

# Article

# *Formica picea* and *F. candida* (Hymenoptera: Formicidae): Synonyms or Two Species?

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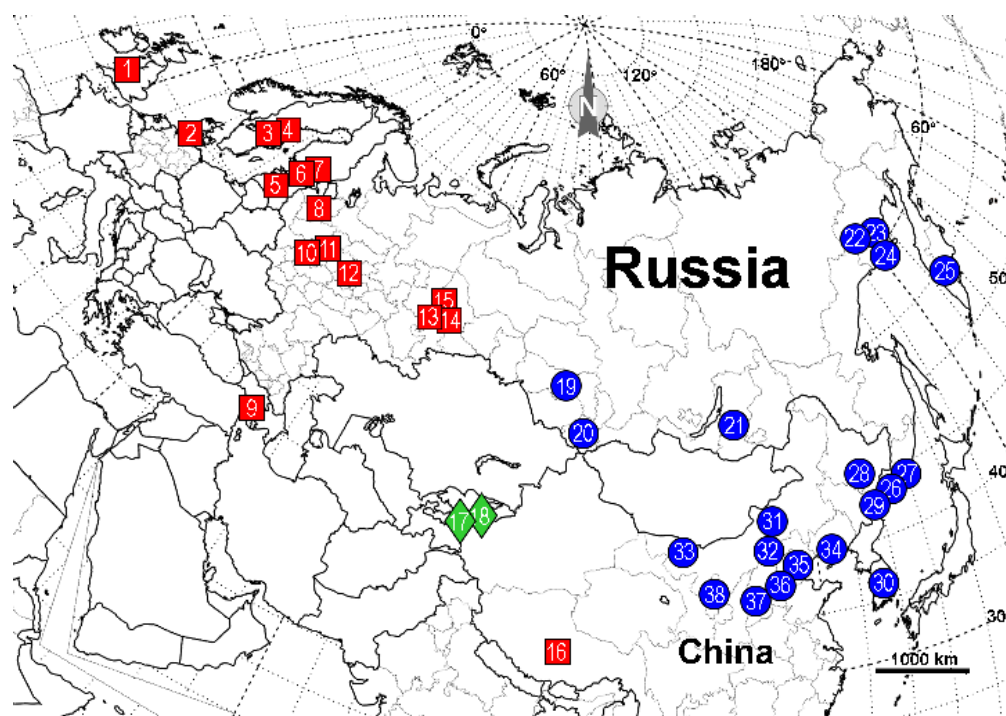
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**Abstract:** Ants of the «*Formica picea*—*F. candida*» complex are widespread across Eurasia. However, it is still a matter of debate if it constitutes one or two species. In this study, we collected a sample of specimens from different parts of Eurasia, sequenced the mitochondrial *cox1* and *cytb* genes, as well as three nuclear loci—*wg*, *Top1*, and ITS2—and analyzed the available published data. We found this complex to contain a new, yet undescribed, taxon that has a large distribution in Siberia and East Asia. Thus, the «*Formica picea*—*F. candida*» complex consists of at least three taxa with distinct distributions.

**Keywords:** *Formica picea*; *Formica candida*; cryptic species; Northern Eurasia; *cox1*; *cytb*

## 1. Introduction

The taxonomic position of many widespread and abundant ant species remains uncertain [1,2]. Among them are the species of the «black bog ant» complex, *Formica picea* Nylander, 1846 and *F. candida* Smith, 1878. Members of this complex are found from West Europe to Kamchatka, as well as from the valleys of northern rivers to Tibet (Figure 1).



**Figure 1.** Specimens of the «*F. picea*—*F. candida*» complex used in this study. Red squares, *F. picea*; green diamonds, *F. candida*; blue circles, *Formica* sp. Specimen numbers refer to Table 1.



