The northward distribution of ants (Hymenoptera: Formicidae) 40 years later: revisiting Robert E. Gregg's 1969 Subarctic collection sites in Churchill, Manitoba, Canada

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Abstract—In 1969, Robert E. Gregg collected five species of ants (Hymenoptera: Formicidae) in three Subarctic localities near the town of Churchill, Manitoba, Canada, which he documented in a 1972 publication in The Canadian Entomologist. To determine whether there have been any additions to the local fauna – as might be predicted to occur in response to a warming climate and increased traffic to the Port of Churchill in the intervening 40 years – we re-collected ants from the same localities in 2012. We identified the ants we collected from Gregg's sampling sites using both traditional morphological preparations and DNA barcoding. In addition, we examined specimens from Gregg's initial collection that are accessioned at the Field Museum of Natural History (Chicago, Illinois, United States of America). Using this integrative approach we report seven species present at the same sites Gregg sampled 40 years earlier. We conclude that the apparent increase is likely not due to any arrivals from more southerly distributed ants, but to the increased resolution provided by DNA barcodes to resident species complexes with a complicated history. We provide a brief synopsis of these results and their taxonomic context.

Introduction

The Subarctic is experiencing increasingly frequent warm weather; for example, western Hudson Bay air temperatures have increased by 2–3 °C over the past 50 years (Gagnon and Gough 2005), and near Churchill, Manitoba, Canada the decade between 1997 and 2007 saw six of the 10 hottest years on record (and all of the top five) (Fernandez-Triana *et al*. 2011). These temperature changes may be linked to faunal shifts observed over the past half century (Fernandez-Triana *et al*. 2011).

In 1969, Robert E. Gregg (1972) sampled ants (Hymenoptera: Formicidae) near the tree-line in northern Manitoba in the vicinity of the town of Churchill. He collected at three sites along a small north-south transect (~6–7 km) that featured sites of exposed tundra and bluffs immediately along Hudson Bay (near the mouth of the Churchill River), boggy tundra, and boggy boreal forest. The ant community that he uncovered was not diverse but was abundant in some cases near the southern end of his investigation. In total, Gregg found five ant species in the Churchill region: *Camponotus herculeanus* (Linnaeus) (Formicinae), *Formica neorufibarbus algida* Wheeler (Formicinae), *Leptothorax canadensis kincaidi* Pergande (Myrmicinae), *Leptothorax canadensis* Provancher (Myrmicinae), and *Myrmica brevinoidis* Emery (Myrmicinae). Francouer (1983) re-examined Gregg's specimens, now held at the Field Museum of Natural History in Chicago (Illinois, United States of America),

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and noted that the specimens originally identified as *L. canadensis kincaidi* were *L. acervorum* Fabricius, and *M. brevinoidis* were actually *M. alaskensis* Wheeler.

As climate changes across the world, the importance of comparing contemporary collections to any measure of "historical" material is critical – as it allows us to determine whether there have been shifts in species composition (Tingley and Beissinger 2009). Documenting the presence or absence of any biotic change is particularly important when collections occur on large ecological ecotones since the isotherms that determine these areas are predicted to move drastically in the coming decades and, further, since the effects of a changing climate are unlikely to be felt in the same manner by all taxa. For example, in Churchill, some groups have seen a substantial change (parasitoid Hymenoptera (Braconidae) – (Fernandez-Triana *et al*. 2011)), while others were not significantly different across collection periods (Diptera – (Renaud *et al*. 2012b)). In 2012, we collected ants from the same locations visited by Gregg in 1969 to determine if the ant community had changed in the intervening time.

