Supercolonies of the invasive ant, *Myrmica rubra* (Hymenoptera: Formicidae) in British Columbia, Canada

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ABSTRACT

Levels of intra-specific aggression between workers and mtDNA sequence comparisons were used to demonstrate that the non-native, invasive ant, *Myrmica rubra* L. has formed supercolonies in southwestern British Columbia. Ants from most, but not all, infested areas act aggressively towards ants from other areas but workers from widely separated locations within two of the largest areas show little aggression towards each other. Comparisons of COX1 mtDNA nucleotide sequences suggest that formation of different supercolonies may have followed possible divergence after a single initial introduction to the province.

Key words: invasive, super-colony, aggression

INTRODUCTION

Worldwide, over 150 species of ants have been introduced into new environments (McGlynn 1999) but a small number have become invasive, i.e., have reduced native ant biodiversity (Holway et al. 2002). Naumann and Higgins (2015), Gargas et al. (2007), and McPhee et al. (2012) have all reported that recently-established populations of *Myrmica rubra* L. in northeastern North America and the Pacific Northwest have all the characteristics of an invasive ant. In southwestern British Columbia *M. rubra* populations have dramatically decreased the incidence and abundance of previously established ants in three different plant communities: a well-drained riparian zone dominated by cottonwood (*Populus balsamifera* subsp. *trichocarpa* (Torrey and Gray) Brayshaw; Salicaceae) and Scotch broom (*Cytisus scoparius* (Linnaeus) Link; Fabaceae); a moister, more shaded community, dominated by red alder (*Alnus rubra* Bongard; Betulaceae), and two exotic blackberries, Himalayan blackberry (*Rubus discolor* Weihe and Nees; Rosaceae), and evergreen blackberry (*Rubus laciniatus* Willdenow); and grassy fields (Naumann and Higgins 2015). They also occur at unusually high densities compared to previously established species. *Myrmica rubra* represented more than 99.99% of the total ant fauna caught in the infested areas, and their capture numbers in the plant communities ranged from 10 to 1300 times the total number of *all* ants collected in corresponding *M. rubra*-free areas. The numbers of several other taxa of insects and non-insect arthropods were also reduced where *M. rubra* was present (also reported by Gargas et al. 2007).

Myrmica rubra is native to Northern Europe and western Asia and was first documented in North America in Massachusetts in 1908 (reviewed in Groden et al. 2005). It has now been reported in all Canadian provinces east of Manitoba and in at least six northeastern United States, and Washington state. Most of the reports are from within the last ten years, suggesting that the North American populations are expanding

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(Wetterer and Radchenko 2011). North American populations have not been observed to produce flying females (Hicks 2012), so spread is suspected to occur via the transport of garden products and by colony budding. The species likely established in southwestern British Columbia over 20 years ago but went relatively unnoticed for several years (Higgins 2013). *Myrmica rubra* comes to the attention of the public mostly because of a painful sting and high densities. Stinging is an unusual feature among the ant species listed from British Columbia (Naumann et al. 1999) and can make yard and garden work difficult and cause distress for pets. There is also concern that these ants may be interfering with the successful nesting of some birds (Higgins 2013). Robinson et al. (2013) estimated that the economic cost of this species in British Columbia could reach $100 million/year if it spreads across its potential range in the province.

The formation of supercolonies may contribute to the ability of invasive ant species to monopolize resources. An ant supercolony can be defined as a population of ants that exists over a large, contiguous area and in which ants move freely between nests and appear to show no aggression to conspecifics. (Haines and Haines 1978; Moffett 2012). Ants within a supercolony are so numerous that is impossible for all members of the colony to interact in their lifetime (Pedersen et al. 2006).

Holway et al. (2002) give a thorough review of the reported interactions between invasive and native ants worldwide. Especially common are reports that invasive species show greater efficiency at exploiting resources. Better resource utilization could be due to a larger force of workers, i.e., more scouts and more foragers to recruit, and/or physical aggression toward other species at a food item (Garnas et al. 2014). Supercolonies are typically seen only in non-native populations and are typified by being multiple-nested, multiple-queened, and lacking distinct behavioural boundaries among physically separate nests. This sort of colony organization has allowed a small number of non-native invasive species such as the Argentine ant, *Linepethema humile* (Mayr) (on all continents except Antarctica); the little fire ant, *Wasmannia auropunctata* (Roger) (in Africa, the Americas, and some Pacific islands); and the African big-headed ant, *Pheidole megacephala* (Fabricius) (all continents except for Antarctica), to attain high local abundances and consequently to dominate entire habitats (Holway et al. 2002). The 'Large Supercolony' of *L. humile* in California spans 1,000 km in distance (Moffett 2012). An apparent absence of intraspecific aggression within such supercolonies may free up time and energy for other uses.

