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Population structure of *Myrmica rubra* (Hymenoptera: Formicidae) in part of its invasive range revealed by nuclear DNA markers and aggression analysis

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Abstract

Myrmica rubra (Linnaeus, 1758) is considered an invasive ant in North America. The genetic relatedness and its dispersive abilities were investigated using microsatellite analysis of colonies on the island of Newfoundland, Canada. The genetic diversity of Newfoundland *M. rubra* was low, likely as the result of founder effect. Colonies located near each other (< 1 km) generally had low pairwise F_{ST} values, which we interpret as considerable mixing of colonies, while populations that are spatially separated showed higher F_{ST} values and very little mixing. Aggression analysis was also used to determine the relatedness of populations. In this analysis, considerable aggression was observed among *M. rubra* worker ants from the different localities, and it depended on the level of genetic relatedness. Calculation of Pearson correlation coefficients showed a positive correlation in aggressive behavior with genetic relatedness. The molecular and aggression analyses of *M. rubra* populations from Newfoundland localities indicate that these ants are not unicolonial over large areas but supercolonial with colonies genetically similar and with aggression reduced toward con-specifics at the local scale (< 1 km).

Key words: Invasive ant, *Myrmica rubra*, dispersal, aggression, Formicidae.

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Introduction

The European Fire Ant, *Myrmica rubra* (Linnaeus, 1758), is native to Eurasia where it is commonly referred to as the common red or ruby ant (Wetterer & Radchenko 2011). It is a small, red to dark-red ant that has a nasty deposition for its aggressive stinging behavior toward any vertebrate that gets too close to the nests. This species exhibits characteristics of typical invasive ants (Garnas & al. 2007) and has the potential to cause considerable ecological and economic damage in the areas where it becomes established. The first record of its introduction to North America was near Boston, Massachusetts, USA, in 1900 (Wheeler 1908). Since that time, it has slowly increased its range northeastwardly and presently has a fragmented distribution in seven US states (Massachusetts, New York, Rhode Island, Vermont, New Hampshire, Maine and Washington) (Wetterer & Radchenko 2011). Moreover, it has also become established in seven Canadian provinces including Ontario, Quebec, New Brunswick, Nova Scotia, Prince Edward Island (Wetterer & Radchenko 2011), British Columbia (Naumann & Higgins 2015), and the island portion of Newfoundland and Labrador (Hicks 2012). The introduction of this ant into new localities is thought to be caused by the human movement of contaminated soil from nursery stock (Groden & al. 2005). However, this has been challenged recently by Hicks & al. (2014) who suggested that the mechanism of introduction into eastern Newfoundland was likely by contaminated ballast soil directly from Britain many years ago. This is a well-documented mechanism for the introduction of many plants and animals to North America (Lindroth 1957, Cooper 1981).

Colonies of *Myrmica rubra* have multiple queens (polygynous) and consist of multiple smaller nests comprising a large nest (polydomous) (Elmes 1973). Nuptial flights of *M. rubra* occur in its native range but are localized events (Donisthorpe 1915, Hubbard & Nagell

1976, Elmes 1991). Nuptial flights had not been recorded in North America until 2010 when Hicks (2012) observed them in Newfoundland, Canada. Groden & al. (2005) did not find convincing evidence of nuptial flights in Maine, USA, even after intensive study, and it lead them to suggest that mating flights are most likely infrequent and that new queens probably mate and remain within their nests. Groden & al. (2005) and Elmes (1973) indicated that with reduced long-range flight dispersal in its invasive and native ranges, dispersal of *M. rubra* colonies is likely accomplished by budding. This behavior is known from other invasive ant species (Markin 1970, Tsutsui & Suarez 2003). It is interesting to note that Horton (2011), while not observing any nuptial flights of the ants in Halifax, Nova Scotia, did collect many alate queens in the nests during July. Hicks (2012) observed swarming of male *M. rubra* in Newfoundland, Canada, but did not, at that time, see any winged queens in the swarms or in the nests, and suggested that the male only swarming behavior is a mechanism to maintain genetic variability (Hicks 2012). However, on two occasions an alate queen and male were observed coupled on the ground under large swarms during two separate years (B.J. Hicks , unpubl.). Data from Steiner & al. (2006) suggests that the dispersal of the males and mating with queens from distant nest does occur. Donisthorpe (1915) mentioned that the polygyny of *M. rubra* nests in Britain was caused by seeking of original nests by females which had been fertilized near their own nests. In Europe, mating swarms of *M. rubra* are generally considered to occur closer to the nest site and this has led some to suggest long-distance dispersal of females rarely occurs (Elmes 1991, Elmes & Clarke 1981). Molecular studies using allozymes have shown that there is restricted dispersal of *M. rubra* females (Walin & al. 2001, Seppä & Pamilo 1995). *Myrmica rubra* in Finland showed significant genetic differentiation between populations (F_{ST} = 0.2) (Seppä & Pamilo 1995). The authors claimed that new nests are founded close to the mother nest by budding of existing nests, a view taken by Groden & al. (2005) for the ant in North America. Additionally, Steiner & al. (2006) used a combination of data from microsatellites of nuclear DNA and mitochondrial DNA to show that dispersal by females was not as great as dispersal by males. Sorvari (2017) showed that in the native range of *M. rubra* gene flow between colonies occurs by the acceptance of mated alien queens into these colonies. He suggested that this tendency for *M. rubra* to readily accept mated alien queens may increase the genetic diversity of colonies and may be one of the factors for *M. rubra* invasion success in parts of North America. However, there is no evidence to corroborate this statement from North America.