Methods

As part of a field course in Arctic Ecology offered by the University of Guelph (Guelph, Ontario, Canada) through the Ontario Universities Program in Field Biology, the instructors and students (22 people total) used descriptions from Gregg (1972) (including his Fig. 2) to revisit the exact sampling locations that Gregg had sampled (1 site/day between 9 and 11 August 1969) on 10 July 2012 (south to north: Goose Creek (bog), Welcome Sign (tundra), and Cape Merry (tundra)) (Fig. 1). Due to descriptions and aspects of the local environment that were likely to be invariant between collection times (*e.g.*, intersections of railway and road, peninsula) we were confident in sampling the same sites that Gregg did in 1972. At each collection locality we actively searched for ants using standard myrmecological collection methods (actively turning woody debris and stones, breaking twigs, pulling apart moss and lichen hummocks, and sampled ants using feather weight forceps and aspirators) for three hours within an area bounded by ~100 × 100 m. In addition to the ant fauna, we recorded the non-grass vascular plants and documented the habitat at each site using high-resolution, panoramic photographs captured using a GigaPan (www.gigapan.org) system (Smith *et al.* 2013). The resultant panoramic photographs are publically available (http://www.gigapan.com/galleries/9086/gigapans).

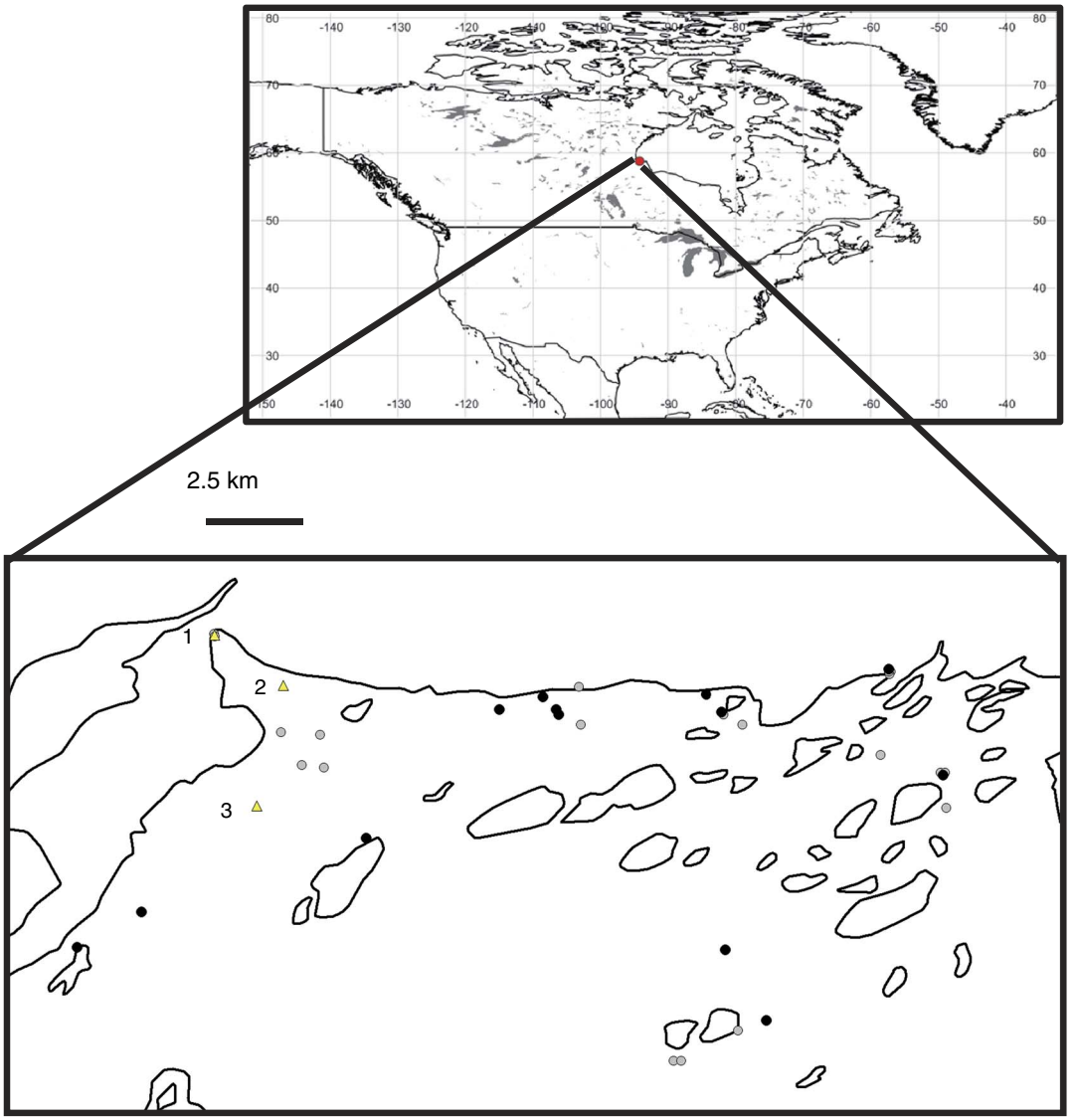
Collected ants were separated into genera and then morphospecies. Ant species were identified using available taxonomic resources (Creighton 1950; Francoeur 1973, 1983, 1997; Fisher and Cover 2007). From these, we selected representatives to be DNA barcoded (~10/morphospecies). These individuals were then point-mounted, photographed, and a single leg (the point-side middle leg) was removed during preparation to serve as template for the total genomic extraction. DNA extractions were prepared from this tissue using a glass-fibre extraction protocol (Ivanova *et al.* 2006), and the DNA was resuspended in 30 µL of dH_2O. We amplified the DNA barcode region of cytochrome *c* oxidase I (COI) (a 658-base pair region near the 5′ terminus of the COI gene) using the insect primers LepF1 (5′-ATTCAACCAAT CATAAAGATATTGG-3′) and LepR1 (5′-TAA ACTTCTGGATGTCCAAAAAATCA-3′) and uni-directionally sequenced the amplicons using LepR1. All laboratory information associated with the individual sequences can be retrieved from the Barcode of Life Data System (BOLD, www.barcodinglife.org (Ratnasingham and Hebert 2007)) using the Process ID (sequence accessions). All sequence data and detailed collection information is available on BOLD in the public dataset: Arctic Ecology Student Projects 2012 (dx.doi.org/10.5883/DS-AEDNA12) and in Table 1.