The purpose of this study was to gain a better understanding of how *M. rubra* has come to dominate its new habitat in BC by determining if supercolony formation has occurred. The number of behviourally and genetically distinct colonies may give insights into whether there has been a single successful introduction or more than one.

METHODS AND MATERIALS

This study was carried out using ants from seven geographically distinct populations of *M. rubra* in southwestern BC. Prior to this study, there was no indication of whether those populations are the product of a single introduction or more than one. The frequency of aggressive interactions between workers was used to determine whether ants from the different areas, and from within them, treated each other as nestmates. The degree of genetic similarity of workers from the same seven areas was estimated by comparing nucleotide sequences of the mtDNA gene for cytochrome oxidase subunit I. It was hoped that this would provide a molecular level confirmation of any patterns of relatedness suggested by the behavioural data.

i) Sourcing and rearing the ants. Colonies of several hundred workers and at least two queens were collected in the last week of May and first week of June 2014 from nests within seven areas of infestation: Sea Island (Richmond), Fraser River Park (Vancouver), Inter River Park (North Vancouver), University of BC (Point Grey), Chilliwack, south Burnaby, and Oak Bay on Vancouver Island. It is unlikely that *M.*

rubra has been established in each area for the same length of time. The source colonies for this study were not identical in size, and not all workers were captured but the assumption was made that this would not have an important influence on the behaviour or individual workers. Each captured colony was maintained, in a laboratory, in a soil-free, 34 x 23 x 8 cm lidded, plastic tub which contained an aluminum foil-covered, 13 x 9.5 x 5 cm plastic container that acted as the nest. The internal box contained multiple folds of moist paper towel. Each colony was given a supply of water (a water-filled test tube stopped with a cotton ball), 1:1 honey water mixture, apple slices, and recently killed meal worms, and kept on a 12:12 h light-dark cycle.

ii) Inter-nest worker aggression between infestation areas. The level of aggression in interactions between ant workers of the same species has often been used as a proxy for levels of genetic difference – i.e. as a method of determining nest mate recognition – and many types of bioassays have been reported (Roulston et al. 2003). The recognition system that ants use for identification with a colony and rejection of aliens is based on shared cues, typically a colony-specific odour blend generated by queens or workers (d'Ettorre and Lenoir 2010), although food and other environment factors can have an influence (Liang and Silverman 2000). Our aim was to use the level of aggression between workers from the seven different areas as a correlate of the degree of genetic similarity. To minimize the confounding effects of foods and odours brought into the lab colonies from their original environments, all colonies were maintained for at least one week prior to being used for bioassays. It was assumed that several weeks in the lab would not diminish the tendency for ants from different colonies to fight, which we defined as ants locked together as they grasped each other with their mandibles.

Methods to measure the level of intraspecific aggression were similar to Roulsten et al. (2003) and are summarized as follows. Sets of workers from each area of infestation were matched with workers from a nest from each of the other areas. There were eight to ten replicates (trials) for each pair. For each trial, five foragers from each of two colonies were transferred to a fluon-coated 250 ml glass beaker which acted as a neutral arena. The number of ants engaged in fights was recorded during five-second scan surveys carried out once every minute for 10 minutes. For comparisons, we used the average (of 10 observations) percentage of ants involved in fights at one time across all colony pairs. For half of the replicates, the first five ants into the arena came from one of the colonies within each pair; for the other half, they came from the second colony. Controls consisted of bioassays of two groups of five ants from the same colony.

The aggression bioassays were repeated a minimum of four weeks after capture, i.e., during the second week of July, 2014. This was meant to test both that the initial one-week latent period in the lab had been long enough to remove the effects of environment, and that maintenance in the lab did not result in loss of aggression. This length of delay was chosen because laboratory colony populations were beginning to decline at that time. Colonies collected from Oak Bay were not included in this particular test because of diminished worker numbers.