McGlynn (1999) showed that 147 ant species were found outside of their native ranges and shared the characteristic of being tramp species (i.e., closely associated with human activities). Some of the tramp species such as *Linepithema humile* (Mayr, 1868) (Argentine ant), *Wasmannia auropunctata* (Roger, 1863) (small fire ant), *Pheidole megacephala* (Fabricius, 1793) (big-headed ant), and *Solenopsis invicta* Buren, 1972 (imported fire ant) initially showed unicoloniality in their invasive ranges (Ross & Keller 1995, Ross & al. 2003, Tsutsui & al. 2000, Tsutsui & Case 2001, Giraud & al. 2002, Holway & al. 2002, Vogel & al. 2010). In these causes, unicoloniality was believed to be a social structure where colony boundaries over wide areas were broken and there was an accompanied loss of intraspecific aggression between individuals. Some authors (i.e., Garnas & al. 2007) have used multicolonial to describe colonies who defend their territories and are aggressive to other nests (even ones that are next to it). Studies have challenged the unicolonial description of invasive ants and suggest that the ants are organized into mutually aggressive supercolonies (Vogel & al. 2009). Supercolonies comprise large networks of polydomous-polygynous nests that exhibit no within but strong between-supercolony aggression (Vogel & al. 2009). The supercolonial nature of the Argentine ants in its native range was shown by Vogel & al. (2010) who suggested they were pre-adapted for invading new habitats. Huszár & al. (2014) suggests two principal methods for supercolony ant formation: (1) it emerges from a single founding colony that expands locally by budding and whose ants would then mate exclusively within the colony resulting in a low genetic diversity and zero genetic differentiation, (2) it arises from smaller colonies merging into large polydomous colonies where substantial genetic diversity and differentiation should be observed.

In its native range, some *Myrmica rubra* populations in northern Europe exhibit elevated levels of intraspecific tolerance (Seppä & Pamilo 1995, Seppä & Walin 1996). These populations show localized patch dominance, reproduce by budding, and show restricted gene flow locally. Fürst & al. (2012) indicated that *M. rubra* in its native range exhibits increased aggression with increasing distance between colonies and that the aggression was correlated with genetic relatedness and cuticular hydrocarbons. Meanwhile, an increase in genetic diversity lead to a reduction in aggression and the aggression was reduced among workers of colonies with high queen numbers (Fürst & al. 2012). Following this, Huszár & al. (2014) showed that *M. rubra* supercolonies do occur in its native range. They showed that Danish *M. rubra* supercolonies had high numbers of co-breeding queens with low nestmate relatedness, suggesting that they may have resulted from the merger of smaller colonies.

In its invasive range, *Myrmica rubra* exhibited patch dominance (Groden & al. 2005). Initially, it was observed that the *M. rubra* populations of Maine (USA) showed low incidence of intraspecific aggression and that the populations resembled the characteristics observed in supercolonies of *L. humile*. However, Garnas & al. (2007) did not show supercoloniality over a large area but did record reduced aggression at a local scale of < 10 m. Garnas & al. (2007) rejected the idea that *M. rubra* is supercolonial in its invasive range and thought that, in fact, it exhibited multicolonial organization. Chen & al. (2018) recently showed that *M. rubra* in its invasive range in northeast-

Tab. 1: The sampling locations for *Myrmica rubra*, their geographical coordinates, and remarks on distances to other sampling locations. NL = Newfoundland and Labrador.