We assessed the degree to which our sampling effort approached the estimated site maximum for alpha diversity (based on the techniques described above) using the incidence matrices in Table 1 and a sample-based rarefaction completed using EstimateS and 1000 randomisations (v9.1; Colwell and Elsensohn 2014).

Identification of provisional species within BOLD

The barcode index number (BIN) (Ratnasingham and Hebert 2013) is a unique alpha-numeric applied to significant sequence clusters in BOLD – clusters with unique and resilient sequence divergence from other BINs of ~2%. In the ants

Fig. 1. Ant sampling localities in the Churchill region. Yellow triangles represent the Gregg, 1969 sampling locations with numbers corresponding to site names in Table 1. Grey circles are the ant collections made in 2005/2006, while black circles are the 2010 ant collections that were analysed in Stahlhut *et al*. (2013).



(and other insect groups) the BIN and the ~2% range of divergence has proven a useful proxy for formally named species (Smith *et al*. 2005, 2009, 2014; Fisher and Smith 2008).

Analyses on Churchill ants collected before 2012

Recent collections (2005–2010). In addition to the Gregg exact site re-sampling comparison between two time periods, we also used the DNA barcodes to compare the diversity we recovered in the Gregg-resampling of 2012 to the diversity of ants we had previously collected in the wider Churchill region between 2005 and 2010 (Fig. 1) (Stahlhut *et al*. 2013). Collection methods and localities are described in Smith *et al*. (2009) and Stahlhut *et al*. (2013) (dx.doi.org/10.5883/DATASET-HYMCHUR1).

For a sub-set of the collection (from 2006), we analysed the prevalence of *Wolbachia* Hertig

(Rickettsiaceae) in somatic tissue-derived total genomic DNA extracts using the polymerase chain reaction-based *wsp* assay as described in Smith *et al*. (2012). This analysis was completed since the differential presence of a *Wolbachia* infection can lead to large intra-specific mitochondrial divergences.

