

Phylogenetic Relationships Based on DNA Barcoding Among 16 Species of the Ant Genus *Formica* (Hymenoptera: Formicidae) from China

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Abstract

In this study, we sequenced fragments of cytochrome oxidase subunit 1 (CO1), internal transcribed spacer 1 (ITS1), and internal transcribed spacer 2 (ITS2) genes from 150 specimens belonging to 16 species of the ant genus *Formica* from China. *Odontoponera transversa* from Ponerinae and *Polyergus samurai* from Formicinae were added as distant relative and close relative outgroups, respectively. Neighbor-joining, maximum parsimony, and Bayesian interference methods were used to analyze their phylogenetic relationships based on CO1 gene sequence as well as combined sequence data of CO1 + ITS1, CO1 + ITS2, and CO1 + ITS1 + ITS2. The results showed that nine *Formica* species (i.e., *Formica sinensis*, *Formica manchu*, *Formica uralensis*, *Formica sanguinea*, *Formica gagatoides*, *Formica candida*, *Formica fusca*, *Formica glauca*, and *Formica* sp.) formed monophyletic clades, which in agreement with the results based on morphological taxonomy. By comparing the results of DNA barcoding and morphological taxonomy, we propose that *Formica aquilonia* maybe a junior synonym of *F. polyctena* and that cryptic species could likely existed in *Formica sinae*. Further studies on morphology, biology, and geography are needed to confirm this notion.

Key words: *Formica*, DNA barcode, phylogenetic relationship

The ant genus *Formica* was established by Linnaeus in 1758 based on the type species *Formica rufa*. This large genus contains 261 extant species or subspecies and is mainly distributed in Nearctic and Palearctic regions (Bollton 2016). In China, 44 species and 1 subspecies were recorded and are reported to be mainly distributed in the northern area (Ran and Zhou 2012).

Formica has a disorganized taxonomy, and many of its species are similar to each other. Some species differ only by color, glossiness, or number of erect hairs, and many synonyms have been used to describe species in this genus. For example, *Formica dluskyi* is a junior synonym of *Formica manchu* (Seifert 2000), and *Formica imitans* is a junior synonym of *Formica cinerea* (Seifert 2002). However, the descriptions of some species have remained controversial. For instance, *Formica transkaucasica* was considered a junior synonym of *Formica candida* (Bollton 1995), but it was later regarded as a species based on morphologic metrology and genetics data (Seifert 2004). Similarly, *Formica glauca* was considered a junior synonym of *Formica cunicularia* (Atanassov and Dlussky 1992), but some myrmecologists thought otherwise (Seifert 2007, Czechowski et al. 2012).

DNA barcoding is a diagnostic technique that uses DNA sequence(s) for species identification (Savolainen et al. 2005). Tautz

et al. (2003) reported that traditional identification can be replaced by DNA identification and that a standard DNA fragment can be a reference for species identification. Hebert (2003) was the first to propose the use of DNA barcoding for species identification. DNA barcoding can be fast and accurate in identifying and discovering of cryptic species, as well as in the assessing biological diversity. Moreover, this method can compensate for the deficiencies in morphological identification.

With the development of molecular analysis techniques, the phylogenetic relationships of Formicidae have also been explored using NDA sequence data. Fisher et al. (2008) used DNA barcoding to revise Malagasy species of *Anochetus* and *Odontomachus*, two similar genera, and found three new species that could be separated from all other species and formed different clades in the neighbor-joining (NJ) tree. Jansen et al. (2009) used DNA barcoding to distinguish all of the species of Nearctic *Myrmica* ants. Similarly, Seppä et al. (2011) used two closely related *Formica* ants, *Formica fusca* and *Formica lemani*, to determine the consistency of morphological and genetic data with chemical data. They found that model-based Bayesian interference clustering facilitates perfect separation of the species without any indication of hybridization. Moreover, their results showed that *F. fusca* and *F. lemani* did not share any mitochondrial haplotypes

and were perfectly separated in the phylogenetic tree. In addition, DNA barcoding has revealed cryptic species, which are difficult to distinguish using traditional morphological taxonomy (Seifert et al. 2009, Smith et al. 2009, Donoso 2012, Ng'endo et al. 2013).

In the present study, we selected 150 specimens belonging to 16 closely related species of the ant genus *Formica* to sequence their cytochrome oxidase subunit 1 (CO1), internal transcribed spacer 1 (ITS1), and internal transcribed spacer 2 (ITS2) fragments. Then, the NJ, maximum parsimony (MP), and Bayesian interference (BI) methods were used to construct phylogenetic trees based on the CO1 gene and the combined sequence data of CO1 + ITS1, CO1 + ITS2, and CO1 + ITS1 + ITS2. Finally, their phylogenetic relationships were analyzed through DNA barcoding.