Within Infestations. The level of inter-nest aggression was also measured between nests from within two of the largest areas of infestation, Sea Island in Richmond and Fraser River Park in Vancouver. At Sea Island, four nests were collected at approximately 500 m intervals along a 2 km transect line; at Fraser River Park, three nests were collected approximately 300 m apart along a 1 km transect line. As before, the nests were reared in the laboratory, as described above. Aggression bioassays were carried out one week after the establishment of the nests; n = 6-10 for each pairing.

ii) The Genetic similarity of ants from different M. rubra populations. Differences in mtDNA nucleotide sequences were measured as a way to determine if there had been a single successful introduction of *M. rubra* into BC, or more than one. It was also hoped that mtDNA differences could allow for discernment of ants from different areas, i.e., different possible supercolonies. All mtDNA samples were collected from workers from the nests used for the aggression bioassays.

DNA was extracted from ants using a modified procedure of Schlipalius et al., 2001. This procedure allowed for the use of whole insects combined with particular primers that limit the possibility of contamination with microbial DNA. Individual frozen ants were removed from storage at –80ºC and immediately crushed in the bottom of a 1.5 ml Eppendorf tube with an extraction buffer consisting of 30µl of boiling 5% Chelex in TE. Each tube was then placed into a boiling water bath for 15 min and centrifuged at 13,000 rpm for 10 min. 20 µl of the supernatant was removed from each sample, and put into storage at –20ºC for later use as template DNA in PCR reactions.

The PCR primer pair LC1490 and HCO2198 (Folmer et al. 1994) were used for the amplification of a 710 bp partial coding sequence of mitochondrial cytochrome oxidase subunit I (COX I). Primers were custom synthesized by INVITROGEN/ Life Technologies ™. PCR was done using 2 µl of PCR buffer, 1 µl of 1 µM of primer solution, 1 µl of Taq polymerase (Amplitaq, from Life Technologies), 1 µl of a 2 mM dNTP, and 1 µl ant DNA, with dH_2O added to a total volume of 20 µl. PCR was run on a Techne Techgene thermal cycler. The program settings were: initial denaturation at 95ºC for 2 minutes; 30 cycles of the following: 30 seconds at 94ºC, 45 seconds at 50ºC, 2 minutes at 72ºC, and a final extension for 5 minutes at 72ºC. Successful amplification of single 710 bp DNA from all ants was confirmed by agarose gel electrophoresis (data not shown) and purified using a QIAquick PCR Purification Kit from QIAGEN. DNA was sequenced using the Sanger method on an Applied Biosystems 3730 DNA analyzer at the NAPS Unit at the University of British Columbia, Vancouver, BC.

Formica sinensis. Wheeler cytochrome oxidase subunit partial coding sequence (Accession EU983580) was used as an outgroup to determine the order of descent among DNA sequences. Phylogenetic analyses were done using MEGA7 (Kumar et al. 2016). Sequences were imported into MEGA7 as fasta files and MUSCLE was used to generate an alignment using the ALIGN CODONS option. Phylogenetic trees were generated using the Maximum Likelihood Estimation (Tamura 1992; Felsenstein 1985). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algortihms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The analysis involved 17 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions with less than 95% site coverage were eliminated – i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 438 positions in the final dataset.

RESULTS

Aggression Bioassays. With the exception of one pairing of localities, ants from nests originating in different areas of southwestern BC showed high levels of worker-worker aggression (Table 1). This included ants from Sea Island and Fraser River Park, which are separated only by an arm of the Fraser River. The exception was a lack of aggression observed when ants from Inter River Park in North Vancouver encountered ants from Point Grey (University of British Columbia, Vancouver). The patterns of fighting between workers from different localities did not change when tested again a further four weeks after the nests were brought into the laboratory (Table 2).

There was comparatively little fighting between ants from nests *within* the Sea Island or Fraser River Park *M. rubra* populations, even when nests were as much as 2 km apart (Table 3).

Genetic Comparisons. Figure 1 shows that the nucleotide sequences of the COXI subunits of the ants from the different outbreak areas fell into two groups. The North Vancouver and Point Grey ants were within the same group. Different samples from Fraser River Park in Vancouver fell within either group.

