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Distinct colony boundaries and larval discrimination in polygyne red imported fire ants (*Solenopsis invicta*)

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Abstract

Evaluating the factors that promote invasive ant abundance is critical to assess their ecological impact and inform their management. Many invasive ant species show reduced nestmate recognition and an absence of boundaries between unrelated nests, which allow populations to achieve greater densities due to reduced intraspecific competition. We examined nestmate discrimination and colony boundaries in introduced populations of the red imported fire ant (*Solenopsis invicta*; hereafter, fire ant). Fire ants occur in two social forms: monogyne (colonies with a single egg-laying queen) and polygyne (colonies with multiple egg-laying queens). In contrast with monogyne nests, polygyne nests are thought to be interconnected due to the reduced antagonism between non-nestmate polygyne workers, perhaps because polygyne workers habituate the colony to an odour unique to *Gp-9*^{b}-carrying adults. However, colony boundaries and nestmate discrimination are poorly documented, particularly for worker-brood interactions. To delimit boundaries between field colonies, we correlated the exchange of a ^{15}N-glycine tracer dissolved in a sucrose solution with social form. We also evaluated nestmate discrimination between polygyne workers and larvae in the laboratory. Counter to our expectations, polygyne colonies behaved identically to monogyne colonies, suggesting both social forms maintain strict colony boundaries. Polygyne workers also preferentially fed larval nestmates and may have selectively cannibalized non-nestmates. The levels of relatedness among workers in polygyne colonies was higher than those previously reported in North America (mean ± standard error: 0.269 ± 0.037). Our study highlights the importance of combining genetic analyses with direct quantification of resource exchange to better understand the factors influencing ant invasions.

KEYWORDS
invasive ant, isotope tracer, microsatellites, nestmate recognition, population genetics, sharing

1 | INTRODUCTION

Invasive ants often reach extremely high densities, outcompete and prey upon native species, disrupt mutualisms, and lower ecosystem biodiversity (Berman et al., 2013; Holway, 1998; Holway et al., 2002; LeBrun et al., 2013; McGlynn, 1999; Porter & Savignano, 1990). Understanding the factors that promote the success of invasive ants is critical to discerning and managing their ecological impacts. Characteristics that are thought to play a role in the success of many invasive ant species include reduced nestmate recognition and an absence of boundaries between unrelated nests (Eyer & Vargo, 2021; Holway et al., 2002; Passera, 1994). By avoiding the costs of colony defense and competition against neighbouring conspecifics, these invasive ant species can reach higher densities (Giraud et al., 2002; Porter et al., 1992) and achieve greater ecological dominance by more effectively outcompeting other species (Holway et al., 2002; Holway & Suarez, 2004; LeBrun et al., 2013). For example, the number of Argentine ant workers (*Linepithema humile*) was approximately 50-fold higher in sites where nests were interconnected compared with sites where nests defended distinct boundaries and competed with conspecifics (Holway & Suarez, 2004). Consequently, interconnected nests of Argentine ants more effectively outcompeted native ants, as native ant species richness was reduced by over 50% compared with sites where nests competed with each other (Holway & Suarez, 2004). Reduced intraspecific competition has also been implicated in the success and ecological impacts of other invasive and noninvasive social insects (Hanna et al., 2014; Korb & Foster, 2010; Perdereau et al., 2015; Wilson et al., 2009).

Colony boundaries and nestmate recognition are typically delimited using worker aggression assays, but there is increasing evidence that workers can discriminate nestmates from non-nestmates without an aggressive response (Breed, 2003). For example, although Argentine ant workers do not aggressively attack non-nestmates from within the same supercolony (Giraud et al., 2002; Tsutsui et al., 2000), they spend more time antennating non-nestmates than nestmates (Björkman-Chiswell et al., 2008), indicating nestmate recognition despite a lack of aggression. Perhaps as a consequence of nestmate recognition, sharing between Argentine ant nests was consistently limited to distinct clusters of nests within a single supercolony over a three-year-period (Heller et al.,2008). By preferentially sharing food resources with nestmates over non-nestmates, workers may increase their inclusive fitness, particularly if nestmates are more related to them (Hamilton, 1964; Helanterä et al., 2009). Because aggression bioassays do not always reliably indicate nestmate recognition and colony boundaries, it is important to use alternative methods to assess intraspecific interactions in the field, such as genetic analyses and direct quantification of resource exchange between nests (Ellis et al., 2017). Assessing some of the more subtle interactions between and within nests improves our understanding of the factors enhancing ant invasions and has important implications for invasive ant management.

We examined worker interactions between nestmates and non-nestmates in red imported fire ants (*Solenopsis invicta*; hereafter fire ants). Fire ants occur in two social forms: the polygyne form (i.e., colonies with multiple egg-laying queens) and the monogyne form (i.e., colonies with only a single egg-laying queen; Gotzek et al., 2007; Ross, 1993; Ross et al., 1996; Tschinkel, 2006). These two social forms are under the control of an inversion-based supergene, which spans over 13 Mb of a "social chromosome" (Muers, 2013; Wang et al., 2013). This social chromosome contains over 400 protein-coding genes (including Gp-9, which has been used as a marker to estimate the social form of the colonies), and experiences greatly reduced recombination (Arsenault et al., 2020; Yan et al., 2020). Interestingly, the fire ant supergene (and all the complex traits associated with it) exhibits two haplotypes (*SB* and *Sb*), which are passed on via Mendelian inheritance (Arsenault et al., 2020; Keller & Ross, 1998; Ross & Shoemaker, 2018; Wang et al., 2013). Colonies bearing the *Sb* supergene haplotype express the polygyne phenotype (i.e., colonies accept multiple *SB*/*Sb* queens and reject any *SB*/*SB* queens), whereas colonies bearing exclusively the *SB* haplotype express the monogyne phenotype (i.e., colonies accept only one *SB*/*SB* queen and reject all *SB*/*Sb* queens; Arsenault et al., 2020; Gotzek & Ross, 2008, 2009; Ross & Keller, 2002). Moreover, supergene control appears to be complete, as social organization is independent of genetic diversity within the colony (Gotzek & Ross, 2008), as well as nongenetic factors such as environmental odours, queen reproductive status, and prior social experience of the workers (Gotzek & Ross, 2007; Ross & Keller, 2002).

Because colonies require only a small number (10%–15%) of workers of the *Sb* haplotype to express the polygyne phenotype, workers appear to regulate social organization (Gotzek & Ross, 2008). One possible mechanism for worker control includes habituating the colony to an odour unique to *b*-carrying adults (Gotzek & Ross, 2008). This hypothesis is supported by a lack of nestmate recognition between polygyne workers in the field (Vander Meer et al., 1990) and a supposed absence of colony boundaries within polygyne populations throughout their invaded range in the USA (Bhatkar & Vinson, 1987). As a consequence, North American polygyne fire ant populations are often referred to as unicolonial (e.g., Greenberg et al., 1992; Holway et al., 2002; Morel et al., 1990; Plowes et al., 2007; Porter et al., 1992; Vander Meer et al.,1990). The exchange of workers and resources between nests in polygyne fire ants is thought to correspond with a greater abundance compared with the monogyne form due to reduced intraspecific competition (Porter et al., 1991). For example, polygyne mounds were over twice as abundant on average compared with monogyne mounds in Texas (mean ± standard error [SE]: 680 ± 475 polygyne mounds/ha vs. 295 ± 240 monogyne mounds/ha; Porter et al., 1991). The greater abundance of the polygyne form may increase the likelihood of ants interacting with and preying upon native species, thereby increasing their ecological impact (Allen et al., 2004; Porter & Savignano, 1990).