Number	Locality	Latitude; longitude	Remarks
1	Canada, NL, Carbonear, Bemister's Hill	47°44'30.24" N; 53°13'28.48" W	185 m from 2&3; 125 m from 4&5; 900 m from 6&7; 43 km to St. John's
2	Canada, NL, Carbonear, Bond Street	47°44'30.58" N; 53°13'19.55" W	80 m from 3; 130 m to 4&5
3	Canada, NL, Carbonear, St. James Church	47°44'33.20" N; 53°13'20.46" W	180 m from 4&5
4	Canada, NL, Carbonear, Tucker's Lane 1	47°44'27.44" N; 53°13'23.90" W	1 m from 5; 800 m from 6&7
5	Canada, NL, Carbonear, Tucker's Lane 2	47°44'27.45" N; 53°13'23.82" W	
6	Canada, NL, Carbonear, Water Street 1	47°44'23.14" N; 53°12'46.28" W	20 m from 7
7	Canada, NL, Carbonear, Water Street 2	47°44'23.39" N; 53°12'47.40" W	
8	Canada, NL, Corner Brook, Country Road	48°56'34.80" N; 57°58'12.83" W	2 km from 9; 374 km from Carbonear
9	Canada, NL, Corner Brook, Glynmill Inn	48°56'48.83" N; 57°56'33.49" W	
10	Canada, NL, Seal Cove Seaspray Crescent	47°27'37.73" N; 53°05'12.61" W	26 km from St. John's; 32 km from Carbonear; 400 km from Corner Brook
11	Canada, NL, St. John's, Lloyd Crescent	47°32'19.73" N; 52°44'42.90" W	1.25 km from 12; 5.5 km from 13; 14 km from 14
12	Canada, NL, St. John's, Bowring Park	47°31'40.90" N; 52°44'53.36" W	15 km from 14
13	Canada, NL, St. John's, Torbay Road	47°34'52.87" N; 52°42'17.61" W	6.5 km from 12
14	Canada, NL, Torbay, Falkirk Place	47°39'35.48" N; 52°42'58.61" W	
15	Germany, Görlitz, Görlitz Cemetery	51°09'42.00" N; 14°59'04.00" E	1200 km from 16; 4750 km from Newfoundland
16	UK, England, Worth Matravers	50°35'53.39" N; 02°03'04.56" W	3600 km from Newfoundland

ern USA showed no increased aggression based on the distance between colonies. They suggest that neighboring *M. rubra* colonies compete with one another and restrict their territories. This may be why Garnas & al. (2007) found ants to be multicolonial over shorter distances (> 10 m between colonies). Naumann & al. (2017) suggested that *M. rubra* formed at least two supercolonies in British Columbia (Canada). One of these supercolonies was several kilometres across and no intraspecific aggression was observed.

The purpose of the present research is to use microsatellites of DNA analysis to determine the relatedness of populations both within localized areas of the island of Newfoundland, Canada, and compared to more distant localities (i.e., England, UK). In addition, aggression tests between workers of populations will be conducted. By comparing results of both analyses we wish to elucidate the coloniality of the ant *M. rubra* in the Newfoundland part of its invasive range.

Methods

Sample locations: Worker ants from the colonies were collected and preserved in 100% ethanol at each location to be used for the molecular analysis. The ants were identified using an unpublished key of André Francoeur (Université du Québec, Chicoutimi, Canada). Verification of the identifications was by A. Francoeur. Voucher specimens are deposited in the Newfoundland collection at the Canadian Forestry Service in Edmonton, Alberta, Canada. For the aggression experiments, one entire colony from each locality was excavated into 2 L plastic tubs and returned to the lab. Table 1 gives the localities sampled and the geographic distances among colonies. Worker ants preserved in 100% ethanol were obtained from single colonies from England and Germany (see Tab. 1 for locality information).

DNA extraction and microsatellite PCR: DNA was extracted from the legs of 157 ants using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Toronto, On-

Tab. 2: Details of microsatellite loci used to screen *Myrmica rubra* ants in this study, including primer sequences, dinucleotide repeat pattern, fluorescent label applied and multiplex grouping for PCR.