Gregg collections (1969). Select specimens from Gregg's initial collection are accessioned at the Field Museum of Natural History. We photographed and examined some of these and subsampled a small number (12) for DNA extraction and analysis. We designed four reverse primers to be used with the general insect forward primer LepF1 amplify two short fragments of COI for the ants in general and *Formica neorufibarbis* Emery in particular (Primers sequences are 5′ to 3′. 145 base-pair amplicons: CO1-211-Formicidae-reverse: ATAATTTTTTTAATRGTWATACCTT and CO1-211-neorufibarbis-reverse: ATGGTAT TACTATGAAAAAAATTAT; or 213 base-pair amplicons: CO1-281-Formicidae-reverse: GGAT CTCCTGATATAGCCTATCCTCG, and CO1-281-neorufibarbis-reverse: CGAGGATAAGCTA TGTCGGGAGATCC.

Results

When we revisited the Churchill sites sampled by Gregg in the summer of 1969 we located nests of all species in all collection sites. Using morphological and genetic methods, we found evidence for seven species of ants (Table 1 and Fig. 2). Two of these species were members of species complexes known to be widely distributed across the Nearctic (*F. neorufibarbis* and *Leptothorax muscorum* Nylander). In each case we found evidence for multiple DNA barcode clusters, suggesting the presence of multiple species under the same name.

Most of the seven species were found in all three sites. Exceptions were *Myrmica alaskensis* and *F. neorufibarbis* (BOLD:AA1470) (found only in the most southerly bog site) and two of the *Leptothorax* species (*L. muscorum* complex and *L. acervorum)* (each found only at one or both of the two more northerly tundra-like sites) (Table 1).

Sampling the broader Churchill area (over the years 2005–2010 yielded three additional species compared to our geographically restricted Gregg resampling (Fig. 3). As in the species set recovered in the 2012 Gregg resampling, the new species from across the wider Churchill area were also DNA barcode divergent and not apparently morphologically different, and likely further members of the *F. neorufibarbis* and *L. muscorum* complexes as well as one further species of *Myrmica* Latreille.

Although sample sizes varied among species (*n* ranged from 1 to 303), *Wolbachia* infection was detected in all species, with incidence rates varying between 16% and 100% (Table 2).

Table 1. Two incidence matrices comparing Gregg's 1969 collections and our July 2012 resampling from the same locations in and around Churchill, Manitoba.

		Cape Merry (1)	Welcome Sign (2)	Goose Creek (3)	
	Camponotus herculeanus	1	1	1	Gregg collections
	Formica neorufibarbis	1	1	1	
	Leptothorax acervorum	1	1	0	
	Leptothorax muscorum	1	1	1	
	Myrmica alaskensis	0	0	1	
BOLD:AAA2372	*Camponotus herculeanus*	1	1	1	2012 collections
BOLD:AAA1469	*Formica neorufibarbis*	1	1	1	
BOLD:AAA1470	*Formica neorufibarbis*	0	0	1	
BOLD:AAB1385	*Leptothorax muscorum* complex	1	1	1	
BOLD:AAB7202	*Leptothorax muscorum* complex	1	1	0	
BOLD:ACE8522	*Leptothorax acervorum*	0	1	0	
BOLD:AAA1866	*Myrmica alaskensis*	0	0	1	

Note: Matrices are presented separately since, without barcoding specimens from the historic collections, we are uncertain which BIN-divergent record to align with which morphologically determined historic collection.

Fig. 2. Accumulation curve for ant collections made in Churchill in 2012 at sites originally collected by Gregg in 1969. Dashed lines represent the 95% confidence interval, and grey bars represent the standard deviation of the expected number of species (S(est)) based on 1000 simulations in EstimateS (v9.1).