Despite the assumption that polygyne nests are highly interconnected (see Bhatkar & Vinson, 1987), the physical exchange of workers and resources between polygyne nests in the field is poorly documented. Moreover, although polygyne workers from different nests do not aggressively attack each other in bioassays (Vander

Meer et al., 1990), their interactions within the nest are relatively unknown, particularly in the case of worker–brood interactions. Prior research on within-colony interactions has focused almost exclusively on worker–queen interactions (DeHeer & Ross, 1997; Gotzek & Ross, 2008; Ross & Keller, 2002), but worker–brood interactions are also critical to colony dynamics and can differ from worker-queen interactions. For example, although *Formica argentea* workers in polygyne colonies show no preference towards related or unrelated queens, they preferentially care for brood that are more closely related to them (Snyder, 1993). Within-colony relatedness between polygyne fire ant workers is often near zero throughout their invaded range in the USA (DeHeer & Ross, 1997; Goodisman et al.,2007; Ross, 1993; Ross & Fletcher, 1985; Ross et al., 1996), but workers may increase their inclusive fitness by preferentially caring for more related brood.

Our study tests fundamental assumptions about inter- and intracolonial interactions in introduced populations of fire ants. First, we compared colony boundaries between the two social forms in the field. To delimit boundaries between colonies, we quantified the exchange of a ^{15}N-glycine tracer dissolved in a sucrose solution and correlated this exchange with colony genetic structure. Using a labeled resource in combination with genetic data allows for two different ways to define colony boundaries (Ellis et al., 2017). We also examined polygyne brood-tending behaviors towards nestmates and non-nestmates in the laboratory. By studying interactions between and within colonies of *S. invicta*, we further elucidate the primary factors influencing the ecology and success of this invasive species.

2 | MATERIALS AND METHODS

2.1 | Between-nest interactions

To quantify sharing between nests in the field, we treated selected nests in each site with a stable isotope tracer and quantified its movement into neighbouring nests (Figure 1). Stable isotope tracers employ naturally occurring, nonradioactive forms of biologically relevant elements, such as nitrogen. The heavier isotope of nitrogen (^{15}N) occurs rarely in nature, so by artificially "spiking" a food with an appropriate concentration of this heavy isotope, we can trace the movement of this isotope through consumers and identify the flow of nutrients through an ecosystem (Fry, 2006). We ensured that only the treated nests had access to the isotope tracer, so if a neighbouring untreated nest showed unnaturally high levels of ^{15}N, this would indicate an exchange of either workers or resources between the treated and untreated nests (i.e., no boundaries between nests). In a preliminary laboratory experiment, we detected highly elevated levels of ^{15}N as quickly as 24 h after feeding fire ant workers an isotope tracer, and these isotope values decreased steadily over a 72 h period (Kjeldgaard, 2020). These preliminary results indicate that the tracer must have been relatively recently consumed to be detected in workers, which would allow for an effective measure of the movement of marked food.

Sampling was conducted between August and October 2019 in six field sites in Texas, USA (Appendix S1). Habitats ranged from restored grasslands (sites O, A, and B) to mowed fields (sites C, T, and T2). Mounds were used as a proxy for individual nests. At this stage of the experiment, we could not determine if distinct mounds/nests belonged to the same polydomous colony. After searching each field site and identifying all active fire ant nests, we selected three clusters of four to five nests at each site. One nest within each cluster was selected as the treatment nest. Clusters were separated by at least 50 m within each site to avoid potential sharing between clusters (Figure 1). Mounds were selected to represent varying distances within each cluster (between 0.4 and 29.07 m from the treated nest, with an average distance of 7.65 ± 0.72 m) to determine any effect of distance on sharing between nests.

Similar to other studies (Goodisman et al., 2007), several fire ant nests disappeared or moved over the course of the sampling period. As a consequence, we were unable to find three nests (one nest from site T, one from O, and one from T2) after the treatment period. Each of these were untreated nests within different clusters, so their removal did not affect the number of clusters analysed in each location. In total, we sampled from 73 fire ant nests across six sites, with 12 nests in Site A, 13 in Site B, 12 in site C, 11 in Site O, 13 in Site T, and 12 in Site T2.

2.2 | Treatment with the tracer

We fed workers from each treated nest a nitrogen tracer mixed in sugar water. A solution with 102 mM of ^{15}N-labelled glycine (98 atom%; Sigma-Aldrich, Inc.) and 61.5 mM of unlabeled sucrose was created using distilled water. This concentration was determined based on a preliminary laboratory experiment with a small number of fire ant workers and an approximation of colony sizes in the field, which can exceed 250,000 workers within a single nest (Tschinkel, 2006). The solution was mixed in bulk at the beginning of the field experiment and frozen between uses to avoid mold growth. We filled 1 ml microtubes with 1 ml of the solution and stoppered each with cotton. Three of these vials were left on the surface of each treatment nest and replaced every other day for 14 days. Vials were placed directly on the mound surface to ensure that only the treated nest fed on the solution. Each treatment nest was fed a total of 160 mg of ^{15}N-labeled glycine in 21 ml of sugar solution over a 14-day period. Fire ant workers were observed feeding on the solution, and there was evidence of nest building over the vial opening, indicating worker foraging.

We collected workers from all treated and untreated nests once immediately before feeding the tracer to treated nests and once at 14 days after beginning the treatment. Workers were collected by disturbing a small section of each nest and aspirating 40–50 workers for isotopic and genetic analyses (see Section 2.4). Workers were frozen at −10°C. We then transferred 10–30 workers per nest to 95% EtOH for storage prior to DNA extraction and left the remaining workers for stable isotope analysis. Workers for stable isotope

