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A multiple-gene phylogeny reveals polyphyly among eastern North American *Aphaenogaster* species (Hymenoptera: Formicidae)

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Twenty-three *Aphaenogaster* species (Hymenoptera: Formicidae) occur in North America. While morphology and ecology define most species, the species limits of a group in the Eastern United States are unclear. In particular, the morphological and behavioural characters of *A. carolinensis*, *A. picea* and *A. rudis* overlap. These observations suggest that these three species are not monophyletic. We therefore tested the monophyly of *Aphaenogaster* in the context of molecular phylogenetic analyses. We used DNA data from five genes: CO1, CAD, EF1αF2, long-wavelength rhodopsin and wingless, to reconstruct phylogenies for 44 *Aphaenogaster* and outgroup species. In the resulting trees, reconstructed using parsimony and Bayesian inference, species boundaries associated with well-supported monophyletic clades of individuals in most of the 23 North American *Aphaenogaster* collected from multiple locations. However, some clades were unresolved, and both *A. picea* and *A. rudis* were not monophyletic. Although this may indicate that clades of multiple species represent fewer but morphologically varied species, given the short branch lengths, the lack of resolution may reflect the fact that these ants have recently radiated, and a lack of gene lineage sorting explains the non-monophyly of species. Additional biological information concerning pre- and postmating barriers is needed before a complete revision of species boundaries for *Aphaenogaster*.

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Introduction

The number of recognized ant species worldwide increases each year. Hölldobler & Wilson (1990) estimated that there were 8800 described species. By 2007, Fisher & Cover (2007) reported 12 000, and currently AntWeb (http://www.antweb.org) posts almost 16 000 valid species and subspecies. Ants are ubiquitous in many ecosystems, and ecologically dominant as predators, scavengers and herbivores (Wilson & Hölldobler 2005). Although ants make up approximately 2% of the known global insect fauna, they comprise at least one-third of its biomass (Wilson & Hölldobler 2005). In the tropics, they can make up to 94% of the biomass of tropical rainforest canopies (Davidson *et al.* 2003). Consequently, ants play an important role in the environment and their study depends on a thorough understanding of their diversity.

The woodland ant genus *Aphaenogaster* includes important seed dispersers in North American forests, and has been the focus of a number of ecological and evolutionary studies (Lubertazzi 2012; Warren & Chick 2013; Bewick *et al.* 2014). Previous systematic studies focused on species descriptions (Longino & Cover 2004; Kiran *et al.* 2008; Shattuck 2008); however, the magnitude of species diversity of *Aphaenogaster* is unclear due to few and conserved distinguishing morphological characters.

Aphaenogaster have expanded frontal carinae that partially or wholly cover the antennal insertions (Creighton 1950). All members of this genus have 12-segmented antennae

with a long scape, well-developed eyes, a two-segmented petiole, and (usually) distinct propodeal spines (Coovert 2005). Approximately 18 morphological characters vary among 23 *Aphaenogaster* species (Creighton 1950; Umphrey 1996; Coovert 2005). Ward (1985) replaced total body length with Weber's length. Recently, DeMarco & Cognato (2015) identified an additional 15 diagnostic characters, including the cephalic index, shape of the base of the antennal scape, length of propodeal spines and sculpturing on the head and thorax. The base of the antennal scape is particularly important, due to the wide range of shapes observed in different species. Current identification keys are based on the workers. Genitalia have not been described for most species, except in Boudinot (2013), and original species descriptions have insufficient information concerning queens and males.

The study of ant diversity has largely been based on morphological characters, although in the last decade molecular characters provided crucial information for the determination of generic and species limits (LaPolla *et al.* 2010; Branstetter 2012; Moreau & Bell 2013). Early genetic studies revealed variability in chromosome number and enzymatic variation among different ant species, which suggested potential taxonomic utility of molecular characters (Whelden & Haskins 1953; Imai 1966; Tomaszewski *et al.* 1973; Pamilo *et al.* 1975). Indeed, chromosomal and allozyme variation exist for *Aphaenogaster* species and among populations (Crozier 1977). For example, *A. rudis* Enzmann, from the coastal plains of the United States, had a chromosome number of $n = 20$ and nearly fixed for an esterase allele, while montane specimens were either $n = 18$ or $n = 22$ and had variable allele frequencies (Crozier 1977).