Table 1

Mean percentage (± SD) of ants from different pairs of colonies engaged in fights after one week of laboratory rearing. The ants were from nests in seven different areas of southwestern British Columbia. FR Park = Fraser River Park, Vancouver. Data with different superscripted letters are significantly different ($p < 0.05$; ANOVA and LSD multiple comparison tests; $F = 104.6$; $df = 20$; $P<0001$. Same-nest comparison data were not included in the statistical analysis).

	Oak Bay	Burnaby	Chilliwack	UBC	N Van	FR Park	Sea Is
Sea Is	74(12)^{e}	51(22)^{c}	72(28)^{ef}	79(24)^{efg}	44(26)^{b}	62(19)^{d}	0
FR Park	87(7)^{hi}	81(13)^{fgh}	83(12)^{gh}	84(5)^{gh}	80(13)^{efg}	0	
N Van	78(12)^{efg}	52(6)^{c}	80(13)^{efg}	0(0)^{a}	0		
UBC	92(27)^{efg}	54(20)^{c}	90(7)^{i}	0			
Chilliwack	55(16)^{c}	83(6)^{gh}	0				
Burnaby	79(10)^{efg}	0					
Oak Bay	0						

Table 2

Mean (± SD) percentage of ants from different pairs of colonies engaged in fights after six weeks of laboratory rearing. The ants were from nests in six different areas of southwestern British Columbia. FR Park = Fraser River Park, Vancouver; nests from a seventh locality (Oak Bay) were not tested at the six week interval. Data with different superscripted letters are significantly different ($p < 0.05$; ANOVA and LSD multiple comparison tests; $F = 94.7$; $df = 12$; $P<0.0001$)). *Insufficient ants.

	Burnaby	Chilliwack	UBC	N Van	FR Park	Sea Is
Sea Is	69(20)^{de}	53(21)^{c}	61(16)^{d}	64(14)^{d}	21(15)^{b}	0
FR Park	51(18)^{d}	73(10)^{ef}	79(4)^{f}	77(11)^{f}	0	
N Van	43(23)^{c}	83(7.5)^{f}	3(8)^{a}	0		
UBC	*	*	0			
Chilliwack	63(21)^{d}	0				
Burnaby	0					

DISCUSSION

Invasive populations of *M. rubra* have formed at least two large, multi-nest supercolonies in BC, and it is reasonable that the same phenomenon has occurred in the other distinct areas of infestation. The one on Sea Island is several kilometers across and, as individual nests are often less than 5m apart, must contain thousands of nests and millions of individual ants. This type of colony organization may be contributing to the displacement of native ants and other epigaeic species that was reported by Naumann and Higgins (2015).

Based on aggression bioassays, most of the major outbreak areas of *M. rubra* in southwestern BC represent different super-colonies, but workers from UBC and North Vancouver interact as if they are nest mates, suggesting either a relatively recent common origin, or that one site was the source of the founding population of the other.

Table 3

Mean (± SD) percentage of ants from different pairs of colonies within the same outbreak areas; SI = Seal Island; FP = Fraser River Park) that engaged in fights after one week of laboratory rearing. The ants were from nests separated by approximately 500 m (SI) or 300 m (FR) intervals along a transect line.

	SI1	SI2	SI3	SI4
SI4	2(7)	3(6)	0	0
SI3	3(6)	2(6)	0	
SI2	13(10)	0		
SI4	0			

	FP1	FP2	FP3
FP3	0	4(6)	0
FP2	0	0	
FP1	0		

Levels of aggression between ants from different colonies are frequently used as a proxy for levels of genetic difference (Roulsten et al. 2003). In this study, patterns of aggression did not change markedly after a minimum of four weeks in the lab, suggesting that it was not chemical cues associated with the original environments that led to recognition of individuals from different locations, but rather colony-specific odor blends generated by queens or workers (d'Ettorre and Lenoir 2010), and likely to be genetically based.

We did not find enough molecular diversity in COXI to be able to distinguish between different populations of *M. rubra* in southwestern BC but the significant separation into two groupings, with ants from Fraser River Park common to both, suggest that an original introduction into BC may have occurred near there, and that divergence of this subunit occurred later. The observation that ants from within the Fraser River outbreak treat each other as nest mates argues against two genetically unique introductions. The similarity of the COXI sequences of the non-aggressive ants from UBC and North Vancouver provides further evidence that those two groups of ants are particularly closely related. Hicks et al. (2012), also using mtDNA, reported evidence that *M. rubra* populations on Newfoundland have come from at least four distinct sources, including the UK and the Northeastern USA. We do not yet have enough data to speculate on the possible source of the *M. rubra* populations in BC.