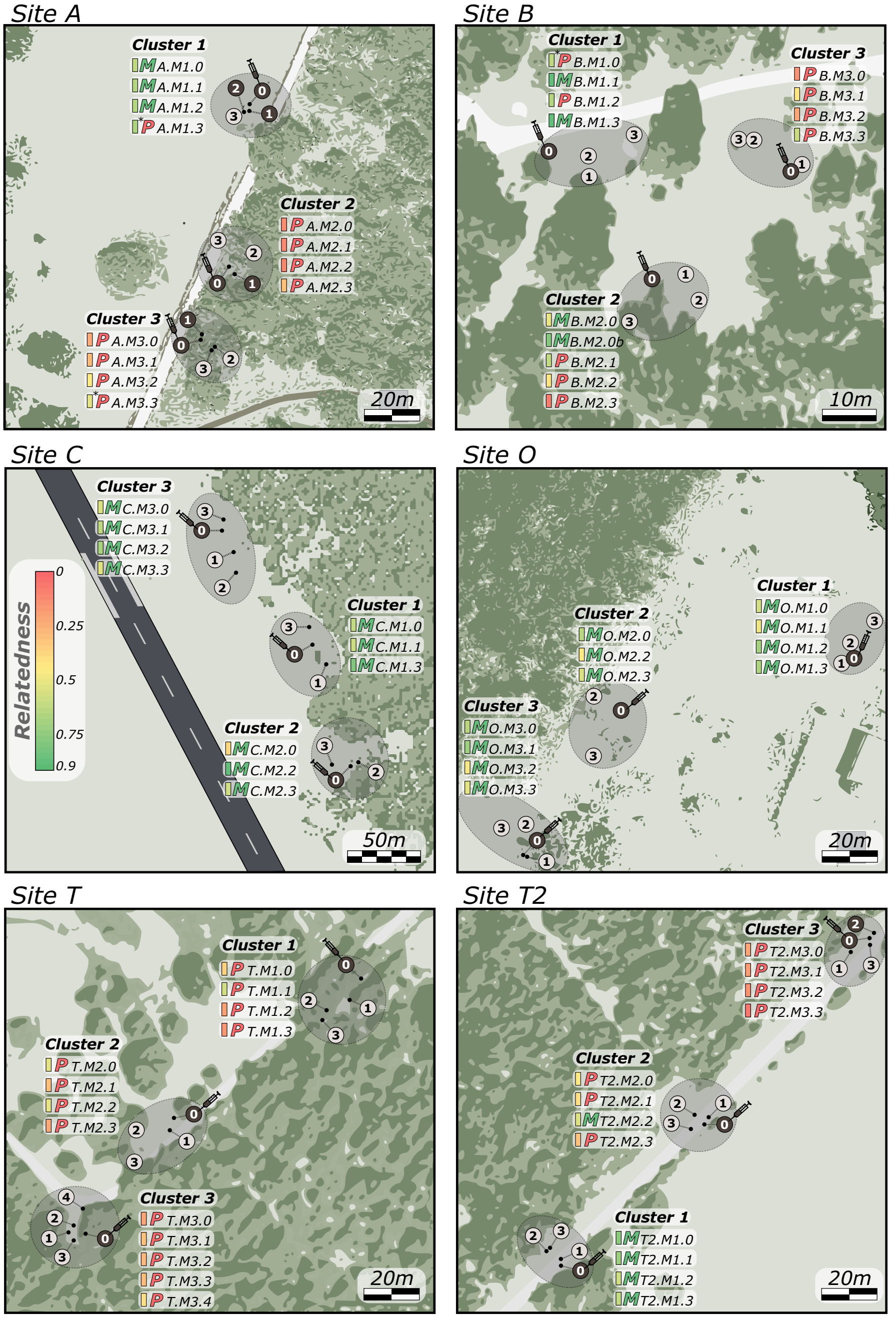


FIGURE 1 Map of sampled nests within each field site. Nests treated with an isotope tracer are labeled as "0" within each cluster. Dark grey circles indicate that worker isotope values increased after 2 weeks of treatment (i.e., δ^{15}N values of workers were greater than 20‰), whereas light grey circles indicate that isotope values did not change (mean natural abundance δ^{15}N values before tracer treatment: 5.00‰ ± 0.15‰). The social form according to *Gp-9* results (P, polygyne; M, monogyne), as well as within-nest relatedness are specified for each mound [Colour figure can be viewed at wileyonlinelibrary.com]

analysis were never stored in EtOH to avoid possible effects of EtOH on isotopic signatures (Tillberg et al., 2006).

2.3 | Stable isotope analyses

Workers were placed in an oven at 60°C until dry (approx. 24–48 h) and then stored in airtight vials prior to processing. The abdomens of all ants were removed prior to weighing to avoid the effects of stomach contents on isotopic signatures (Tillberg et al., 2006). To achieve appropriate weights for each sample, 5–10 workers per sample were pooled and chopped in glass vials to fine homogeneous powders using small scissors. Approximately 0.400 mg of each sample was weighed into tin capsules (Costech Analytical Technologies Inc.) using a microbalance (Mettler Toledo). All samples were analysed at the Texas A&M University Stable Isotopes for Biosphere Science Laboratory (https://sibs.tamu.edu/) using a Delta V Advantage Isotope Ratio Mass Spectrometer coupled with a Costech Elemental Analyser and Thermo ConFlo IV Universal Interface (Thermo Fisher Scientific). All baseline samples (collected before nests were fed the tracer) were analysed before any post-feeding samples to ensure that natural abundance values were not influenced by memory effects from the high levels of ^{15}N in spiked samples. Nitrogen isotope ratios are presented in δ notation:

$$\delta^{15}\mathrm{N}\,(‰) = [(R_{\mathrm{sample}} - R_{\mathrm{standard}})/R_{\mathrm{standard}}] \times 10^3$$

where R_{sample} is the $^{15}N/^{14}N$ ratio of the sample and $R_{standard}$ is the $^{15}N/^{14}N$ ratio of the atmospheric N standard (Coplen, 2011; Mariotti, 1983). Precision was 0.1‰.

To verify that our isotope tracer methods could detect resource sharing over 30-m distances, we conducted a proof-of-concept experiment using a population of tawny crazy ants (*Nylanderia fulva*) near College Station, Texas. This species forms a single supercolony throughout its invaded range in North America, in which workers regularly share collected resources with each other and occupy transitory nests (Eyer et al., 2018; Wang et al., 2016). Our results from this experiment confirmed that the isotope tracer is highly successful at detecting sharing in unicolonial populations at distances that were relevant for our study (i.e., up to 28.4 m from the treated area; Kjeldgaard, 2020) using the same biomass of workers for isotope analysis.

2.4 | Genetic analyses

Genetic analyses were used to infer the social structure (i.e., monogyne or polygyne) and genetic relatedness within and between each fire ant nest analysed. For each nest, eight workers were randomly selected for DNA extraction. DNA was extracted from individual workers following a modified Gentra-PureGene protocol (Gentra Systems).

To determine the social form of each nest, we pooled the DNA extracted from the eight individual workers per nest and screened this pooled sample for the presence of the *Gp-9^b^* allele, exclusively present in polygyne colonies (e.g., only present in individuals with the *Sb* haplotype; Arsenault et al., 2020; Krieger & Ross, 2002; Ross & Keller, 1998). A PCR reaction was performed on each pooled sample using the specific primer pair 24bS and 25bAS (Valles & Porter, 2003). This primer pair amplifies a 423-bp amplicon, and a successful amplification denotes the presence of the *Gp-9*^{b} allele, thereby characterizing the workers as polygyne. Amplifications were performed according to the protocol described in Valles and Porter (2003) and visualized on a 1% agarose gel. In all, we identified 38 monogyne and 35 polygyne nests across all six fire ant sites using the *Gp-9* method, of which 10 monogyne and 8 polygyne nests were treated with the isotope tracer.