Locus	Primer sequence (5' - 3')	Repeat	Dye	Reference	Multiplexgroup
MS26	F: GAG TAT GCG AAG ATG TCC A R: GAA TAG TTA GGG TTT GCT G	$(GCA)_n$	6-FAM	Azuma & al. (2005)	I
MS86	F: GAT AGC AGA TAA AAC GA R: AGT CGT GAG TAC AAC AT	$(GA)_n$	VIC	Azuma & al. (2005)	I
MS3.62	F: CAG AAA AGT CGT ATC C R: GTA GTA ATG CCG TTC A	$(CT)_nTT(CT)_n$	NED	Azuma & al. (2005)	I
MP67	F: GTT CTC TGA GCT TCT CTC CTA C R: TCC TTT CTT CTA CCT CTT ATG AG	$(AC)_n$	VIC	Herbers & Mouser (1998)	II
Msc7	F: GCT TTA ATT CCG GGA CAC TC R: AAA GGC GAT TAA ACG TGG TG	$(TG)_n$	PET	Zeisset & al. (2005)	II
Myrt4	F: CGT GCA TGC GAG CAT TCC GT R: CGG CGA TGC AAG TAC GTC	$(GT)_nGC(GT)_n$	6-FAM	Evans (1993)	-

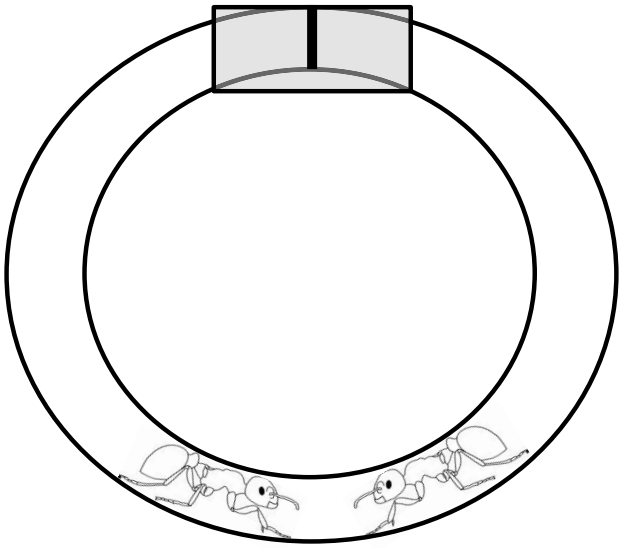

Fig. 1: Arena for testing aggression between individual worker ants from different colonies and from the same colonies (controls).

tario, Canada) following the Tissue Protocol. Legs were digested overnight, and DNA was resuspended in two consecutive 75 µL elutions, for a final volume of 150 µL of DNA. Additional DNA that was extracted from 95 *Myrmica rubra* ants of a previous study (Hicks & al. 2014) was also analysed for a total of 252 samples.

Extracted DNA was screened at six microsatellite loci (see Tab. 2) in a combination of two multiplex PCRs (polymerase chain reactions), while one locus, Myrt4, was amplified individually. Each reaction contained 0.6 µM of each primer, with the exception of Msc7 primers for which 0.4 µM was used. In addition to primers, all reactions contained 1X QIAGEN Multiplex PCR Master Mix, 0.5X QIAGEN Q-Solution (Qiagen Inc.), and 1 µL of DNA template. All PCR experiments included a no-template control.

Thermal cycling was performed in a GeneAmp 9700 Thermal Cycler (Applied Biosystems Inc., Foster City, California, USA). Multiplex I was amplified using the following conditions: 95 °C for 15 minutes, followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 45 seconds and 72 °C for 1 minute, and a final extension of 60 °C for 30 minutes. Multiplex II was amplified with the following conditions: an initial denaturation of 95 °C for 15 minutes, 35 cycles of 94 °C for 30 seconds, 53 °C for 45 seconds, and a final extension at 60 °C for 30 minutes. Myrt4 was amplified with an initial denaturation of 95 °C for 15 min, 35 cycles of 94 °C for 30 seconds, 60 °C for 45 seconds and 72 °C for 1 minute, and a final extension of 60 °C for 30 minutes.

Microsatellite loci were run with an internal standard (LIZ500, Applied Biosystems Inc.) on an Applied Biosystems 3730 DNA Analyzer using GeneScan software (Applied Biosystems Inc.), and analysed using Peak Scanner Software v1.0 (Applied Biosystems Inc.). Alleles were scored by two independent readers.