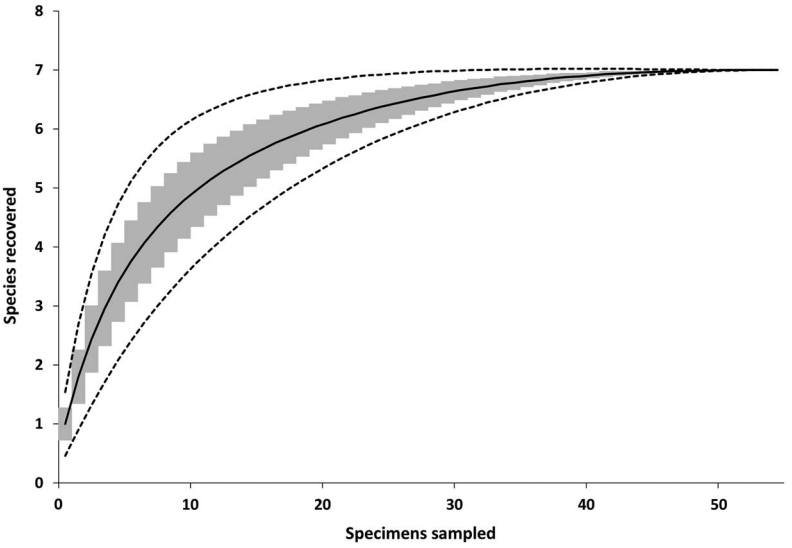
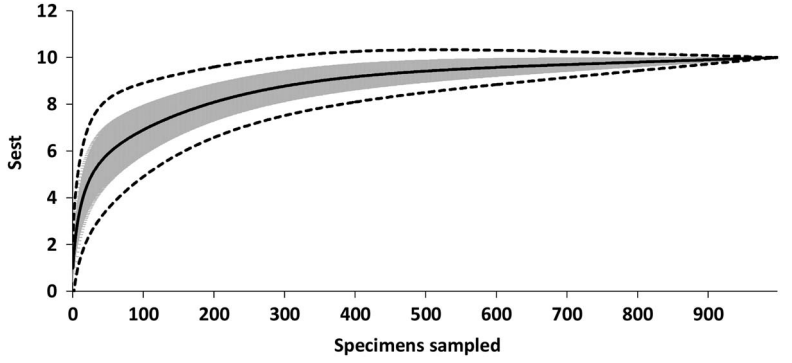


Fig. 3. Accumulation curve for all ant collections made in Churchill (2005–2012). Dashed lines represent the 95% confidence interval, and grey bars represent the standard deviation of S(est) based on 1000 simulations in EstimateS (v9.1).



Gregg's 1969 collections from the Field Museum of Natural History

Specimens appear to have been originally preserved in a low concentration in ethanol and at room temperature and DNA appear to be degraded. This scenario is not uncommon in museum collections and so our strategy was to design primers that amplified shorter fragments that would be less likely to be degraded (while also retaining species identification characteristics). To date, based on nearly 100 amplification trials, we have been able to amplify small fragments of COI DNA from only two species (*C. herculeanus* and *L. muscorum*) and so the direct DNA to DNA comparison of the contemporary collection to Gregg's collections is not possible here.

Discussion

In 1969 Gregg documented the occurrence of five species of ants in the vicinity of Churchill,

Table 2. Incidence of *Wolbachia* in Churchill ants determined using the *wsp* polymerase chain reaction assay.

Identification	BIN	Total no.	*wsp* Positives	*wsp* Negatives	% *wsp* Positive
Camponotus herculeanus	BOLD:AAA2372	142	23	30	16.2
Formica neorufibarbis	BOLD:AAA1469	303	242	119	79.9
Formica neorufibarbis	BOLD:AAA1470	34	23	61	67.6
Leptothorax acervorum	BOLD:AAB1385	41	11	14	26.8
Leptothorax muscorum complex	BOLD:AAB7202	17	3	1	17.6
Leptothorax muscorum complex	BOLD:AAB7203	2	1		50.0
Leptothorax muscorum complex	BOLD:ACE8522	1	1		100.0
Myrmica alaskensis	BOLD:AAA1866	14	3	11	21.4

Note: All species tested were found to contain the endosymbiont.

Manitoba – the same ant species that would be expected to be found near the tree-line in Canada (Francouer 1983). Using the integration of traditional methods based on morphology and DNA barcoding, we report seven species present at the same sites Gregg sampled 40 years earlier. We conclude that the apparent increase is more likely due to morphologically cryptic species (that are genetically divergent) rather than the arrival of species from the south. However, it should be noted that since we were unable to DNA barcode the Gregg collections we are unable to conclusively exclude the possibility that the "new" BINS recovered in 2012 were also present 43 years earlier. Gregg (1972) found only a single worker of the carpenter ant, *Camponotus herculeanus*, in the exposed tundra near Cape Merry, but no nests. This differentiates our study as we located nests of all species in all collection sites.