In addition, five microsatellite markers previously developed for *S. invicta* (*Sol11*, *Sol20*, *Sol42*, *Sol49* and *Sol55*; Krieger & Keller, 1997) were amplified for each of the eight individual workers per nest. The allelic polymorphism of these five microsatellites was previously shown to be suitable to delimit colonies of *S. invicta* and infer their colony structure (Krieger & Keller, 1997). The microsatellites were genotyped using the M13-tailed primer method (Boutin-Ganache et al., 2001), consisting of 5′-fluorescently labelled tails with 6-FAM, VIC, PET or NED dyes to facilitate multiplexing. DNA amplifications were performed in a volume of 15 μl including 0.25–1.0 U of MyTaq HS DNA polymerase (Bioline), 2 μl of MyTaq 5× reaction buffer (Bioline), 0.08 μl of each primers, 0.08 of each M13 dye and 1 μl of the DNA template. PCR reactions were carried out using a Bio-Rad thermocycler T100 (Bio-Rad). PCR products were sized against LIZ500 internal standard on an ABI 3500 genetic analyser (Applied Biosystems). Allele calling was performed using Geneious software v.9.1 (Kearse et al., 2012).

For every nest, the social structure result obtained with the *Gp-9* method was confirmed using microsatellite markers, inferring whether all workers from a nest could be assigned to a single queen (carrying one of the two alleles of the mother queen at each microsatellite marker studied). Polygyny was deduced when more than one worker per colony could not be unambiguously assigned to a single queen (see Appendix S2 for results). In addition, we compared the relatedness coefficients (*r*) between monogyne and polygyne nests (as identified using *Gp-9*) using analysis of variance to verify that relatedness coefficients were significantly lower in polygyne versus monogyne nests (i.e., suggesting the reproduction of several unrelated queens) and to determine any differences by site. We also used *t*-tests to establish if relatedness coefficients were significantly different from zero for polygyne nests (i.e., multiple unrelated queens producing workers within a single nest) and 0.75 for monogyne nests (i.e., one singly-mated queen producing workers within a nest). Relatedness coefficients were calculated using the program COANCESTRY v.1.0 (Wang, 2011), according to the algorithm described by Queller and Goodnight (1989). Relatedness coefficients were weighted equally and SEs were obtained by jackknifing over colonies. Relatedness coefficients were also calculated separately for each site to prevent an artificial overestimation of relatedness within colonies due to potential differences in the genetic background between sites.

Colony spatial structure was investigated for the six sites to determine whether distinct nests of *S. invicta*, especially those collected within 5 m of each other, consisted of a single colony (i.e., polydomy) or separate colonies. To answer this question, genotypic frequencies at all nests were compared using a log-likelihood (G)-based test of differentiation using GENEPOP ON THE WEB (Rousset, 2008). Bonferroni's correction was applied to account for multiple comparisons of all pairs (adjusted *p*-value < .0008). Significance was determined using a Fisher's combined probability test.

Colony clustering was visualized for each site by plotting individuals on a principal component analysis (PCA) using the adegenet R package (Jombart, 2008). The clustering of nests into distinct colonies was also represented by Bayesian assignments of individuals into genetic clusters (i.e., colonies; *K*) using STRUCTURE v.2.3.4 (Pritchard et al., 2000). For each site, STRUCTURE simulations were run with values of *K* from 1 to the total number of nests encountered in each site and repeated 10 times for each value of *K*. Each run included a 5×10^4 burnin period followed by 1×10^5 iterations of the Markov Chain Monte Carlo (MCMC). The mean posterior probability LnP(K) (Pritchard et al., 2000) implemented in StructureSelector (Li & Liu, 2018) was used to estimate the most likely number of genetic clusters in each data set. Additional details and results for clustering analysis can be found in Appendices S2 and S3.

2.5 | Within-colony relatedness of fire ants in the literature

We also compiled all published coefficients of within-colony relatedness between workers in the red imported fire ant to compare our results with those in the literature. We searched the Web of Science using the following search terms: “Solenopsis invicta” OR “red imported fire ant” AND population AND microsatellite. Our search generated 87 records, but a large number of these studies focused on a different ant or social insect species. We excluded any study that contained only queen–queen relatedness coefficients, as well as any study of a different species of ant or social insect. We reviewed each record and extracted within-colony relatedness coefficients between workers (means and SE whenever available) and recorded the sampling location. In total, we extracted information from eight studies.

2.6 | Nestmate discrimination within polygyne colonies

To evaluate polygyne nestmate discrimination, we conducted a laboratory experiment testing worker tending behavior towards larvae from the same nest and larvae from a different nest. To do this, we established six single-lineage experimental colonies by collecting mated polygyne queens following mating flights. All colonies were kept in standardized laboratory conditions for at least two years to ensure they were large enough to be divided into smaller experimental colonies (i.e., over 360 workers and 150 larvae). Colonies were confirmed as the polygyne social form by screening workers for the presence of the *Gp-9*^{b} allele using the same methods as described above (see Section 2.4). Once incipient colonies were large enough, we used food dye to label the brood of each colony. We dyed brood by giving workers two separate tubes of 15 ml of water and 15 ml of artificial nectar each containing 0.9 ml of food colouring (McCormick Food Colors & Egg Dye, McCormick & Company, Inc.). We gave three colonies (colonies A, B, and C) yellow food colouring and three colonies (colonies D, E, and F) green food colouring (Table 1). Colours were randomly assigned. During this six-day period of brood dyeing, we did not give the ants any proteinaceous food so that dye would be highly visible in the guts of larvae.

Next, we created experimental colonies by combining 0.1 g of workers (~120 workers) with 50 larvae from the same natal colony as the workers (i.e., nestmates) and 50 larvae from a different colony (i.e., non-nestmates; see Table 1 for complete family combinations). Not all permutations of families were logistically possible in this experiment, so only larval combinations of different colours were combined so that all possible two-colour combinations were created (Table 1). In all, there were 18 experimental colonies.

TABLE 1 Single-lineage colony combinations to determine worker discrimination

	D (Green)	E (Green)	F (Green)
A (Yellow)	AD, DA	AE, EA	AF, FA
B (Yellow)	BD, DB	BE, EB	BF, FB
C (Yellow)	CD, DC	CE, EC	CF, FC

Note: Identity of the family (A, B, C, D, E, F), dyed colour of the brood (yellow or green), and experimental colony combinations provided. Experimental colonies were constructed with 0.1 g workers (~120) and 50 larvae (related brood) from the family indicated by the first letter in the experimental colony combinations. The second letter indicates the family of the other 50 larvae (unrelated brood).

To quantify the feeding of larvae by workers, experimental colonies were given 7.5 ml artificial nectar containing 0.2 g of nontoxic, fluorescent dye (DFDRY-C0 UV Dye from Risk Reactor) for 18 h. After 18 h, we recorded the number of larvae remaining from each family and used a black light to count the number of larvae from each family fed the fluorescent dye. In order to ensure accurate results for potentially variable behaviours, data for experimental colonies were averaged across the three iterations of this experiment. This allowed us to remove within colony temporal variation and estimate the general behaviours of each experimental colony instead of only looking at a single snapshot of their behaviour.