Umphrey (1996) attempted to discriminate a complex group of ten sibling species of the *Aphaenogaster fulva–rudis–texana* complex with karyotypes and morphology. Karyotyping of 223 colonies from 63 localities, mostly in eastern North America, identified 10 genetic forms including *A. rudis*, *A. picea* (Wheeler), *A. miamiana* Wheeler, *A. carolinensis* Wheeler, *A. texana* Wheeler, *A. fulva* Roger and four undescribed taxa. Chaetotaxy and a morphometric analysis using 12 characters including characters such as head width, scape length, spine length, and distance between the spines yielded little additional diagnostic information. While there was some variability in size, shape and colour, this variation was confounded by variation within a colony or species. For example, *A. rudis* was morphologically similar to other species occurring in the same habitat. Umphrey (1996) concluded that karyotypes provided the best, but imperfect, means for species diagnosis. He acknowledged that DNA would ultimately prove useful as a definitive method for separating these groups.

A phylogeny based on DNA characters could define the relationships among *Aphaenogaster* species and further diagnose North American species. A recent Bayesian phylogeny testing the placement of the genus demonstrated non-monophyly of *Aphaenogaster*, as a clade of *Aphaenogaster* species grouped with species in two other genera, *Messor* and *Stenamma* (Brady *et al.* 2006; Branstetter 2012). Ward (2011) suggested that convergent evolution and retention of ancestral similarities were two major factors contributing to this non-monophyly. *Aphaenogaster* monophyly was resolved, in part, with the resurrection of *Novomessor* (DeMarco & Cognato 2015). There are no published phylogenies based on DNA or morphological data that focus on the species relationships within *Aphaenogaster* despite the apparent need (Umphrey 1996; Ward 2011; Lubertazzi 2012; Ward *et al.* 2015), particularly for the '*fulva–rudis–texana*' complex, as described by Umphrey (1996). In this study, we used sequence data from five genes to reconstruct a phylogeny for 44 *Aphaenogaster* and outgroup species. The resulting trees support some previously recognized groups, but also reveal polyphyly among specimens identified as *A. rudis*.

Materials and methods

Previous species concepts for *Aphaenogaster* were mainly morphological and genetic (Crozier 1977; Umphrey 1996). Our phylogenetic approach necessitated a phylogenetic species concept, founded in hypothesis testing (Hey 2006). Thus, we tested the monophyly of the currently recognized *Aphaenogaster* species. Non-monophyly of species suggested the need for the revision of species boundaries.

Ant collecting occurred in the eastern and central US forests and grasslands, and the western forests and deserts. Collection localities represent disjunct populations separated by a distance greater than the ants' dispersal capabilities in one generation (Lubertazzi 2012) (Table 1). For hypothesis-generating purposes, four additional samples were included in the analysis from Costa Rica, Greece, Japan and Madagascar to so to explore the relationships between North American and worldwide *Aphaenogaster*. GPS coordinates were recorded for all sites. Specimens were collected using an aspirator and baits (peanut butter and pecan shortbread cookies) and stored in 100% ethanol. At least 12 ants per nest were collected, and 10 nests were sampled for within a 3 km radius to assess intraspecific variation at a local level. Reproductive forms were collected when possible. Eight representatives from each nest were pinned. Specimens were vouchered in the A.J. Cook Arthropod Research Collection at Michigan State University (Table 1). Other individuals were stored in 100% ethanol at −80 °C for future DNA analysis.