Materials and Methods

Specimen Sampling

The specimens used in this study were collected from the following provinces in China: Hebei, Heilongjiang, Jilin, Liaoning, Inner Mongolia, Ningxia, Shanxi, Shaanxi, Yunnan, and Guangxi (Supp Appendix table [Online only]). *Odontoponera transversa* from Ponerinae and *Polyergus samurai* from Formicinae were added as distant relative and close relative outgroups, respectively. Whole bodies of ant specimens collected in the field were placed in 100% ethanol and maintained at -60°C until further use. Some specimens were used for morphological identification, and they were deposited in the Insect Collection of Guangxi Normal University, Guilin, China.

DNA Extraction, PCR, and Sequence Alignment

Prior to DNA extraction, gasters were removed to minimize contamination from gut bacteria. Total genomic DNA was extracted from the specimens using a previously described protocol (Rawson and Hilbish 1995) and then stored in 100% ethanol. Gene fragments of CO1, ITS1, and ITS2 were amplified using the primers listed in Table 1.

Polymerase chain reactions (PCR) were performed in a total volume of 50 μl containing 25 μl of 2 \times Taq Master Mix, 1 μl of each primer, 1 μl of DNA, and 22 μl of H_2O . The PCR conditions for CO1 gene were as follows: 95°C for 5 min, 35 cycles of 94°C for 45 s, 48°C for 45 s, and 70°C for 90 s; and a final extension at 72°C for 10 min. The PCR conditions for the ITS regions consisted of 94°C for 2 min, 40 cycles of 94°C for 20 s, 51°C (ITS1), and 52°C (ITS2) for 30 s, and 72°C for 15 s; and a final extension at 72°C for 10 min. PCR products were purified using a DNA purification kit (Sangon Biotech, Shanghai, China) and were sequenced from both directions by Shanghai Generay Biotech Co. Ltd.

Phylogenetic Analyses

DNA sequences were assembled using SeqMan (DNASTar Inc., Madison, WI; (Swindel and Plasterer 1997) and aligned using CLUSTAL X algorithm (Thompson et al. 1997) with default

parameters. The sequences were first added in the ingroup taxa and subsequently added in the outgroup taxa using the profile alignment option. CO1 nucleotide sequences were translated to amino acid sequences using MEGA 4.0 (Tamura et al. 2007) to eliminate sequences with stop codons. Single-gene (CO1, ITS1, ITS2), two-gene (CO1 + ITS1, CO1 + ITS2), and three-gene (CO1 + ITS1 + ITS2) data sets were then assembled followed by analysis of phylogenetic relationships among taxa using NJ, MP, and BI.

NJ trees were built using MEGA 4.0 (Tamura et al. 2007) with the K2P molecular evolutionary model. Estimates of nodal support on distance trees were obtained using bootstrap analyses (1000 replications). Unweighted MP analysis was performed using PAUP* Version 4.0b10 (Swofford 2002) with a heuristic search with random sequence addition (10 replicates each) and the TBR branch-swapping algorithm. BI tree was constructed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) with the best-fitting model (GTR + I + G) selected from Modeltest 3.7 (Posada and Crandall 1998) based on the akaike information criterion (Posada and Buckley 2004). Markov chains were run for 100,000 generations with sampling for every 100th generation. After finishing the runs, the first 250 trees were discarded as burn-in.

Results

DNA Sequence Composition

Sequence alignment of the three combined data of CO1, CO1 + ITS1, and CO1 + ITS2 yielded data matrices with 2844 bp, 343 variable sites, 306 parsimony-informative sites, and 37 singleton sites. Of the three combined data, CO1 was 658-bp long and had 155 variable sites, 147 parsimony-informative sites, and 8 singleton sites. ITS1 the longest with 1134-bp long and had 87 variable sites, 62 parsimony-informative sites, and 25 singleton sites. ITS2 was 1053-bp long and had 112 variable sites, 88 parsimony-informative sites, and 24 singleton sites. The base composition of the three gene fragments varied among the species. On average, the CO1 fragment was heavily AT biased (72.1%: A, 30.6%; T, 41.5%; G, 10.7%; C, 17.2%). However, the ITS fragments were heavily GC biased; ITS1 had a GC value of 59.3% (A, 21.7%; T, 19.0%; G, 30.8%; C, 28.5%), whereas ITS2 had a GC value of 60.3% (A, 20.9%; T, 18.8%; G, 30.5%; C, 29.8%; Table 2).