The apparent increase we report in ant diversity at Churchill is more likely to be due to changes in systematics (Francouer 1983; Heinze 1993) and the resolution of species-level diversity afforded by DNA barcoding (Smith *et al*. 2008, 2009) than to the intervening arrival of more southerly distributed species (Fernandez-Triana *et al*. 2011). The two new species recorded in our 2012 collection, one *Formica* Linnaeus and one *Leptothorax* Mayr species, are members of species complexes that are difficult to identify morphologically. Furthermore, we did not detect any species that others have predicted could soon be added to the northern ant fauna due (in part) to increased human activity in the area (Francouer 1997) (*e.g.*, *Tapinoma sessile* (Say)).

Crypsis in apparent species complexes: *Formica neorufibarbis* and *Leptothorax muscorum*

Formica neorufibarbis. Churchill ants that were morphologically identified as *F. neorufibarbis* contained two DNA barcode BINs (BOLD: AA1469 and BOLD:AAA1470) that were 4.74% divergent (K2P distance). In addition to these DNA barcode divergences, the BINs were characterised by a single evident fixed base pair difference within rDNA (D2B region of 28S) (Smith *et al*. 2009), and each provisional species are infected with *Wolbachia.* Infections from different *Wolbachia* strains can still cause reproductive isolation between host populations, even when both populations maintain *Wolbachia* (Hurst and Jiggins 2005; Smith *et al*. 2012). The *Wolbachia* test here was polymerase chain reaction-based and so no direct comparison of sequence divergence is possible. Thus while the specific effects of *Wolbachia* on these provisional ant species remain unknown, the bacteria may affect host fitness and the mitochondrial divergence of the ant host. Alternatively, the observed genetic divergence may be due to other mechanisms, such as speciation in allopatry during prior glacial cycles. *Formica neorufibarbis* BOLD:AA1469 was recovered from all three Gregg re-sampling sites, while *F. neorufibarbis* BOLD:AA1470 was only recovered in the farthest south site of Gregg's sampling (Goose Creek bog). Each ant keys out (using Creighton (1950) or Francoeur (1973)) to *F. neorufibarbis*; however, BOLD:AA1469 is a smaller, dark, and concolorous ant, while BOLD:AA1470 is larger and bicoloured with a mesosoma that is lighter in colour than the head

or gaster. If we were to align these BINs with existing, but synonymised names, BOLD:AA1469 would be the synonymised subspecies *F. gelida* Wheeler and BOLD:AA1470 would be *F. neorufibarbis*. Our evidence suggests that these names should be reconsidered for resurrection to species status.

Although, both species of *Formica* collected in the Gregg resampling key to *F. neorufibarbis* they are nearly 5% divergent in the DNA barcode region. The combination of a deep mtDNA divergence congruent with an evident fixed difference in an independent gene region (Smith *et al.* 2009), a shared *Wolbachia* infection, morphological differences, and an apparently sympatric distribution supports the hypothesis that these *F. neorufibarbis* BINs are, in fact, different species. Before 1973, *F. neorufibarbis* contained two additional subspecies (*F. neorufibarbis gelida* and *F. neorufibarbis algida*) (Francoeur 1973). However, others felt that Creighton's subspecies were "untenable" (Brown 1955), and in 1973 Francouer synonymised them as representing geographical variants of the same species.

When we compared the Churchill *F. neorufibarbis* BINs to those found when barcoding Francoeur's collection in BOLD (http://www.barcodinglife.org/index.php/Public_SearchTerms; search "public data" for "Francoeur"), we found that each BIN was present in geographically broad collections, while one BIN (BOLD:AA1469) was more common than the other (note that each BIN can be searched directly on BOLD to see the breadth of this distribution). Gregg (1963) reported that the subspecies *F. gelida* was more characteristic of high-altitude or northern distributions than the *F. neorufibarbis* ants. Interestingly, we note that while Gregg knew *F. gelida*, he did not identify it as being present in his Churchill collections.