Table 1 Number of varying nucleotide positions (number of varying sites in a given sequence) and uncorrected *p*-distance per gene within the *Aphaenogaster rudis* clade

Gene	Total base pairs	Parsimony uninformative	Parsimony informative	Variability (%)	Interspecific *p*-distance range/average	Intraspecific *p*-distance range/average
CO1	650	16	105	16.10	0.1959–0.2051/0.2026	0.0000–0.1529/0.0535
CAD	817	25	53	6.10	0.0500–0.0575/0.0528	0.0000–0.0285/0.0076
EF1αF2	517	15	14	2.70	0.0000–0.0128/0.0051	0.0000–0.0260/0.0087
LWR	557	29	4	0.07	0.0000–0.0128/0.0051	0.0000–0.0216/0.0060
WG	428	9	6	0.14	0.0029–0.0072/0.0051	0.0000–0.0558/0.0108

A molecular data set was assembled using genetic loci identified in a previous study of ant phylogeny (Brady *et al.* 2006), including the nuclear protein-coding genes wingless, long-wavelength rhodopsin, elongation factor-1αF2 and the mitochondrial protein-coding gene COI. The nuclear protein-coding gene CAD was also used (Ward *et al.* 2010). These loci were chosen because they represent a variety of phylogenetically informative nucleotide positions for intraspecific to intergeneric relationships (Brady *et al.* 2006; Ward *et al.* 2010). DNA was extracted from 22 of 23 currently recognized species of *Aphaenogaster* ants plus outgroups for a total of 123 samples using a silica-based spin column procedure (QIAamp; Qiagen Inc., Santa Clara, CA), following the manufacturer's tissue protocol (Table S1). Specific regions of mitochondrial and nuclear DNA were amplified via polymerase chain reaction (PCR). All PCR cocktails consisted of a total volume of 25 μL and included 14.25–17.25 μL ddH20, 2.5 μL 10× PCR buffer (Qiagen), 1.0 μL 25 mM MgCl2 (Qiagen), 0.5 μL dNTP mix (Qiagen), 2–5 μL DNA template and 0.25 μL HotStar Taq DNA polymerase (Qiagen). PCRs were performed as specified by DeMarco & Cognato (2015). After PCR amplification, unincorporated deoxyribonucleotide triphosphates (dNTPs) and oligonucleotides were removed from PCRs with ExoSAP (http://www.usbweb.com/category.asp?cat=pcr&id=78200) and directly sequenced on an ABI 3700 automated sequencer using a BigDye (Applied Biosystems, Inc., Foster City, CA) fluorescent chemistry reaction. Both sense and antisense strands, including introns and exons, were sequenced for all individuals. Data for all five genes were not generated for some specimens because of amplification difficulties with CAD for *Camponotus* and *Formica* samples.

Phylogenetic analysis was performed using the computer software PAUP* (Swofford 2003) with a heuristic search to find the most parsimonious phylogeny. Bootstrap analysis used resampling, with 1000 replicates. Bremer support was performed with TreeRot v.2.0 (Sorenson 1999) with partition Bremer support for all genes. A phylogeny was inferred using maximum likelihood to maximize the probability of the data given the tree with RAxML (Stamatakis 2014) via the CIPRES Gateway (Huelsenbeck & Ronquist 2001; Miller *et al.* 2010) with 1000 bootstrap replicates. A phylogeny was also inferred with Bayesian analysis based on posterior probabilities with MrBayes via the CIPRES Gateway (Huelsenbeck & Ronquist 2001; Miller *et al.* 2010). We followed guidelines to make credible Bayesian inferences (Huelsenbeck & Ronquist 2001; Bollback 2002). Data were partitioned by gene and codon position (Castoe *et al.* 2004), with models of evolution applied independently to each partition (Nylander *et al.* 2004). For the Bayesian analysis, we used MrModeltest 3.7 (Nylander 2004) for the selection of partition-specific substitution models for the nucleotide data using the Akaike Information Criterion to decrease the potential of overparameterization of the models. The best-fit model for all genes for the Bayes phylogeny was GTR + I + G.

