoff et al. (1984) has reported and we have observed directly, *Polyergus* raiders appear to be flexible in the targets they choose during raids, often encountering other targets on the way to the original *Formica* nest their scout was leading them to. This means that raiders are capable of "switching" from transit behaviour to actual raiding (i.e., attacking a nest) at any moment, and they are not behaviourally constrained to keep running until they reach a target nest. Both these examples suggest that *Polyergus* are behaviourally capable of impromptu attacks on rivals they meet while raiding, so our finding that they can also abstain from attacking conspecifics is interesting. One tantalizing possibility for further investigation is that host similarity, possibly including relatedness, affects parasite nestmate recognition such that raids that cross recognize each other as nestmates.

Parasites' interactions with each other and their hosts have implications for speciation, co-evolution, virulence, population growth, and competition (Bull, 1994; Buschinger, 1989; d'Ettorre & Heinze, 2001; Foitzik, DeHeer, Hunjan, & Herbers, 2001; Thompson, 2005). We show that patterns of host use affect gene flow in a sympatric parasite population, but that not all of this genetic structure can be explained by host use. We also demonstrate the opportunity for kin-selected interactions but find little evidence that they are more important than local competition in this system. Our results are a valuable starting point for future work on parasite-parasite interactions, parasite and host genetic structure, parasite mating and dispersal behaviour, parasite competition, and host phylogenetics.

ACKNOWLEDGEMENTS

We thank the following field and laboratory assistants who assisted in finding nests, collecting specimens, and assisting with

molecular work: Skyler Hackley, Matt Freitas, Yasmine Akky, Andrew Frostholm, Beatriz Nobua Behrmann, Mallory Hee, Jessica Simms, Steven Cooper, Hannah Nolan, and Don Brown. We thank Phil Ward, Candice Torres, and James Trager for sharing their taxonomic expertise and natural history insights that were instrumental in the design of this study. We thank UC Berkeley's Sagehen Creek Field Station, and the University of California Natural Reserve System for providing the ideal natural environment to conduct this study. We also thank station managers Jeff Brown and Faerthen Felix for facilitating our research and residency there for the duration of this study. We thank Neil Tsutsui, Giacomo Bernardi, and Barry Sinervo for feedback on experimental design and earlier versions of this manuscript. This work was supported by UC Natural Reserve System Mathias grants, a UC Santa Cruz STARS grant, UCSC Ecology and Evolutionary Biology department graduate summer research grants, and a Sigma Xi grant-in-aid of research.

AUTHOR CONTRIBUTIONS

J.R.S. designed and performed the research, analysed the data, and wrote the paper. J.Y. performed the research and edited the paper. B.L. designed the research and wrote the paper.

DATA AVAILABILITY STATEMENT

All data including microsatellite genotypes, estimated relatedness values, nest locations, host species determinations, calculated distances between parasite nests, and raid distances are openly available in Dryad at: https://datadryad.org/stash/dataset/doi:10.5061/dryad.wstqjq2h9 (Sapp, Yost, & Lyon, 2020).

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How to cite this article: Sapp JR, Yost J, Lyon BE. The socially parasitic ant *Polyergus mexicanus* has host-associated genetic population structure and related neighbouring colonies. *Mol Ecol*. 2020;29:2050–2062. https://doi.org/10.1111/mec.15468