Aggression experiments: The collected ant colonies were kept in the laboratory at ambient temperature and humidly and fed sugar water and pieces of tuna for protein. The ants were used in experiments within one week of sampling. Aggression was assessed by placing two ants into a circular arena compose of a 15 cm long piece of Nalgene® (Fisherbrand, Ottawa, Ontario, Canada) tubing (3.18 mm internal diameter and 1.59 mm wall thickness) (Fig. 1). Worker ants from different colonies were tested and ants from the same colonies were tested as a control. After the ants were placed into the arena the ends were sealed together using a transparent plastic sleeve to make a circular runway. As the ants walked around the runway and encountered each other, their interactions were recorded. Observations included overt fighting (grabbing and stinging) or passing (indifference to each other's presence). Observation of each encounter was for 5 minutes and 20 encounters occurred for each test. Fresh ants and a new piece of tubing was used for each encounter.

Data analysis: The software Micro-Checker v2.2.3 (van Oosterhout & al. 2004) was used to determine whether null alleles were present at any of the loci used in this study. Loci were checked for departures from Hardy-Weinberg and linkage equilibrium using Arlequin v3.5

(Excoffier & Lischer 2010). Measures of population diversity (expected heterozygosity, H_E) and differentiation (pairwise F_{ST}) were also calculated using Arlequin. Calculation of the Pearson product moment correlation between aggressive behavior and genetic relatedness, plus the Mann-Whitney test for differences in genetic diversity between the colonies was completed in Minitab (version 15). Significance of values was determined at the $p > 0.05$ confidence level.

Results

Microsatellite profiles were obtained for 252 ants, of which 221 (87%) were complete profiles and 31 were partial profiles. The level of missing data across the entire set was 2.81%. No locus showed significant departure from linkage equilibrium in any sample, at $\alpha = 0.05$ after Bonferroni correction for multiple tests. One locus (Msc7) showed possible evidence of null alleles in one population (Country Road, Corner Brook), indicated by significant $H_O < H_E$ at $\alpha = 0.05$, after Bonferroni correction. Across all loci, no sample showed a significant departure from $F_{IS} = 0$ after Bonferroni correction for multiple tests. However, for both Corner Brook populations F_{IS} was positive but significantly so only for Glynmill before Bonferroni adjustment ($F_{IS} = 0.120$, $p = 0.166$ for Country Road, and $F_{IS} = 0.366$, $p = 0.003$ for Glynmill). In addition, F_{IS} was also significantly positive for Lloyd Crescent and Torbay Road before Bonferroni adjustment ($F_{IS} = 0.300$, $p = 0.015$ for Lloyd Crescent, and $F_{IS} = 0.420$, $p = 0.014$ for Torbay Road).

Tab. 3: Genetic diversity (expected heterozygosity, H_E) of different populations of *Myrmica rubra* in its native and invasive range, revealed by 6-locus microsatellite profiles.

Population	H_E
1. Carbonear, Bemister's Hill	0.27
2. Carbonear, Bond St	0.24
3. Carbonear, St. James Church	0.24
4. Carbonear, Tucker's Lane 1	0.23
5. Carbonear, Tucker's Lane 2	0.24
6. Carbonear, Water St 1	0.16
7. Carbonear, Water St 2	0.25
8. Corner Brook, Country Rd	0.46
9. Corner Brook, Glynmill Inn	0.41
10. Seal Cove, Seaspray Cres	0.22
11. St. John's, Lloyd Cres	0.25
12. St. John's, Bowring Park	0.18
13. St. John's, Torbay Rd	0.31
14. Torbay, Falfirk Pl	0.22
15. Germany	0.28
16. UK	0.45