Results

All analyses recovered similar phylogenies and the Bayesian phylogeny was mostly resolved. Most species represented by more than one individual were monophyletic and had relatively high branch support, except *A. rudis*, *A. picea*, *A. huachucana* and *A. uinta* (Figs 1, 2 and S1a–c). In general lower Bremer support coincided with lower bootstrap values. The parsimony tree differed compared to the likelihood and Bayesian trees with a polyphyletic *A. texana* (Figs 1, 2 and S1a–c). The likelihood and Bayesian trees differed by the positions of *A. carolinensis*, and the *A. ashmeadi* (Emery) and *A. treatae* Forel clades (Figs. 2 and S1a–c). As indicated by the partition Bremer values, COI provided most of the support with a total score of 751.1 followed by EF1αF2 with a score of 142.9 (Figs 1 and S1a–c, Table S1, Supporting information). The other genes (CAD at −73.1, LWR at −5.7 and WG at −6.1) provided little support or conflicted with CO1 and EF1αF2 as indicated by negative values. An intron was missing from *A. carolinensis* and *A. miamiana* CAD sequences.

Nucleotide variability, the number of parsimony informative sites, and *p*-distances as compared among the indi-

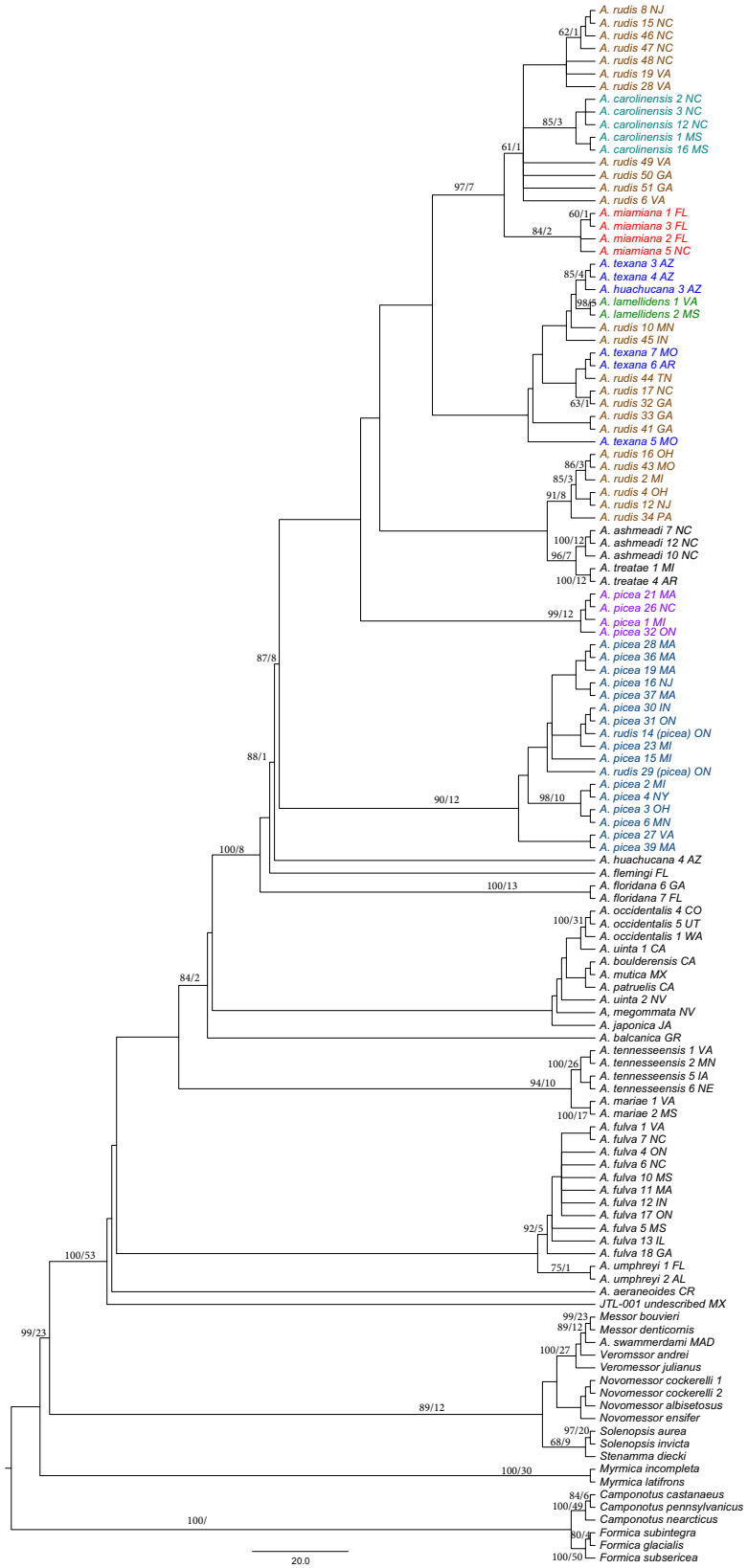

Fig. 1 One of 64 525 MPT reconstructed for 123 taxa of *Aphaenogaster* and outgroups with DNA data and analysis of five genes in PAUP*. Bootstrap/Bremer support values displayed on the branches. Bootstrap values of <60% and Bremer support values of <2 were not shown on the phylogeny. *A.*, *Aphaenogaster*. Specimen numbers and two-letter codes for state/province/country collection localities are displayed next to each sample. Clades with <50% bootstrap values were unresolved in the strict consensus of all most parsimonious trees. Colours indicate morphologically defined taxa.