Phylogenetic Trees

Phylogenetic analyses showed that the outgroups *P. samurai* and *O. transversa* were well resolved from the *Formica* taxa at the base of the tree. Based on the four datasets (CO1, CO1 + ITS1, CO1 + ITS2, and CO1 + ITS1 + ITS2), all of the phylogenetic trees constructed using NJ, MP, or BI methods were clustered into nine clades. The CO1 and CO1 + ITS NJ trees, MP trees, and BI trees are shown in Figs. 1–3, respectively.

Figures 1–3 show the formation of nine clades in the phylogenetic trees. In clade I, *F. sinensis* formed a clade and then clustered

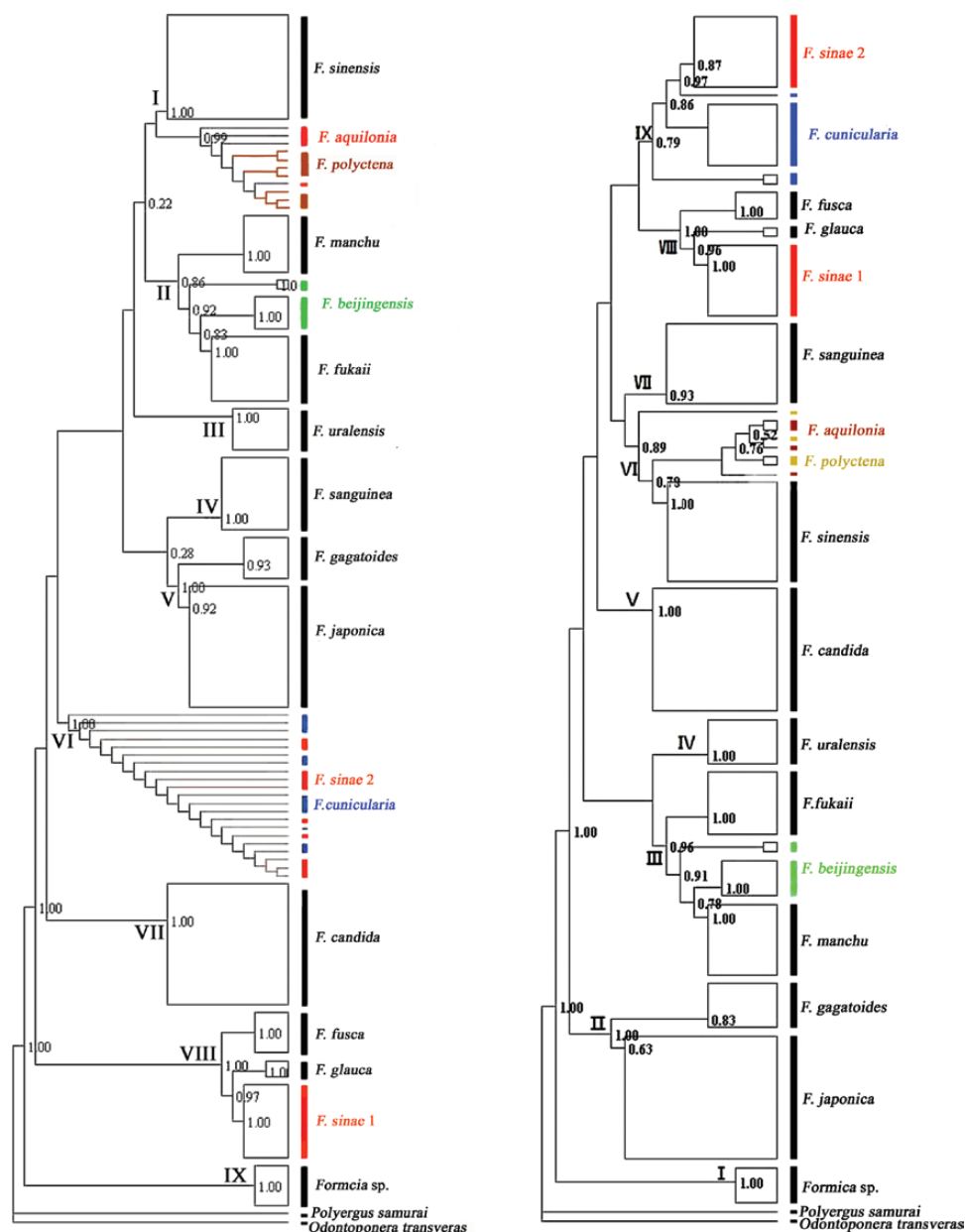
Table 1. Primers used to amplify CO1, ITS1, and ITS2 fragments