Tab. 4: Pairwise F_{ST} values among colonies of *Myrmica rubra* in its invasive and native range. All values are significantly positive at $\alpha = 0.05$ after Bonferroni correction, except those bolded. The locality numbers correspond to those in Tables 1 and 3.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1															
2	**0.13**														
3	**0.04**	**0.03**													
4	**0.04**	0.19	**0.10**												
5	**0.03**	0.16	**0.08**	**0.00**											
6	**0.07**	0.40	0.25	0.19	0.18										
7	**0.02**	0.16	**0.09**	**0.00**	**0.01**	0.17									
8	0.28	0.33	0.32	0.33	0.34	0.40	0.31								
9	0.35	0.44	0.39	0.39	0.39	0.43	0.37	0.21							
10	0.40	0.45	0.44	0.44	0.42	0.53	0.47	0.37	0.43						
11	0.46	0.44	0.43	0.50	0.48	0.59	0.46	0.33	0.41	0.61					
12	0.55	0.45	0.51	0.58	0.54	0.72	0.51	0.39	0.53	0.69	0.22				
13	0.29	0.40	0.37	0.34	0.33	0.39	0.37	0.30	0.27	**0.05**	0.55	0.64			
14	0.35	0.39	0.41	0.40	0.38	0.47	0.42	0.38	0.43	**0.04**	0.60	0.65	**0.03**		
15	0.47	0.50	0.53	0.52	0.50	0.61	0.48	0.35	0.40	0.51	0.42	0.46	0.39	0.44	
16	0.36	0.42	0.37	0.40	0.40	0.46	0.38	0.27	0.24	0.39	0.45	0.28	0.44	0.36	0.46

Tab. 5: Aggression experiments of *Myrmica rubra* worker ants from different colonies in Newfoundland, Canada.

Colony 1	Colony 2	Aggression	F_{ST}
Carbonear, St James	Carbonear, Water St	1 / 20	0.09
Carbonear, St James	St. John's, Bowring	17 / 20	0.51
Carbonear, Tucker's Lane 1	Carbonear, Water St	14 / 20	0.25
Carbonear, Tucker's Lane 1	Carbonear, Tucker's Lane 2	0 / 20	0.00
Carbonear, Tucker's Lane	Corner Brook, Country Rd	19 / 20	0.34
Carbonear, Tucker's Lane	Corner Brook, Glynmill Inn	20 / 20	0.39
Carbonear, Tucker's Lane	Seal Cove	19 / 20	0.39
Carbonear, Tucker's Lane	St. John's, Torbay Rd	19 / 20	0.34
Carbonear, Tucker's Lane	Torbay, Falkirk Pl	20 / 20	0.40
Carbonear, Water St	St. John's, Bowring	15 / 20	0.51
Corner Brook, Country Rd	Corner Brook, Glynmill Inn	18 / 20	0.21
Corner Brook, Country Rd	Seal Cove	18 / 20	0.37
Corner Brook, Country Rd	St. John's, Bowring Park	19 / 20	0.39
Corner Brook, Country Rd	St. John's, Torbay Rd	19 / 20	0.30
Corner Brook, Country Rd	Torbay, Falkirk Pl	20 / 20	0.38
Corner Brook, Glynmill Inn	Seal Cove	19 / 20	0.43
Corner Brook, Glynmill Inn	St. John's, Bowring Park	18 / 20	0.53
Corner Brook, Glynmill Inn	St. John's, Torbay Rd	19 / 20	0.27
Corner Brook, Glynmill Inn	Torbay, Falkirk Pl	18 / 20	0.43
Seal Cove	St. John's, Bowring Park	20 / 20	0.69
Seal Cove	St. John's, Torbay Rd	17 / 20	0.05
Seal Cove	Torbay, Falkirk Pl	15 / 20	0.04
St. John's, Bowring Park	St. John's, Torbay Rd	19 / 20	0.64
St. John's, Bowring Park	Torbay, Falkirk Pl	20 / 20	0.65
St. John's, Torbay Rd	Torbay, Falkirk Pl	0 / 20	0.03
Carbonear, Tucker's Lane	*Formica subaenescens*	20 / 20	-

The genetic diversity was generally low (H_E < 0.3) in most of the ant samples (Tab. 3). The exceptions were the two Corner Brook sites (H_E = 0.46 and 0.41) and the UK sample (H_E = 0.45).

When looking at pairwise F_{ST}, values close to zero indicate that two populations are genetically similar and that there is considerable mixing of individuals between them. Typically, pairs of populations with F_{ST} > 0.2 are considered genetically distinct with limited mixing occurring (Wright 1943). Pairs of populations of *Myrmica rubra* sampled around the town of Carbonear generally had lower F_{ST} values (F_{ST} = 0-0.40, average 0.111; Tab. 4). This suggests that these colonies were likely from the same parent colony. Similarly, there were low F_{ST}s among St. Johns, Torbay Rd, Seal Cove, and Torbay, Falkirk Pl, indicating that these populations were related (average F_{ST} = 0.04; Tab. 4). Meanwhile, populations that were spatially separated showed higher F_{ST} values. For Corner Brook, Glynmill Inn, and St. John's, Bowring Park, a pair of populations separated by > 400 km, F_{ST} = 0.59. The largest value determined was F_{ST} = 0.72 between Carbonear, Water Street, and St. John's, Bowring Park, populations separated by 45 km. Other populations that were spaced apart also had high pairwise F_{ST} values (see Tab. 4). However, between the two populations in the Corner Brook area F_{ST} = 0.21, indicating that there was not much mixing between these nearby localities.