2.7 | Data analysis

We used logistic regression to determine the effects of spatial distance, genetic differentiation (using pairwise F_{ST} values), social form, within-nest relatedness coefficients between workers, and site on whether or not untreated nests shared with the treated nest. To do this, we constructed generalized linear models with a binomial distribution using the glm function in base R statistical software v3.6.1 (R Core Team, 2019). Distance from the treated nest, pairwise F_{ST} values (compared between the treated and untreated nests), social form of both treated and untreated nests (i.e., monogyne or polygyne), within-nest relatedness coefficients between workers in both treated and untreated nests, and site were treated as independent variables. The sharing status of the untreated nests (i.e., “shared with the treated nest” or “did not share with the treated nest”) was the dependent, binary variable. Nests that were identified as having shared with the treated nest had δ^{15}N values greater than 20‰, as these values were far higher than any natural abundance isotope values observed at our field sites (mean natural abundance δ^{15}N values before tracer treatment: 5.00‰ ± 0.15‰). Untreated nests could only have attained δ^{15}N values greater than 20‰ by freely exchanging workers and/or resources with the treated nest. All other nests were designated as “did not share with the treated nest.” All plots were generated using ggplot2 (Wickham, 2016).

Data from the laboratory experiment were analyzed in R statistical software using paired *t* tests. Percentage data were arcsine-square-root transformed prior to analysis. All graphs were produced with untransformed data. A more detailed description of the methods can be found in Appendix S4.

3 | RESULTS

3.1 | Between-nest sharing and colony genetic structure

All treated nests showed elevated δ^{15}N values, indicating that our methods were successful in enriching the isotope values of individual nests. Counter to our expectations, treated nests shared very little with neighbouring nests, regardless of social form (Figure 2a) and within-nest relatedness between workers (Appendix S5). Five of the 52 untreated nests showed evidence of sharing with the treated nest (two monogyne and three polygyne). Sharing was independent of the social form of the treated nest ($\chi^2 = 0.0091$, df = 1, $p = .924$), the social form of the untreated nest ($\chi^2 = 0.0001$, df = 1, $p = .992$), and by the interaction between these variables ($\chi^2 = 0.0061$, df = 1, $p = .938$). Moreover, sharing was independent of within-nest relatedness between workers in the treated nest ($\chi^2 = 0.0681$, df = 1, $p = .794$), by relatedness between workers in the untreated nest ($\chi^2 = 0.7718$, df = 1, $p = .380$), and by the interaction between these variables ($\chi^2 = 0.000$, df = 1, $p = 1.000$).

There was a significant effect of distance on whether or not sharing was detected in untreated nests ($\chi^2 = 10.0858$, df = 1, $p = .001$). All untreated nests with elevated δ^{15}N values were within 5 m of the treated nest (Figure 2b). However, not all nests within 5 m of the treated nest shared (Figure 2b, Appendix S6), indicating that distance was not the only component influencing sharing between nests. There were 13 untreated nests within 5 m from the treated nest that did not share with the treated nest, one of which was only 0.4 m from the treated nest. Sharing between nests did not vary by site ($\chi^2 = 2.0408$, df = 5, $p = .843$).

Treated nests were genetically indistinguishable from the untreated nests with which they shared and were probably part of the same polydomous colony. Pairwise F_{ST} values between the treated and untreated nests were significantly lower on average and near zero in nests that shared (mean and SEs: 0.028 ± 0.015) than in nests that did not share (0.228 ± 0.021; $\chi^2 = 14.5562$, df = 1, $p < .001$; Figure 2c). Likewise, all untreated nests that shared were not genetically differentiated from the treated nest according to the log-likelihood (G)-based test of differentiation. Genetic clustering of