Gene	Primer	Sequence(5'–3')	References
CO1	LCO1490	GGTCAACAAACATAAAGATATTGG	Ji et al. 2003
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	
ITS1	18s F1	TACACACCGCCCGTCGCTACTA	
	5.8s B1d	ATGTGCGTTTCRAAATGTCGATGTTCA	
ITS2	5.8s Fc	TGAACATCGACATTTTGAACGCACAT	
	28s B1d	TTCTTTTCCTCCSCTTAYTRATATGCTTAA	

Table 2. Composition of nucleotide sequences

Genes (bp)	Cs	V	Pi	S	A (%)	T (%)	G (%)	C (%)	A + T (%)	G + C (%)
CO1(658)	503	155	147	8	30.6	41.5	10.7	17.2	72.1	27.9
ITS1(1134)	1013	87	62	25	21.7	19.0	30.8	28.5	40.7	59.3
ITS2(1053)	891	112	88	24	20.9	18.8	30.5	29.8	39.7	60.3

Cs (conserved sites); V (variable sites); Pi (parsimony-informative sites); S (singleton sites).

**Fig. 1.** NJ trees of 16 *Formica* species.

with *F. aquilonia* and *F. polycтена* to form a lineage. The latter two species were mixed and could not be separated. In clade II, *Formica beijingensis* divided into two branches, one branch clustered with *F. manchu* to form a lineage and another branch clustered with *Formica fukaii* to form another lineage. The two lineages grouped in a clade and clustered with clade I, which then clustered with clade III formed by the monophyletic species *Formica uralensis*. Clade IV

was also formed by the monophyletic species *Formica sanguinea*. Clade IV clustered with clade V, which was formed by *Formica gagatoides* and *Formica japonica*. Clade VI included *Formica siniae* and *F. cunicularia*, which were mixed and could not be separated. Clade VII was formed by the monophyletic species *F. candida* and grouped with clade VI. Moreover, *F. fusca*, *F. glauca*, and *F. siniae* grouped with clade VIII and then clustered with clade IX, which