Leptothorax muscorum. Although the name *L. muscorum* is still widely applied, it is likely to be a complex of closely related species (Francouer 1997). For instance, using chromosome enumeration, Loiselle *et al.* (1990) found that there were at least four species hidden within the name *L. muscorum* as used by Brown (1955). Furthermore, some historical collections that were originally identified as *L. muscorum* subsequently had these identifications corrected to *L. acervorum,* a species formerly thought to be restricted to the Palaearctic, but is now known to occur in northern Canada (including northern Québec and Churchill based on Francouer's (1983) re-examinations of previous collections). In Churchill, our sampling over more than five years, has recovered evidence for *L. acervorum* (BOLD:ACE8522) and for two further species that key out as members of the *L. muscorum* complex (BOLD:AAB1385 and BOLD:AAB7202).

The important role of DNA barcodes in comparing time periods in Arctic collections

In the recent past, there have been other examples of sampling the arthropods from the Churchill area using DNA barcodes (Araneae – Blagoev *et al.* (2013); Diptera – Renaud *et al.* (2012a); Ephemeroptera, Plecoptera, and Trichoptera – Zhou *et al.* (2009); Coleoptera – Woodcock *et al.* (2013); and Hymenoptera – Smith *et al.* (2009), Fernandez-Triana *et al.* (2011), Stahlhut *et al.* (2013). In a few cases (Fernandez-Triana *et al.* 2011; Renaud *et al.* 2012b), these recent collections were compared with historical collections. In the case of the Hymenoptera (specifically the subfamily Microgastrinae (Braconidae)) there were dramatic changes in the community between samples collected over a 50-year period. However, in the case of the Diptera (specifically the family Muscidae) there were no changes observed.

The increasing number of comparisons between historical collections and contemporary collections in the Canadian Arctic or Subarctic has recorded incidences of change and stasis (including for the Diptera (Subarctic – Renaud *et al.* 2012b) and Hymenoptera (High Arctic – Timms *et al.* 2013)). In each of these two studies, they found no significant differences between sampling periods in spite of demonstrably large changes in climate. However, it is important to note that in these cases the analyses used expert-identified specimens (via morphology alone) as their taxonomic grain size (no genetic evidence). In many cases with many insect groups (including both parasitoids and flies) it is increasingly accepted that species boundaries can be most accurately estimated using an integrative approach that includes both morphology and DNA, as many species are morphologically quite cryptic and perhaps variant in only qualitative character states (Rodriguez *et al.* 2012). Thus, it is interesting to contrast Renaud *et al.* (2012b) and

Timms *et al.* (2013) with the findings of Fernandez-Triana *et al.* (2011), who used both DNA and morphology-based species estimates to compare historical and contemporary collections and found differences in the community composition. Renaud *et al.* (2012b) and Timms *et al.* (2013) both note that the Fernandez-Triana *et al.* (2011) finding may have been skewed by large intra-specific differences being analysed as representative of inter-specific differences, which is true. However, what is missing from their analysis is a recognition that by not barcoding any (Timm's) or only half (Renaud) of their chrono-sequence, their analyses would be unable to capture species differences in community composition hidden by cryptic species. We call attention to this methodological difference (barcoding only one of multiple, chrono-sequences) here as it is germane to our work. In the case of our comparison of the ant fauna at Churchill over 40 years, we have barcoded the 2012 ant collections – but not the Gregg collections due to degraded DNA. Thus, we cannot exclude the possibility that in our own analysis, we have missed morphologically cryptic species present only in the 1969 collections but absent in the contemporary collections. However, we consider this unlikely given that we recovered precisely the same morphospecies as Gregg and have no evidence of additional species that have migrated northward. Moreover, the additional species we have documented are largely morphologically cryptic and divergent in COI, and thus we cannot reject the hypothesis that these new species are simply intraspecific variants.

Conclusions

The ant fauna in the subarctic locality surrounding Churchill, Manitoba does not appear to have significantly changed since the area was surveyed in the late 1960s. The fauna is dominated by species known to be characteristic of the tree-line and tundra environment in Canada and is characterised by taxonomically difficult species complexes. As a locality with a long history of hosting scientific inquiry, there is great value in continuing to regularly survey the insect and arthropod fauna in and around the Churchill region, as the response of this diverse community is unlikely to be simple.

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