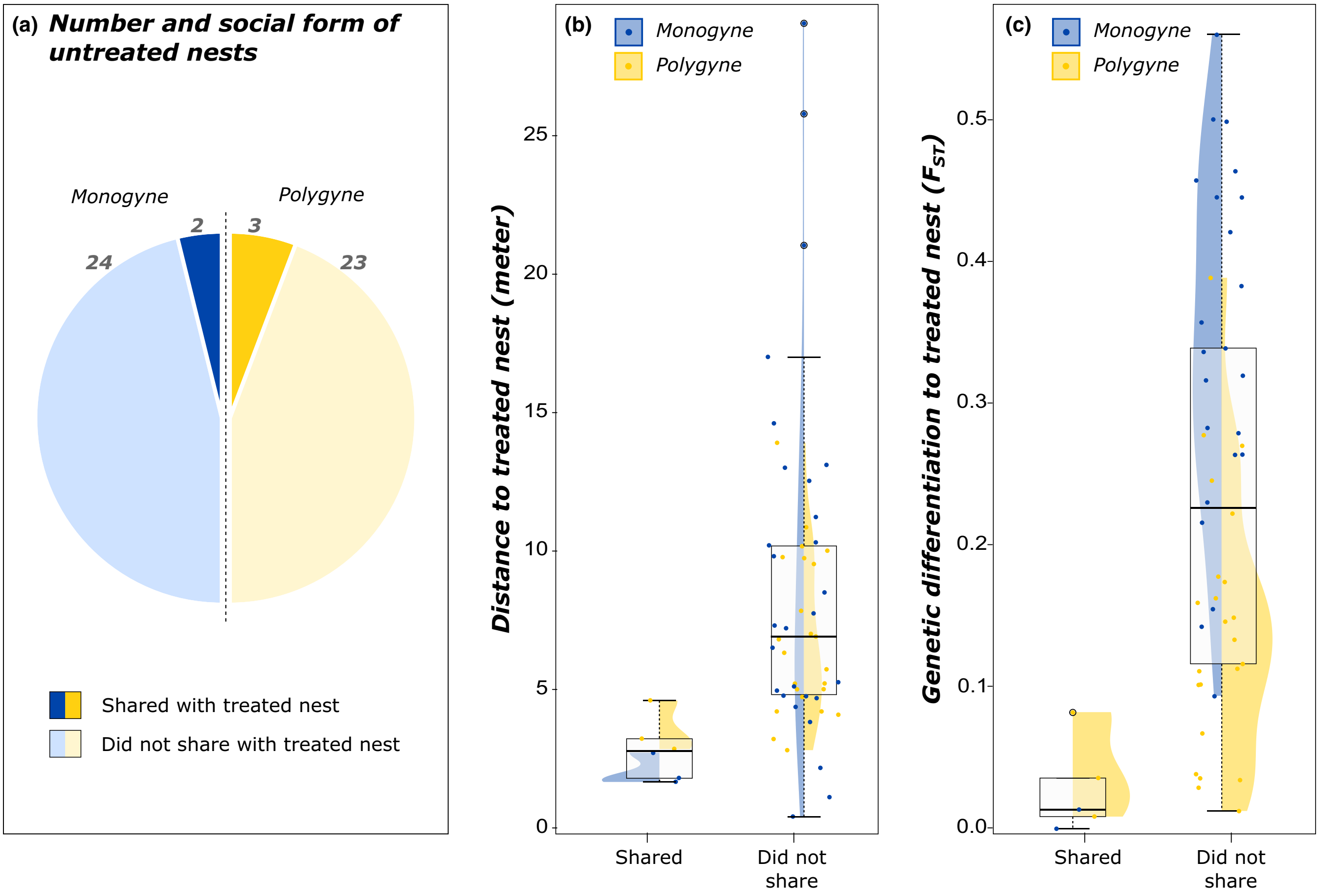


FIGURE 2 (a) Very few untreated nests shared with treated nests, regardless of social form. Plot includes the status of an isotope tracer in untreated nests by social form according to *Gp-9* results. (b) All untreated nests that shared were within 5 m of the treated nest. Plot includes status of an isotope tracer in untreated nests by distance in metres to the treated nest. (c) All untreated nests that shared showed very low pairwise F_{ST} values with the treated nest. Plot includes status of an isotope tracer in untreated nests by pairwise F_{ST} values compared to the treated nest. Results shown are from all six sites. Nests with δ^{15}N values greater than 20‰ indicated that untreated nests exchanged workers and/or resources with the treated nest ("Shared"), and the values of all other nests indicated that untreated nests did not exchange workers or resources with the treated nest ("Did not share") [Colour figure can be viewed at wileyonlinelibrary.com] nests using PCA and STRUCTURE analyses is provided for each site in Appendix S3. There was no significant interaction between distance and pairwise F_{ST} values ($\chi^2 = 0.3395$, df = 1, $p = .560$). Pairwise F_{ST} between polygyne nests within each site was lower than between monogyne nests (mean and SEs of 0.144 ± 0.008 and 0.356 ± 0.010 respectively), but there was no significant interaction between pairwise F_{ST} and social form ($\chi^2 = 0.0000$, df = 3, $p = 1.000$) on sharing between nests. For each site, the number of alleles, as well as observed and expected heterozygosity are provided for each microsatellite marker in Appendix S7.

3.2 | Within-colony relatedness and comparison with the literature

At our sites, within-nest relatedness was significantly higher in monogyne nests (mean and SEs: 0.644 ± 0.024) than in polygyne nests (0.269 ± 0.037; $F_{1,60} = 75.832$, $p < .001$). However, relatedness in polygyne nests at our sites was significantly greater than zero ($t_{33} = 7.249$, $p < .001$; 95% confidence interval [CI]: 0.193–0.344). Relatedness in monogyne nests was significantly lower than 0.75 ($t_{35} = -4.368$, $p < .001$; 95% CI: 0.595–0.693), consistent with our genetic results showing that some colonies (18/38) were headed by a multiply mated queen (Appendix S8), as has been reported previously (Fritz et al., 2006; Lawson et al., 2012). Within-nest relatedness did not vary by site ($F_{5,60} = 1.781$, $p = .130$), nor did the within-nest relatedness of each social form vary by site ($F_{3,60} = 0.382$, $p = .766$). In all but one nest that were defined as monogyne using *Gp-9*, the presence of a single reproducing queen was confirmed using microsatellite markers, as all workers could be assigned to a unique queen. The reproduction of multiple queens was confirmed in all but three polygyne nests.

Based on our analysis of within-colony relatedness coefficients between workers reported in the literature, no other study has analysed within-colony relatedness between fire ant workers in Texas, USA (Figure 3). Of the five studies conducted in Georgia, USA, four reported coefficients in polygyne populations that were not significantly greater than zero, and one reported a coefficient that was significantly greater than zero (mean: 0.16; Ross et al., 1993; Figure 3). Studies conducted on introduced polygyne populations in Australia and Taiwan revealed relatedness coefficients higher than zero (Richlands, Australia: 0.246; Chiayi, Taiwan: 0.1444; Taoyuan, Taiwan: 0.1122). Likewise, within-nest relatedness coefficients of polygyne fire ants in their native range of Argentina were also greater than zero (Corrientes: 0.24; Formosa: 0.15).

3.3 | Nestmate discrimination within polygyne colonies

After 18 h in experimental colonies with workers, significantly more nestmate brood remained compared with non-nestmate brood (Figure 4a; $t_5 = 3.883$, $p = .012$). The number of non-nestmate brood diminished by 27% while nestmate brood diminished by only 14%. No dead larvae were found in the colonies.

Likewise, the feeding of larvae by workers differed depending on the relationship between workers and brood. A higher percentage of nestmate brood were fed compared with non-nestmate brood (Figure 4b; $t_5 = 3.246$, $p = .023$).

4 | DISCUSSION

Here we provide evidence that polygyne fire ants in Texas can and do discriminate between nestmate and non-nestmate workers and brood. Sharing between nests of both social forms was limited to nests within the same polydomous colony, indicating that fire ants maintain strict colony boundaries regardless of social form. Polygyne workers also preferentially fed larvae from the same natal colony and may have cannibalized non-nestmates during times of stress. One hypothesis for worker control of colony social form is that polygyne workers may habituate the colony to odours unique to *b*-carrying adults (Gotzek & Ross, 2008). Based on our results, however, this hypothesis is unlikely. Finally, within-nest relatedness between polygyne workers in the field was higher than those previously reported in North America (DeHeer & Ross, 1997; Goodisman et al., 2007; Ross, 1993; Ross & Fletcher, 1985; Ross et al., 1996; but see Ross, 1993) and much more similar to those found in native populations (Ross et al., 1996) where polygyne workers display well-developed nestmate recognition (Chirino et al., 2012). Past studies have referred to polygyne fire ant populations as unicolonial (e.g., Greenberg et al., 1992; Holway et al., 2002; Morel et al.,1990; Plowes et al., 2007; Porter et al., 1992; Vander Meer et al., 1990), but our results suggest a multicolonial structure, which has important implications for the ecology and management of this species.

Counter to our expectations, we detected distinct colony boundaries between almost all nests in the field regardless of social form (i.e., polygyne nests were no more likely to share than monogyne nests; Figure 2a) and within-nest relatedness between workers (Appendix S5). The nests that did share with each other were probably part of the same polydomous colony, as suggested by their low genetic differentiation and small spatial distance between nests (Figure 2). Despite previous assumptions that polygyne fire ant populations are highly interconnected, several other studies have found evidence of boundaries, at least on some level, between polygyne colonies (Goodisman et al., 2007; Krushelnycky et al., 2010; Weeks et al., 2004). For example, polygyne nests in Georgia, USA, showed distinct genotypic frequencies and worker weight profiles, suggesting that workers and queens are not moving freely between nests (Goodisman et al., 2007). Moreover, although polygyne workers do not aggressively attack non-nestmates like their monogyne counterparts (Vander Meer et al., 1990), workers will antennate and occasionally bite non-nestmates in the laboratory, indicating nestmate recognition (Obin et al., 1993). There is also evidence of exploitative competition between polygyne nests in the field (Weeks et al., 2004).