Results of the aggression experiments supports the molecular data. There was no aggression between worker ants from the same colonies (data not shown). As a positive control, encounters of *Myrmica rubra* workers and the outgroup species *Formica subaenescens* workers always ended in aggression. There was considerable aggression between *M. rubra* worker ants from the different localities and the level of which depended on their genetic relatedness. Calculation of Pearson correlation coefficients showed a positive correlation of aggressive behavior with genetic relatedness (R^2 = 0.661; p < 0.001). Ants from the Carbonear area populations were genetically related (see the low F_{ST} values in Tab. 4) and showed low aggression

(Tab. 5). Meanwhile, aggression was considerably higher between Carbonear colonies and St. John's, Bowring Park ants. The F_{ST} values calculated for these two populations showed them to be the least related (F_{ST} = 0.72) and were spatially separated by 43 km. The two Corner Brook populations were also genetically distinct (F_{ST} = 0.21) and aggression was observed between them. Corner Brook Glynmill Inn and St. John's, Bowring Park ants were genetically differentiated (F_{ST} = 0.53), separated by 400 km and had considerable aggression (18 / 20).

Discussion

Population genetic diversity and structure: The microsatellite genetic diversity of Newfoundland *M. rubra* was generally low in most populations likely caused by founder effects. This is consistent with results from Hicks & al. (2014) who showed that the Newfoundland populations of ants contained only one mitochondrial DNA haplotype each. Hicks & al. (2014) concluded from the mitochondrial sequence data that this ant species was introduced to Newfoundland from Britain at least four times and they suggested that the introductions probably predated the original record of Wheeler (1908) and generally occurred by the soil ballast mechanism that was proposed by Lindroth (1957).

In Hicks & al. (2014), the two Corner Brook populations were each defined by a unique haplotype not observed in any other population. In the present study, the microsatellite diversity of the two populations in Corner Brook is higher than the rest of the ants from Newfoundland and similar to the sample from Britain. This data supports Hicks & al. (2014) that these two populations, in particular, represent more recent introductions from Britain that did not occur by the typical ballast traffic mechanism. Furthermore, the positive F_{IS} observed in each of the two Corner Brook populations also potentially supports more recent founder events leading to populations not yet in genetic equilibrium. Alternatively the higher diversity and positive F_{IS} could be due to recent admixture from two or more source populations, however the mitochondrial data do not support this interpretation.

On the other coast of the island, the genetic diversity of ants at St. John's, Torbay Road, is higher than the other ants from eastern Newfoundland. In this case, these ants are more diverse genetically because they are likely a mixture of local (British) ants and ants introduced from the USA. Hicks & al. (2014) showed that ants at this location have a haplotype observed only in ants found in eastern USA and they suggested that the ants were introduced to St. John's, Torbay Road, after a USA air force base was established near that site in the 1940s. The St. John's, Torbay Road, population was the only one shown to contain two mitochondrial haplotypes, also consistent with mixed sources.

Low genetic diversity was also observed in the sample from Germany, but this is unlikely related to the Newfoundland ants. Historically, there was very little connection between the two regions, as was suggested by Hicks & al. (2014), nor is there any association between the locations suggested by the mitochondrial data.

The pairwise F_{ST} measure were fairly low among the populations around the Carbonear area and thus indicate considerable mixing (see Tab. 4). All of these populations are spatially close, ranging from 1 to 900 m apart. Meanwhile populations that were more separated were significantly differentiated, such as Carbonear populations compared with those near St. John's.