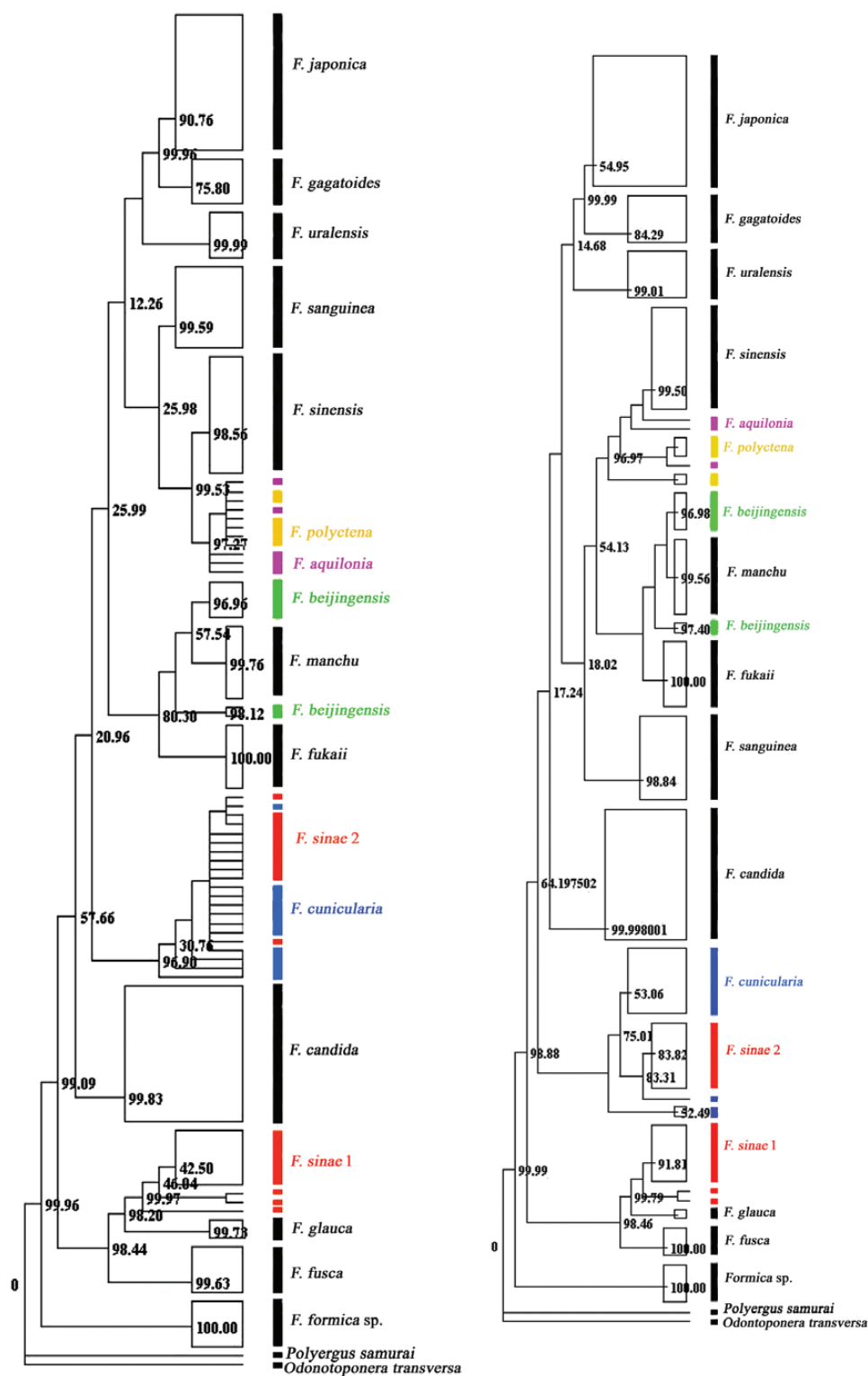


Fig. 2. MP trees of 16 *Formica* species.

was formed by an undescribed *Formica* sp. Interestingly, *F. sinensis* was divided into two fixed groups with no morphological differences. In the trees based on CO1 gene sequences, *F. sinensis* group 2 mixed with *F. cunicularia* in a branch, whereas these species separated in the trees based on CO1 + ITS sequences. The K2P distance in *F. sinensis* is shown in Table 3. The intragroup distances of *F. sinensis* group 1 and *F. sinensis* group 2 were 0–0.6% and 0–0.3%, respectively. However,

the intergroup distances of *F. sinensis* group 1 and *F. sinensis* group 2 were 6.8–7.1% and 6.8–7.1% or 7.1–7.4%, respectively.

Discussion

CO1, ITS1, and ITS2 from 150 specimens belonging to 16 species of the ant genus *Formica* from China were used as the target genes



Fig. 3. BI trees of 16 *Formica* species.

for DNA barcoding and analysis of the phylogenetic relationship among the species. Our results showed that CO1 was heavily AT biased compared with other insects (Simon et al. 1994). However, ITS1 and ITS2 were heavily GC biased compared with those reported in fish (Chow et al. 2006) and insects (Hung et al. 2004, Trizzino et al. 2009). The sequences of CO1 had useful variations and therefore yielded better phylogenetic trees than ITS1 and ITS2. CO1 had a success rate of 99.33%, whereas both ITS1 and ITS2 barcodes showed significantly lower success rates (ITS1: 93.33%, ITS2: 91.33%, and CO1 + ITS: 90.67%). Single ITS1 or single ITS2 was not suitable for phylogenetic analyses for the genus *Formica*, because these genes have high similarity and their transition and transversion almost reached saturation. The power of barcoding can be improved by multiple-gene analysis because of its capacity to increase genetic diversity (Dai et al. 2012). We found that *F. siniae* and *F. cunicularia* were divided into two branches in the NJ tree of CO1 + ITS1 + ITS2 trees, whereas these genes mixed into one branch in the CO1 trees.

Formica sp. resembles *F. wongi* and *F. yessensis* but differs in characteristics, such as gaster with abundant hairs, alitrunk, and petiole with no hair, and upper margin of petiole concave. *Formica* sp. can be separated from others in the genus as shown in all of the figures. Further studies on their DNA barcoding are needed because

CO1, ITS1, and ITS2 sequences of *F. wongi* or *F. yessensis* are lacking in the National Center for Biotechnology Information database.

For *F. manchu*, among all of the specimens of NMG030 in the same nest, 17 specimens had erect hair at the end of the first gastral segment; 7 had erect hair at the end of the second gastral segment; and 1 had erect hair at the end of the third, fourth, and fifth gastral segments. All of the specimens were grouped into the same branch. These results suggest that distinguishing the species based on the erect hairs on different gastral segments is not scientifically reliable.