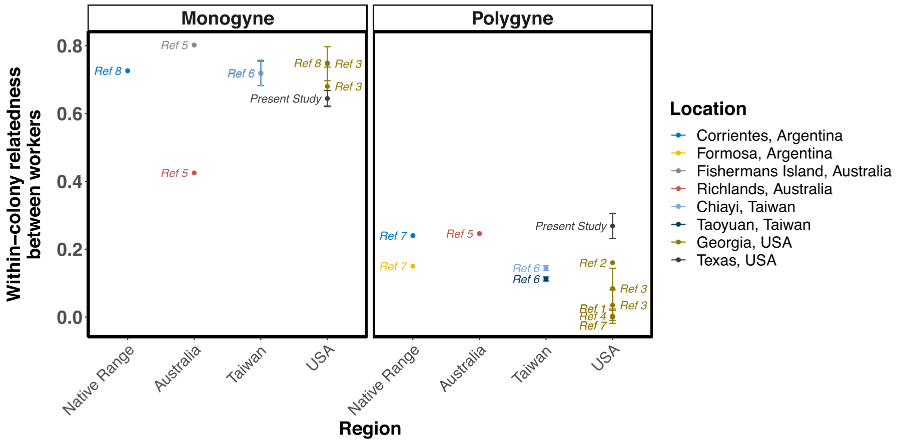
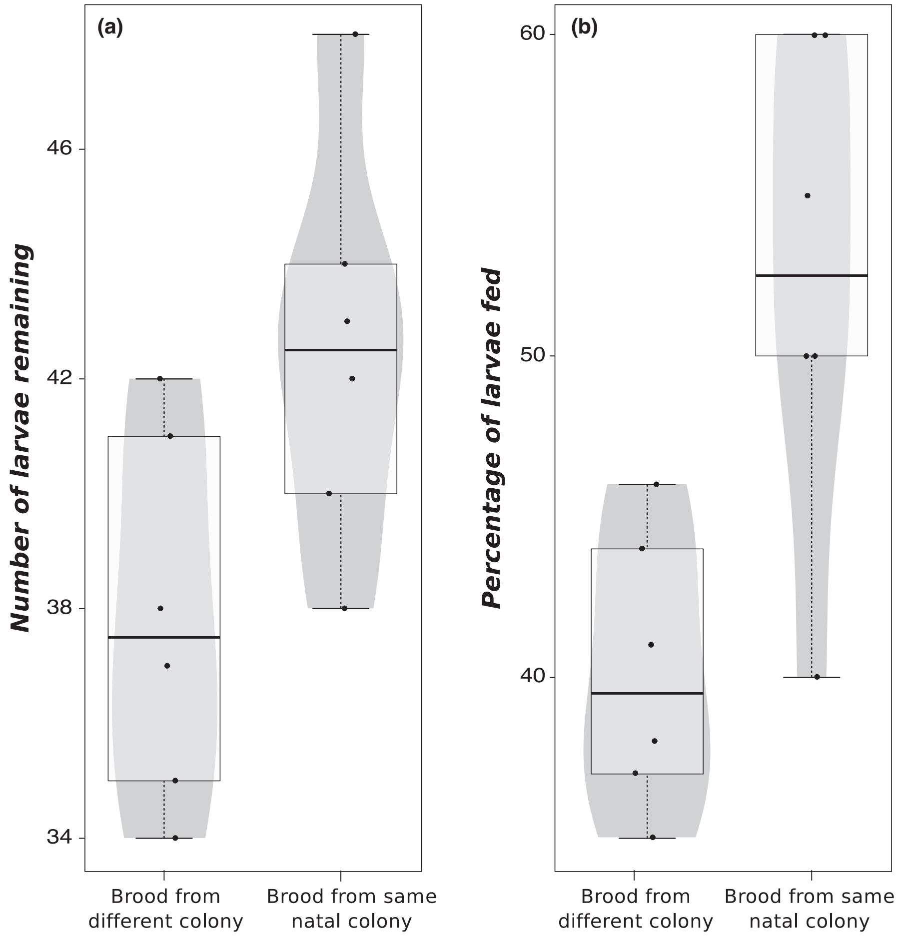


FIGURE 3 Within-colony relatedness coefficients between workers of fire ants (*Solenopsis invicta*) by social form from multiple populations. Data based on results extracted from the literature and results in the present study. Points and error bars (when available) represent mean ± standard error (SE). *Ref 1*, Goodisman et al. (2007); *Ref 2*, Ross (1993); *Ref 3*, Ross and Fletcher (1985); *Ref 4*, DeHeer and Ross (1997); *Ref 5*, Henshaw et al. (2005); *Ref 6*, Yang et al. (2008); *Ref 7*, Ross et al. (1996); *Ref 8*, Ross et al. (1993) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 (a) Number of brood remaining after 18 h with workers. (b) Percentage of brood fed after 18 h with workers



Moreover, boundaries appear to be present at relatively small spatial scales, as many nests of both social forms did not exchange resources despite being within 5 m from each other (Appendix S6). Weeks et al. (2004) found that most labeled polygyne fire ant workers remained within 4 m of their colony. In our study, we found nests with distinct boundaries separated by <1 m, suggesting that nests very close to each other may belong to different colonies. These results imply that fire ants are able to distinguish nestmates from non-nestmates, even when environmental odour cues may be similar from living in close proximity. Heritable and environmental odour cues are thought to be additive in fire ants, but monogyne and polygyne fire ant workers have been shown to distinguish nestmate from non-nestmate despite similar environmental odour cues (Obin, 1986; Obin et al., 1993). It should be noted that nest structure and even nestmate recognition can change seasonally in other ant species (Heller & Gordon, 2006; Katzerke et al.,2006), so we may have detected colony boundaries at such small spatial scales due to our sampling in the late summer/ early fall. It will be important to verify our results at other times of the year to determine if colony boundaries shift temporally.

Our laboratory experiment provides further evidence that polygyne fire ants are able to discriminate between nestmates and non-nestmates, as workers preferentially fed brood from the same natal colony (Figure 4a). Workers may have even preferentially cannibalized non-nestmate brood, because there was a significantly greater reduction in the number of non-nestmate than nestmate brood remaining at the end of the experiment (Figure 4b). Larvae were given to the colonies by placing them outside of the nest dishes and allowing the workers to bring them into the nest, so it is also possible that workers collected greater numbers of nestmate than non-nestmate brood. We found no desiccated larvae, however, in or around the experimental colonies. Instead, we hypothesize that polygyne fire ant workers preferentially cannibalize non-nestmate brood in times of stress. High levels of cannibalism are known from this species (Sorensen et al.,1983; Tschinkel, 1993) and often occur when resources are in short supply (e.g., a lack of proteinaceous food). All colonies were kept in standardized laboratory conditions and fed standardized diets to minimize acquired, environmental identification cues (Obin et al., 1993). As such, heritable odour cues may have played a more important role in worker recognition because nestmate and non-nestmate brood came from two different mothers. Workers that preferentially care for closely related nestmates consequently increase their own inclusive fitness (Hamilton, 1964; Helanterä et al., 2009), but the potential for nepotism must be further investigated using laboratory colonies with multiple queens (rather than individuals as in our experiment). It is important to note that laboratory colonies were kept separated for 2 years, which may have accentuated worker behavior towards non-nestmates. Our results suggest, however, that the presence alone of the *b* allele of the supergene does not determine sharing between workers and larvae in polygyne fire ants.