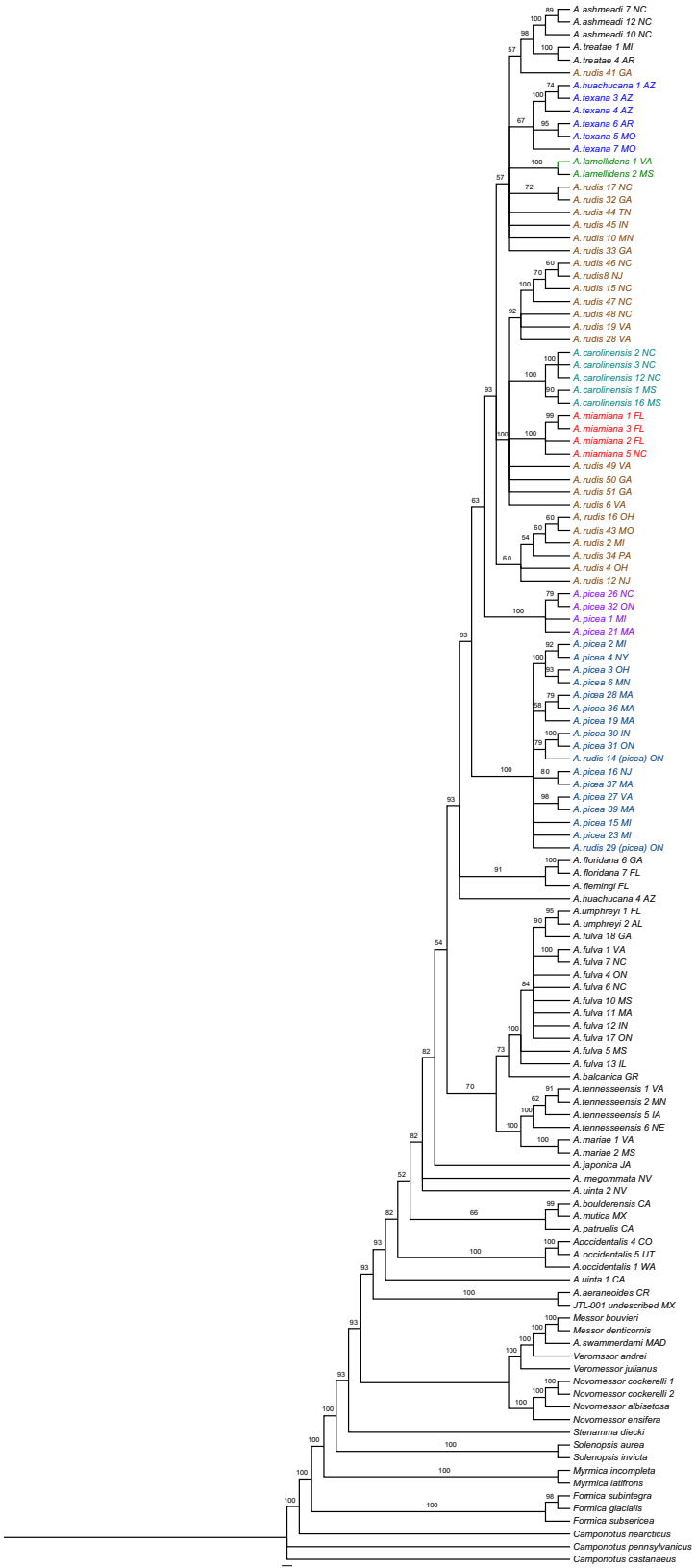

Fig. 2 Bayesian majority rule consensus tree reconstructed for 123 taxa with morphology and five genes found in a Mr. Bayes analysis. Posterior probability values >50% are displayed on the branches. Data were partitioned by gene and codon position and analysed with a best-fit GTR + I + G model, 30 million generations and a burn-in of 7 500 000 generations. *A.*, *Aphaenogaster*. Specimen numbers and states/provinces where collected are displayed next to each sample. (Inset image: lateral habitus of *Aphaenogaster rudis*).

viduals within the *Aphaenogaster rudis* clade differed by gene (Fig. 1, Table 1). The taxa included in this clade were as follows: the four taxa from the small *Aphaenogaster picea* clade, all *A. rudis* except the two within the larger *A. picea* clade, *A. miamiana*, *A. carolinensis*, *A. lamellidens*, *A. texana*, one *A. huachucana*, *A. treatae* and *A. ashmeadi*. The nucleotide differences varied widely from 16% (CO1), 6% (CAD), ~3% (EF1αF2) to <1% for the other nuclear genes (Table 1). The average interspecific COI *p*-distance for the individuals of the *A. rudis* clade is similar to those of *Leptomyrmex* ants (Lucky 2011).

There was strong support for the outgroup taxa in the Formicinae with *Camponotus* and *Formica* sister to the remaining taxa. This was also true for most of the Myrmicinae, including *Solenopsis*, *Stenamma*, *Myrmica* and *Novomessor*. *Veromessor* was sister to the European *Messor* species and *Aphaenogaster swammerdami*. *Aphaenogaster swammerdami* was the only species not within the *Aphaenogaster* clade. *Aphaenogaster araneoides* from Costa Rica and an undescribed species (JTL-001) from Mexico were sister to the other *Aphaenogaster* species. *Aphaenogaster japonica* Forel, from Japan, was within the NA *Aphaenogaster* clade, as was *A. balcanica* (Emery), from Greece.

Most of species collected west of the Rocky Mountains were grouped together near the outgroup species. *Aphaenogaster uinta* Wheeler, like *A. huachucana* Creighton, was polyphyletic. *Aphaenogaster occidentalis* (Emery) from Washington, Utah and Colorado formed a monophyletic clade. There was strong support for the clade including *A. tennesseensis* (Mayr) and *A. mariae* Forel. The clade containing *A. fulva* and *A. umphreyi* was completely separated from the *A. rudis* species complex.