F. polycтена and *F. aquilonia* are in the *F. rufa* group (Collingwood 1979, Douwes 1981). Douwes (1981) found that *F. polycтена* could not be distinguished from *F. aquilonia* by using the head and hind femur color, the number of hairs on the occiput of the head and hind femur, or the shape of upper margin the petiole. Our result supports Douwes' finding; in the samples of HLJ024 collected from the same nest and identified morphologically as *F. aquilonia*, the numbers of hairs on the occiput of the head and hind femur differed. Among the 17 specimens, 15 had hairs on the occiput of the head and 9 with upper margin of the petiole concave. Among the 25 specimens of *F. polycтена*, 10 had hairs on the occiput of the head, 8 on the hind femur, and 15 with upper margin of the petiole concave. In the trees of CO1 and CO1 + ITS1 + ITS2 for *F. aquilonia* and *F. polycтена*,

Table 3. K2P-distance (transitions + transversions) of *F. sinae* group 1 and *F. sinae* group 2 (all of the data were multiplied by 100)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	0																		
2	0	0																	
3	0	0	0																
4	0	0	0	0															
5	0	0	0	0	0														
6	0	0	0	0	0	0													
7	0.2	0.2	0.2	0.2	0.2	0.2	0.2												
8	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2											
9	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2									
10	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.9	6.9	7.1								
11	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.9	6.9	7.1	0							
12	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9	7.1	7.1	7.3	0.3	0.3						
13	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.9	6.9	7.1	0.2	0.2	0.2					
14	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9	7.1	7.1	7.3	0.3	0.3	0.3	0.2				
15	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.3	7.3	7.4	0.5	0.5	0.5	0.3	0.5			
16	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.1	7.3	7.3	7.4	0.5	0.5	0.5	0.3	0.5	0		
17	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9	7.1	7.1	7.3	0.6	0.6	0.6	0.5	0.6	0.2	0.2	
18	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.9	6.9	7.1	0.6	0.6	0.6	0.5	0.6	0.5	0.5	0.6
19	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.9	6.9	7.1	0.5	0.5	0.5	0.3	0.5	0.3	0.3	0.5

Note: Numbers (1–19) in the first column refer respectively to samples of JL003, LN001, HLJ027, NMG006, NMG005, NMG004, HLJ036, SX012, SX011, SX013, HLJ028, HLJ029, NMG007, HLJ030, NMG009, SX032, JL002, HLJ031, and HLJ009. Among them, 1–10: *F. sinae* group 2 and 11–19: *F. sinae* group 1.

all of the specimens sampled in the Heilongjiang Province were mixed in the same branch. Their morphological identification was not possible. Thus, based on morphology and DNA barcoding data, we propose that *F. aquilonia* may be a junior synonym of *F. polycetena*.

Finally, *F. sinae* was divided into two fixed groups in the phylogenetic trees, group 1 fell into a monophyletic clade, while group 2 clustered with *F. cunicularia*, and the genetic distance between the two groups (0–0.6%) was smaller than the intergroup (6.8–7.4%; Table 3). Hebert et al. (2003) proposed a mean intraspecific divergence of 10 times as the standard threshold for differentiating species. Thus, we propose that cryptic species are likely to exist in *F. sinae*. Further studies based on morphology, biology, and geography are needed to confirm this notion.

Supplementary Material

Supplementary material can be found at *Journal of Insect Science* online.

Acknowledgments

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References Cited

- Agosti, D. A. 1994. The phylogeny of the ant tribe Formicini (Hymenoptera: Formicidae) with the description of a new genus. *Syst. Entomol.* 19: 93–117.
- Atanassov, N. and G. M. Dlussky. 1992. *Fauna na Bulgariya* 22. *Hymenoptera, Formicidae*. Fauna Bulg. 22: 1–310.
- Bollton, B. 1995. A new general catalogue of the ants of the world, pp. 506. Harvard University Press, Cambridge, MA.