We also detected greater relatedness within polygyne colonies than previously reported in North America, which may be one reason why we observed distinct boundaries between field colonies. Although relatedness between workers was lower within polygyne nests than within monogyne nests, relatedness coefficients in polygyne nests were much higher (mean and SEs: 0.269 ± 0.037) than those previously observed in other introduced populations in the USA (Figure 3; DeHeer & Ross, 1997; Goodisman et al., 2007; Ross, 1993; Ross & Fletcher, 1985; Ross et al., 1996). Past studies have reported values that were not significantly different from zero due to the reproduction of many unrelated queens within the same nest (but see Ross, 1993), but our results suggest that some workers within polygyne nests in Texas may even be half-sisters (expected r for half-sisters = 0.25) or cousins from highly related mothers (expected r for cousins $=0.75 \times 0.5 \times 0.5 = 0.19$). Interestingly, our relatedness coefficients between workers were more similar to those reported in native polygyne fire ant populations (Figure 3; Ross et al., 1996). In these native populations, polygyne colonies are multicolonial; nestmate queens are highly related (Ross et al., 1996), workers recognize nestmate from non-nestmate (Chirino et al., 2012), and colony densities are 4–7 times lower than those observed throughout North America (Porter et al.,1997). Although we did not measure relatedness between nestmate queens, our behavioral results in the field and in the laboratory support the conclusion that polygyne fire ants in Texas probably function similarly to native conspecifics, in that colonies are multicolonial and could engage in high levels of intraspecific competition. It is important to note that our within-nest relatedness coefficients between polygyne workers were also similar to those reported in Australia and Taiwan (Figure 3; Henshaw et al., 2005; Yang et al., 2008), so it would be interesting to determine if other introduced populations of polygyne fire ant behave similarly to those in Texas and in the native range.

One explanation for why we observed higher relatedness coefficients than those previously documented in the USA could be that the polygyne nests that we surveyed contained fewer queens than those sampled in past studies. Ross (1993) demonstrated that relatedness between workers within polygyne nests in Georgia was negatively correlated with queen number. Geographic variation in colony genetic structure, perhaps due to variation in queen number, may also explain the higher within-nest relatedness and pairwise F_{ST} values in polygyne nests compared with those in other states (DeHeer & Ross, 1997; Goodisman et al., 2007; Ross & Fletcher, 1985; Ross et al., 1996; but see Ross, 1993). Much of the population genetics data of introduced polygyne fire ants in the USA has focused on one or a few geographic regions (DeHeer & Ross, 1997; Ross, 1993; Ross & Fletcher, 1985; Ross & Keller, 1995; Ross et al., 1996). Although fire ant populations in Texas have been shown to vary genetically from other parts of the country (Shoemaker et al., 2006), only a few studies have examined colony genetic structure in Texas (Chen et al., 2003; Ross et al.,1993, 1996), and none that we know of have reported within-colony relatedness between workers (Figure 3). It is also possible that colony genetic structure has changed over time. For example, relatedness was almost twice as high in older compared with younger populations (i.e., over 100 years old vs. 17 years old) in the polygyne ant *F. fusca* (Hannonen et al., 2004). Past studies of polygyne fire ant queens in Texas reported a near zero relatedness between co-occurring queens (Chen et al., 2003; Ross et al., 1996), which should result in similarly low relatedness between workers, but it is possible that within-colony relatedness has increased over the past 20 years. The ecological impact of polygyne fire ants weakened significantly over a 10-year period in parts of Texas (Morrison, 2002), which may have corresponded with a change in genetic structure.

Relatedness alone does not predict sharing between nests, however, as some studies have detected colony boundaries despite very low relatedness between polygyne fire ant nests (e.g., Goodisman et al., 2007). Likewise, several neighbouring nests in our study had low pairwise F_{ST} and relatedness values but did not share with each other (Appendix S6). In other ant species, kinship does not always correlate with a lack of boundaries between nests (Procter et al., 2016). For example, nests of the polygyne ant *F. lugubris* did not share workers or resources with each other despite high genetic relatedness (Procter et al., 2016). Similarly, Argentine ants (*L. humile*) did not freely exchange workers between all nests within a single supercolony, even though there were no detectable genetic differences between nests (Heller et al., 2008). Likewise, gene flow was limited, and some workers were unexpectedly aggressive towards each other within the same supercolony in the unicolonial ant *F. pressilabris*, suggesting that supercolonies do not always function as a single unit (Hakala et al., 2020). This highlights the importance of quantifying colony boundaries using several different methods (Ellis et al., 2017), as genetic relationships do not always reflect the levels of worker and resource sharing. Low relatedness among nestmate workers may also result from extreme polygyny, where workers originate from numerous unrelated queens (Keller, 1995). In this case, each polygyne colony can contain as much genetic diversity as the background population, with nestmate workers being as related to each other than to any random worker within this population, leading to a zero relatedness within the colony (Queller & Goodnight, 1989). Future research should examine the exchange of resources between polygyne fire ant nests in other parts of their invaded range where within-nest relatedness has been reported to be positive (i.e., Australia and Taiwan) or close to zero (i.e., Georgia, USA) to ultimately determine the relationship between sharing and genetic relatedness.

Our study tests fundamental assumptions about polygyne fire ant behavior and suggests that workers can discriminate between nestmate and non-nestmate. Polygyne fire ants are often more abundant than the monogyne form throughout their invaded range (Porter et al., 1991), but our results suggest that their high abundance is not due to a lack of boundaries between neighbouring colonies, at least in parts of Texas. Rather, variation in competitive interactions (e.g., territoriality vs. exploitative competition) or queen dispersal strategies may better explain differences in fire ant abundance between social forms (DeHeer et al., 1999; Weeks et al., 2004). Past research has found that the “social chromosome” determines worker acceptance of queens (DeHeer & Ross, 1997; Gotzek & Ross, 2008; Ross & Keller, 2002) and worker aggression towards non-nestmates (Vander Meer et al., 1990), but our results show that supergene control does not extend to sharing between nests or sharing between workers and brood. Our study also has important implications for fire ant management, as it is often assumed that fields with polygyne fire ants may require less insecticidal bait due to high interconnectedness and sharing between nests (i.e., horizontal transfer). Although this is an effective management strategy in the unicolonial Argentine ant (*L. humile*; Buczkowski & Wossler, 2019), our results suggest that polygyne fire ants are multicolonial and should be managed similarly to their monogyne counterparts. Overall, our findings call for caution in assuming that a lack of clear aggression and genetic differentiation among nests always denotes a collapse of colony boundaries in ants. By directly quantifying sharing between nests in the field, future research can trace and measure the flow of workers and resources among nonaggressive nests. Identifying the network of connectivity among nests within supercolonies will surely provide insights into the factors promoting invasive ant success and will improve our understanding and managing of their ecological impacts.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

MacKenzie K. Kjeldgaard, Micky D. Eubanks, and Edward L. Vargo conceived the study. MacKenzie K. Kjeldgaard collected samples and conducted the stable isotope experiment with assistance from Joanie T. King, Ayumi Hyodo, Thomas W. Boutton, and Micky D. Eubanks. Collin C. McMichael and Alison A. Bockoven conducted the laboratory experiment. Pierre-André Eyer and Joanie T. King performed the genetic analyses. MacKenzie K. Kjeldgaard and Pierre-André Eyer analysed the data. MacKenzie K. Kjeldgaard wrote the manuscript with contributions from Collin C. McMichael, Pierre-André Eyer, Joanie T. King, Ayumi Hyodo, Thomas W. Boutton, Edward L. Vargo, and Micky D. Eubanks.

DATA AVAILABILITY STATEMENT

Microsatellite genotypes, raw stable isotope data, and all raw laboratory experiment data files have been made available on Dryad: https://doi.org/10.5061/dryad.jq2bvq89g.

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