Possible mechanisms for the superior competitive abilities of invasive ant populations include direct aggression, superior recruitment to resources, and higher activity levels. Garnas et al. (2014) reported that *M. rubra* shows both higher levels of recruitment and aggression towards native ant species in Maine, USA; foragers consistently discover baits faster and displace foragers from native species. Foragers from highly populous supercolonies with many dispersed nests would have an advantage at discovering, recruiting to, and exploiting food resources. For example, supercolony-forming *L. humile* have been reported to be more numerous than other species in the same area, and to be a superior interference competitor that displaces native species from contested baits, often via direct physical aggression (Human and Gordon 1996). In addition, lack of aggression between workers over large areas could leave more time and energy for foraging. *Linepithema humile* for example, maintains higher colony activity levels,

forages for longer periods each day, and recruits in greater numbers to food resources than native species (Human and Gordon 1996).

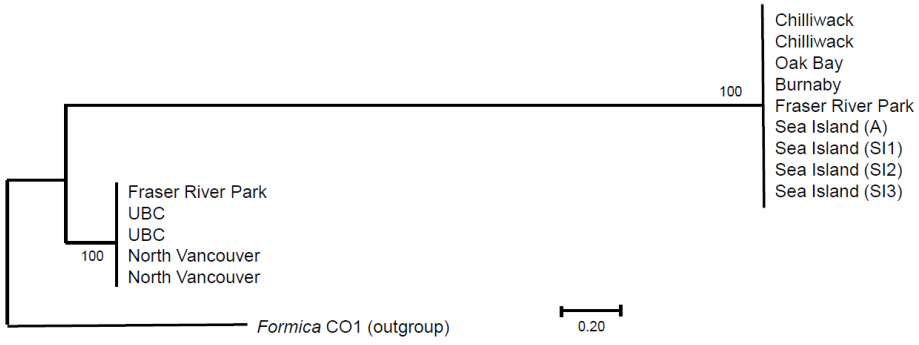

Figure 1. Molecular phylogenetic tree of the COXI subunit of mtDNA from *M. rubra* workers from different outbreak areas of British Columbia. The tree with the highest log likelihood (-1824.1574) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

The proximate and ultimate causes of supercolony formation remain inconclusive. According to Holway et al. (2002), the phenomenon is more common among ant species that are non-native and have become invasive in their newly established environments. They also tend to show relatively small size, omnivory, and a tendency towards multiple queen nests. On the other hand, most of these species exhibit similar life histories in their native ranges (Moffett 2012), and at least one other ant species, *Liometopum occidentale* Emery, may form large (at least one km in diameter), habitat-dominating supercolonies within its home range (Wang et al. 2010). Failure to form large colonies in those areas may be due to constraints by other native species that are aggressive and effective competitors. In other words, it is the release from those competitors in a new region that allows for the formation of supercolonies (Moffett 2012). Supercolony formation in *M. rubra*, as in other supercolony-forming species, may also be related to the fact that virgin queens in North America do not carry out mating flights (Hicks 2012), although they do in their home range. Instead, North American queens mate at or near the nest and then travel a short distance, with a group of workers, to found a new nest. Infestations thus expand relatively slowly via colony budding, and jump to new areas, likely through human activities like the transport of infested nursery products**.** It is possible that lack of contact with conspecifics from other colonies inhibits queen mating flights or fails to stimulate them. If there are no intraspecific competitors in an adjacent area, why risk a mating flight when territory that is likely to be suitable lies right next door? Also, the success of incipient colonies is likely to be higher if the queen is not alone, and if the number of founding workers is greater (reviewed in Holway et al. 2002).

Although it is now possible to add *M. rubra* to the list of invasive ant species that share a suite of behavioural features such as supercolony formation, much work needs to be done to resolve both the details of the *M. rubra*'s establishment in different areas of North America, and the general mechanisms that lead to the formation of ant supercolonies. Do some ants become ecologically dominant because they form supercolonies or does the monopolization of resources by certain species lead to supercolony formation (Hölldobler and Wilson 1977)?

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