- Bollton, B. 2016. A new general catalogue of the ants of the world[EB/OL]. [28 April 2016]. <http://www.antweb.org/description.do?subfamily=formicinae&genus=formica&rank=genus&project=missouriant>.
- Cai, C. C., Z. Y. Yang, H. Xie, and S. L. Pan. 2011. Identification of medicinal plants from *Piper* L. based on rDNA ITS sequences. *Chinese J. Clin. Med.* 18: 136–138.
- Chow, S., T. Nakagawa, N. Suzuki, H. Takeyama, and N. Matsunaga. 2006. Phylogenetic relationships among *Thummus* species inferred from rDNA ITS1 sequence. *J. Fish Biol.* 68 (Supplement A): 24–35.
- Clerc-Blain, J. L., J. R. Starr, R. D. Bull, and J. M. Saarela. 2010. A regional approach to plant DNA barcoding provides high species resolution of sedges (*Carex* and *Kobresia*, Cyperaceae) in the Canadian Arctic Archipelago. *Mol. Ecol. Res.* 10: 69–91.
- Collingwood, C. A. 1979. The Formicidae (Hymenoptera) of Fennoscandia and Denmark. *Fauna Entomol. Scand.* 8: 1–174.
- Czechowski, W., A. Radchenko, W. Czechowska, and K. Vepsäläinen. 2012. The ants of Poland, with reference to the myrmecofauna of Europe. *Fauna Polon. (N.S.)* 4: 1–200.
- Dai, Q. Y., Q. Gao, C. S. Wu, D. Chesters, C. D. Zhu, and A. B. Zhang. 2012. Phylogenetic reconstruction and DNA barcoding for closely related pine moth species (*Dendrolimus*) in China with multiple gene markers. *PLoS One.* 7: e32544.
- Douwes, P. 1981. Intraspecific and interspecific variation in workers of the *Formica rufa* group (Hymenoptera: Formicidae) in Sweden. *Entomol. Scand. Suppl.* 15: 213–223.
- Donoso, D. A. 2012. Additions to the taxonomy of the armadillo ants (Hymenoptera, Formicidae, Tatuidris). *Zootaxa* 3503: 61–81.
- Emery, C. 1920. La distribuzione geografica attuale delle formiche: tentativo di spiegarne la genesi col soccorso di ipotesi filogenetiche e paleogeografiche[M]. *Tipogr. della R. Accad. dei Lincei.* 8: 357–450.
- Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3: 294–299.
- Fisher, B. L., and M. A. Smith. 2008. A revision of Malagasy species of *Anochetus mayr* and *Odontomachus latreille* (Hymenoptera: Formicidae). *Plos One.* 3: e1787.
- Gao, T., H. Yao, J. Song, Y. Zhu, C. Liu, and S. Chen. 2010. Evaluating the feasibility of using candidate DNA barcodes in discriminating species of the large Asteraceae family. *bmc Evol. Biol.* 10: 324.
- Hebert, P. D., A. Cywinska, S. L. Ball, and R. Jeremy. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B Biol. Sci.* 270: 313–321.