Aphaenogaster floridana Smith and *A. flemingi* Smith were sister to the *A. picea* and *A. rudis* clades. *Aphaenogaster picea* individuals were found in two clades, one containing mostly northern *A. picea* samples and the other individuals were in the *A. rudis* clade. *Aphaenogaster rudis* was not monophyletic, and appeared in four clades (Figs 1, 2 and S1a–c). Taxa in the largest *A. rudis* clade included *A. rudis*, *A. carolinensis* and *A. picea*, in addition to *A. miamiana*, *A. lamellidens* Mayr and *A. texana*, and the clade was sister to *A. ashmeadi* and *A. treatae*. The four smaller *A. rudis* clades are separated by colour in the phylogenies.

Discussion

We tested the monophyly of *Aphaenogaster* in the context of a multigene phylogenetic analysis. In the resulting phylogenies, species boundaries associated with well-supported monophyletic clades of individuals for 11 of 22 NA *Aphaenogaster* species. Many of these monophyletic species contained morphological diagnostic characters discovered by previous taxonomic studies. For example, *A. tennesseensis* lacks setae on the mesosoma and gaster and is a nest parasite of *A. rudis* and *A. fulva* (Creighton 1950; Ellison *et al.* 2012). *Aphaenogaster mariae* is an arboreal species with a starburst pattern of striae on the first gastral tergite (Ellison *et al.* 2012). *Aphaenogaster floridana* is the only south-eastern species lacking propodeal spines and nests in sandy soil in pine forests in North Carolina and Florida, while *A. boulderensis* also lacks spines but occurs in the Northwest (Creighton 1950). *Aphaenogaster flemingi* is diagnosed by a shiny exoskeleton and thin propodeal spines (Creighton 1950). *Aphaenogaster fulva* and *A. umphreyi* have upward pointing spines, and can be separated from each other by the reduced eyes in *A. umphreyi* (Deyrup & Davis 1998). There is no pattern to the type or the magnitude of difference among morphological characters that diagnosis species; they can be obvious like the lack of spines or subtle like the pattern of striae. *Aphaenogaster occidentalis* with reduced spines and occurring across the Northwest is also monophyletic. Three other species, with only one representative each are morphologically distinct. *Aphaenogaster mutica* (Pergande 1896) has minuscule spines and a dark gaster; *A. patruelis* is piceous brown (Creighton 1950) and *A. megommata* is a yellow desert species with large eyes for night foraging (Smith 1963).

Polyphyly of the remaining eleven species is an issue of concern because given the criteria of monophyly, our phylogeny suggests the recognition of fewer species. The large clade of *A. rudis* also includes *A. ashmeadi*, *A. carolinensis*, *A. lamellidens*, *A. miamiana*, *A. texana* and *A. treatae*, and the placement of *A. rudis* individuals is scattered in six separate clades among these other species (Fig. 1). Other instances of paraphyly occur with *A. fulva*–*A. umphreyi* (Figs 2 and S1a–c) and polyphyly with *A. texana*–*A. huachucana*, *A. uinta* and *A. picea* (Figs 2 and S1a–c). It is tempting to synonymize these species in order to preserve monophyly. However, many of the included species are well-supported subclades with morphological and behavioural diagnostic characters; for example, the presence and size of lobe at the base of the scape diagnose *A. ashmeadi* and *A. treatae*, which are well-supported monophyletic species (Creighton 1950). In other cases, the diagnostic character is minor, as with the smaller eye, which characterizes *A. umphreyi* from *A. fulva* (Deyrup & Davis 1998). In addition, there are other potential molecular differences that could diagnose species. For example, *A. carolinensis* and *A. miamiana* lack a 217-bp CAD intron as compared to most other *Aphaenogaster* species. The remaining clades of individuals (e.g. *A. rudis*) may represent unrecognized species that await the discovery of diagnostic characters. Morphology of reproductive adults and nest architecture could provide these characters (Tschinkel 2011; Boudinot 2013).