In general, most authors suggest that long-distance dispersal by *Myrmica rubra* queens does not occur and that dispersal is by budding of existing nests (Elmes & Clarke 1981, Elmes 1991, Seppä & Pamilo 1995, Walin & al. 2001, Groden & al. 2005 and Steiner & al. 2006). Once these new colonies are started, we would expect the colony to show significant relatedness as inbreeding would occur. This would especially be the case in its invasive range as intranidal mating occurs and nuptial flights are thought to be absent (Groden & al. 2005). Meanwhile in its native range, the opposite appears to occur. Colonies appear to be genetically differentiated even at the site level (Seppä & Pamilo 1995, Seppä & Walin 1996). Sorvari (2017) showed that mated alien queens are adopted by some nests in its native range resulting low related nestmates. Seppä & Pamilo (1995) showed that *M. rubra* populations on some Finnish islands showed strong differentiation both among sites and between islands. Seppä & Walin (1996) went on to show that populations from Finland are polygynous and showed low relatedness among worker nest mates. In a typical outbreeding population where there is dispersal of queens, one would expect this. Dispersal by males may also contribute to keeping the populations genetically heterogeneous. Steiner & al. (2006) showed that there was considerable dispersal of males and Seppä & Walin (1996) showed that the males that mated with queens in colonies were unrelated and thus concluded that significant outbreeding occurred.

Groden & al. (2005) did not observe nuptial flights and only one alate queen during substantial collecting of this species in Maine. Horton (2011) observed numerous alate queens during the summer in Halifax, Nova Scotia, Canada. In 2012, Hicks observed swarming behavior of *Myrmica rubra* in Newfoundland and located 56 swarms in an area where this species was dominant. These swarms consisted primarily of male ants and only two alate queen and male couplings were observed in the three years that swarms were observed. Male-only swarms are known to occur in other invasive ants (e.g., *Linepithema humile*) (see Markin 1970) and mediate gene flow between spatially separated colonies and prevent inbreeding depression (Passera & Keller 1994).

While the specific role of the male-only swarms observed by Hicks (2012) is unknown, he speculated that it may be a mechanism to help maintain gene flow between distant colonies. The results from the present study do not corroborate this. We found colonies located in Newfoundland more than 1 km apart are not genetically related and thus, the nuptial flights that are known to occur in

Newfoundland are probably not involved in long-distance dispersal of males. However, at the local scale (i.e., the Carbonear colonies) there is considerable gene flow and the swarms may be maintaining a low differentiation of *Myrmica rubra* in the area. This may be a reason contributing to the low F_{ST} values observed between the Carbonear colonies. To the contrary, Seppä & Pamilo (1995) and Seppä & Walin (1996) showed that nearby *M. rubra* populations in its native range showed significant differentiation between them and suggested that outbreeding by dispersing males results in low relatedness among nest mates.

Aggression analysis: Spatially separated colonies show significant aggression and this correlates with the gene flow between colonies. The colonies that are genetically distinct have workers that are able to distinguish ants that are nestmates against those that are from other colonies. Cuticular hydrocarbons are believed to be the factors that ants use for nestmate recognition (Lehav & al. 1999). These hydrocarbons may vary between colonies as a result of intrinsic genetic factors (Holway & al. 1998, Suarez & al. 2002) and extrinsic factors including diet and nesting substrate (Heinze & al. 1996, Singer & Espelie 1996; Liang & Silverman 2000). Holway & al. (2002) reviewed the literature and outlined examples of how several important invasive ants are supercolonial over extensive areas. In these cases, the ants form large polygynous colonies that lack defensive aggression behaviors against other nests in the area. The results from the aggression tests on *Myrmica rubra* colonies from Newfoundland show that the ants from the Carbonear area did not show aggression and we conclude that the colonies located in Carbonear comprise a large supercolony. The male-only swarms observed by Hicks (2012) contained male ants that were produced in the local area and do not migrate over long distances as proposed by Hicks (2012). The aggression observed between Seal Cove ants with Torbay Rd and Torbay, Falkirk Place ants is an anomaly as these populations were shown to be genetically related (shown by the low F_{ST} between the three areas and data from Hicks & al. 2014). It's likely that the distribution of these related ants occurred by recent human-mediated transport. Further aggression testing and cuticular hydrocarbon profile determination will help understand the importance of genetic and environmental influences on the behavior.

The molecular and aggression testing completed on *Myrmica rubra* populations from Newfoundland localities and from elsewhere, supports the research by Garnas & al. (2007) that they are not unicolonial over large areas of its invasive range (at least in Newfoundland). While Garnas & al. (2007) suggest that *M. rubra* is multicolonial in Maine, our evidence suggests that Newfoundland *M. rubra* are supercolonial with genetically related ants exhibiting reduced aggression toward other related ants.

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