- Hurst, G. D. and F. M. Jiggins. 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proc. Biol. Sci.* 272: 1525–1534.
- Hung, Y. T., C. A. Chen, W. J. Wu, C. C. Lin, and C. J. Shih. 2004. Phylogenetic utility of the ribosomal internal transcribed spacer 2 in *Strumigenys* spp. (Hymenoptera: Formicidae). *Mol. Phylogenet. Evol.* 32: 407–415.
- Jansen, G., R. Savolainen, A. L. A. Veps, and K. Inen. 2009. DNA barcoding as a heuristic tool for classifying undescribed Nearctic *Myrmica* ants (Hymenoptera: Formicidae). *Zool. Scri.* 38: 527–536.
- Ji, Y. J., D. X. Zhang, and L. J. He. 2003. Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. *Mol. Ecol. Notes.* 3: 581–585.
- Jorgensen, R. A. and P. D. Cluster. 1988. Modes and tempos in the evolution of nuclear ribosomal DNA: new characters for evolutionary studies and new markers for genetic and population studies. *Ann. Mis. Bot. Gard.* 75: 1238–1247.
- Kuperus, W. R. and W. Chapc. 1994. Usefulness of internal transcribed spacer regions of ribosomal DNA in melanopline (Orthoptera: Acrididae) systematics. *Ann. Entomol. Soc. Am.* 87: 751–754.
- Liu, J. S. and C. L. Schardl. 1994. A conserved sequence in internal transcribed spacer 1 of plant nuclear rRNA genes. *Plant Mol. Biol.* 26: 775–778.
- Ng'endo, R. N., Z. B. Osimo, and R. Brandl. 2013. DNA barcodes for species identification in the hyperdiverse ant genus *Pheidole* (Formicidae: Myrmicinae). *J. Insect Sci.* 13: 27.
- Park, D. S., S. J. Suh, H. W. Oh, and P. D. Hebert. 2010. Recovery of the mitochondrial COI barcode region in diverse Hexapoda through tRNA-based primers. *bmc Genomics.* 11: 423.
- Posada, D. and T. R. Buckley. 2004. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Syst. Biol.* 53: 793–808.
- Posada, D. and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics.* 14: 817–818.
- Ran, H. and S. Y. Zhou. 2012. Checklist of Chinese ants: Formicomorph subfamilies (Hymenoptera: Formicidae) (II). *J. Guangxi Normal Univ. Nat. Sci. Edit.* 30: 81–91.
- Rawson, P. D. and T. J. Hilbish. 1995. Evolutionary relationships among the male and female mitochondrial DNA lineages in the *Mytilus edulis* species complex. *Mol. Biol. Evol.* 12: 893–901.
- Ronquist, F. and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics.* 19: 1572–1574.
- Savolainen, V., R. S. Cowan, A. P. Vogler, G. K. Roderick, and R. Lane. 2005. Towards writing the encyclopedia of life: an introduction to DNA barcoding. *Philos. Trans. r. Soc. Lond. b. Biol. Sci.* 360: 1805–1811.
- Seifert, B. 2000. A taxonomic revision of the ant subgenus *Coptoformica* Mueller, 1923. *Zoosystema.* 22: 517–568.
- Seifert, B. 2002. A taxonomic revision of the *Formica cinerea* group. *Abhandl. Beri. Nat. Görlitz.* 74: 245–272.
- Seifert, B. 2004. The “Black Bog Ant” *Formica picea* Nylander, 1846—a species different from *Formica candida* Smith, 1878. *Myrmecol. Nachrichten.* 6: 29–38.
- Seifert, B. 2007. Die Ameisen Mittel- und Nordeuropas, pp. 1–368. Tauer, lutra Verlags- und Vertriebsgesellschaft, Germany.
- Seifert, B., B. C. Schlick-Steiner, and F. M. Steiner. 2009. *Myrmica constricta* Karavajev, 1934 – a cryptic sister species of *Myrmica hellenica* Finzi, 1926. *Soil Organisms.* 81: 53–76.
- Seppä, P., H. Helanterä, K. Trontti, and J. Martin. 2011. The many ways to delimit species: hairs, genes and surface chemistry. *Myrmecol. News* 15: 31–41.
- Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook, 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* 87: 651–701.
- Smith, M. A. and B. L. Fisher. 2009. Invasions, DNA barcodes, and rapid biodiversity assessment using ants of Mauritius. *Front. Zool.* 6: 31.
- Stahlhut, J. K., J. Fernández-Triana, S. J. Adamowicz, M. Buck, H. Goulet, P. D. Hebert, J. T. Huber, M. T. Merilo, C. S. Sheffield, T. Woodcock, et al. 2013. DNA barcoding reveals diversity of Hymenoptera and the dominance of parasitoids in a sub-arctic environment. *bmc Ecol.* 13: 2.
- Swindel, S. R. and T. N. Plasterer. 1997. SeqMan.//Sequence data analysis guidebook. Springer, New York. 75–89.
- Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Vol. 4. Sinauer Associates, Sunderland, MA.
- Szalanski, A. L., J. A. Mckern, C. Solorzano, and J. W. Austin. 2010. Genetic diversity of ants (Hymenoptera: Formicidae) from the Ozark-St. Francis National Forest, Arkansas, USA. *Sociobiology.* 56: 601.
- Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24: 1596–1599.
- Tautz, D., P. Arctander, A. Minelli, R. H. Thomas, and A. P. Vogler. 2003. A plea for DNA taxonomy. *Trends Ecol. Evol.* 18: 70–74.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25: 4876–4882.
- Trizzino, M., P. Audisio, G. Antonini, A. De Biase, and E. Mancini. 2009. Comparative analysis of sequences and secondary structures of the rRNA internal transcribed spacer 2 (ITS2) in pollen beetles of the subfamily Meligethinae (Coleoptera, Nitidulidae): potential use of slippage-derived sequences in molecular systematics. *Mol. Phylogenet. Evol.* 51: 215–226.
- Wheeler, W. M. 1913. A revision of the ants of the genus *Formica* (Linne) Mayr. *Mus. Comp. Zool. Harvard College.* 53: 379–565.
- Wu, J. 1990. Taxonomic studies on the genus *Formica* L. of China (Hymenoptera: Formicidae). *Forest Res.* 3: 1–8.
- Yarrow, I. H. H. 1955. The British ants allied to *Formica rufa* (Hym., Formicidae). *Trans. Soc. British. Entomol.* 12: 1–48.
- Yasumatsu, K. and W. L. Brown, Jr. 1951. Revisional notes on *Camponotus herculeanus* Linné and close relatives in Palearctic regions (Hymenoptera: Formicidae). *J. Facul. Agri. Kyushu Univ.* 10: 29–44.