It is unlikely that current interbreeding contributed to the observed polyphyly because the distribution of shared haplotypes spans hundreds of kilometres beyond the dispersal capabilities of an individual's life. Reported flight distances for one *Aphaenogaster* species are shorter than 1500 m (Talbot 1966). However, there are a number of alternative reasons for the apparent polyphyly in the *A. rudis* clade of NA *Aphaenogaster*, which would argue against abandoning existing nomenclature without additional evidence. First, there may be an insufficient amount of phylogenetically informative data for complete resolution. Although we sampled five genes known to resolve ant phylogenies, only 1102 of 2967 characters were phylogenetically informative and most of the phylogenetic support derived from COI and EF1αF2 (Figs 1, 2 and S1a–c). The other genes demonstrated little variation and gave little or negative support, which is a pattern observed in other insect phylogenies (e.g. Damgaard & Cognato 2003; Danforth *et al.* 2004) (Table 1). Doubling the number of sampled genes or increasing the number of nucleotides to the 100 000s via phylogenomic methods may help to resolve this issue, as they have provided resolution for other taxa (Peterson *et al.* 2012; Ward & Sumnicht 2012).

It is also possible that recent species radiation could explain the low resolution due to a lack of lineage sorting of gene lineages, as demonstrated with gallwasps (Rokas *et al.* 2003) and *Formica* ants (Goropashnaya *et al.* 2004). Although the estimated age of this genus is 44 million years (Moreau *et al.* 2006), the relatively short branches and minimal COI sequence variation (mean 2.85%) observed for the *A. rudis* clade (not including the larger *A. picea* clade) suggest more recent origins of the species. A possible Pleistocene origin of these species during the expansion and contraction of glaciers could have contributed to the isolation of populations by altitude and latitude in north-eastern United States, as has been shown for many other taxa (e.g. Cognato *et al.* 2003; Maroja *et al.* 2007; Lecocq *et al.* 2013). Pre- and postmating isolating mechanisms such as chromosomal rearrangements may have developed in glacial refugia and contributed to *Aphaenogaster* speciation. Potentially karyotype number may diagnose species boundaries, because much chromosomal variation exists within subfamilies, genera and even *Aphaenogaster* (Umphrey 1996; Menezes *et al.* 2013; Cardoso *et al.* 2014), and could be important in the generating reproductive isolation (Lorite & Palomeque 2010). This is consistent with the observation that distinct karyotypes associate with geographic distributions (Umphrey 1996). For example, populations of western *A. picea* have $n = 17$, while eastern populations have $n = 18$. Our specimens of *A. picea* occur in two clades (Figs 2 and S1a–c) but unfortunately, other than one sample, *A. rudis* (#43), we do not have associated karyotype numbers for our specimens. It is unknown whether members of the *A. rudis* clade with different karyotypes produce viable offspring or whether other isolating mechanisms exist. Identification of these mechanisms and the possibility of a speciation gene, such as those that cause hybrid male sterility in *Drosophila*, could help resolve *Aphaenogaster* species relationships (Gomes & Civetta 2014). Obviously, more study is needed to resolve the non-monophyly of *A. rudis* and other species, to provide diagnostic characters and to determine the existence of pre- or postmating barriers among the species. Thus, a revision of *Aphaenogaster* is premature.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Maximum likelihood tree reconstructed for 123 taxa with DNA data and analysis of five genes in a RAxML analysis. Bootstrap values >50% are displayed on the branches; *A.*, *Aphaenogaster*. Specimen numbers and state/province collection localities are displayed next to each sample. The names of non-monophyletic species correspond to specific colours. *Aphaenogaster rudis* is separated into four smaller clades. (a–c) Bayesian majority rule consensus tree reconstructed for 123 taxa with morphology and five genes in a Mr. Bayes analysis, posterior probability values >50% are displayed on the branches. Data were partitioned by gene and codon position and analysed with a best-fit GTR + I + G model, 30 million generations and a burn-in of 7 500 000 generations. *A.*, *Aphaenogaster*. Specimen numbers and state/province collection localities are displayed next to each sample. (Inset image: lateral habitus of *Aphaenogaster rudis*).

Table S1. *Aphaenogaster* and outgroup specimens with associated localities and GenBank numbers.