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**Table 1.** Specimens used in this study.

| Location               | Species   | cox1                                       | cytb                                       |
|------------------------|---|--|--|
| <i>Formica picea</i>   |   |  |  |
| 1                      | United Kingdom: Cors Goch National Nature Reserve, S. Wales                                       | -  | AY786145<br>(as <i>F. candida</i> ) [3]    |
| 2                      | Denmark: N55.83 E12.56  | LT977411 [4]                               | -  |
| 3                      | Sweden: Trajmossen, Brattforsheden  | -  | AY786144<br>(as <i>F. candida</i> ) [3]    |
| 4                      | Sweden: Varmland  | -  | JX170886 [5]                               |
| 5                      | Estonia: Torma  | -  | AY786147<br>(as <i>F. candida</i> ) [3]    |
| 6                      | Finland: Nylandia, Raasepori, Harpar Stortraesket   | MZ607401 * [6]                             | -  |
| 7                      | Finland: Helsinki, Vihti  | -  | AY786146 *<br>(as <i>F. candida</i> ) [3]  |
| 8                      | Russia: Novgorod oblast   | -  | AY786148<br>(as <i>F. candida</i> ) [3]    |
| 9                      | Armenia: Aragaz mtn, N40.43203 E44.23642  | ON220876                                   | ON228281                                   |
| 10                     | Russia: Moscow oblast, Odintsovo district, raised bog, moss tussock, N55.733 E36.850              | ON220886-87                                | ON228278-79                                |
| 11                     | Russia: Moscow oblast, collected by G. Dlussky in 2000  | -  | AY786149<br>(as <i>F. candida</i> ) [3]    |
| 12                     | Russia: Nizhniy, Novgorod oblast, Kerzhenskiy Natural Reserve, Vishenskoye swamp, N56.467 E44.500 | ON220875                                   | ON228280                                   |
| 13                     | Russia: Sverdlovsk oblast, Revda  | -  | AY786151<br>(as <i>F. candida</i> ) [3]    |
| 14                     | Russia: Sverdlovsk oblast, Yekaterinburg  | -  | AY786150<br>(as <i>F. candida</i> ) [3]    |
| 15                     | Russia: Perm oblast, 30 km SSW of Kachkanar   | -  | AY786152<br>(as <i>F. candida</i> ) [3]    |
| 16                     | China: Quinghai, Tibet  | -  | AY786157<br>(as <i>F. candida</i> ) [3]    |
| <i>Formica candida</i> |   |  |  |
| 17                     | Kyrgyzstan: Alai Valley   | -  | AY786154 **, AY786153 [3],<br>JX170887 [5] |
| 18                     | Kyrgyzstan: Tian-Shan   | -  | AY786155-AY786156 [3]                      |
| <i>Formica sp.</i>     |   |  |  |
| 19                     | Russia: Novosibirsk oblast, Iskitim district, Listvyanskiy town, N54.450 E83.483                  | ON220885                                   | -  |
| 20                     | Russia: Altai Republic, Ongudai town, N50.733 E86.133   | ON220880                                   | ON228273                                   |
| 21                     | Russia: Buryatia Republic, Kizhinga town, N51.84639 E109.91278                                    | ON220883-84                                | ON228270, ON228274                         |
| 22                     | Russia: Magadan oblast, 15 km from Yagodnoye town, bank of Sokhatina river, N62.517 E149.617      | ON220878                                   | ON228277                                   |
| 23                     | Russia: Magadan oblast, 16 km from Yagodnoye town, bank of Detrin river, N62.750 E150.467         | ON220879                                   | ON228271                                   |
| 24                     | Russia: Magadan oblast, Arman town, high floodplain, N59.667 E150.117                             | ON220872                                   | ON228275                                   |
| 25                     | Russia, Kamchatka oblast, N53.19 E158.48  | LT977377<br>(as <i>F. candida</i> ) [4]    | -  |
| 26                     | Russia: Primorye krai, Lazo town, Lazovka river, N43.38056 E133.89778                             | ON220881                                   | ON228276                                   |
| 27                     | Russia: Primorye krai, Sinii Utes town, N43.06222 E131.35472                                      | ON220882                                   | ON228272                                   |
| 28                     | China: Harbin, Helongjiang  | KX665031-32<br>(as <i>F. candida</i> ) [7] | -  |

Table 1. Cont.

| Location  | Species                          | cox1  | cytb  |
|---|----------------------------------|---|---|
| 29  | China: Yanbian, Jilin            | KX665026-27<br>(as <i>F. candida</i> ) [7]          | -   |
| 30  | South Korea                      | MT800217<br>(as <i>F. candida</i> ) [8]             | -   |
| 31  | Xilin Gol League, Inner Mongolia | KX665022,<br>KX665024 (as <i>F. candida</i> ) [7]   | -   |
| 32  | Ulanqab, Inner Mongolia          | KX665023,<br>KX665025 (as <i>F. candida</i> ) [7]   | -   |
| 33  | Helan Mountains, Inner Mongolia  | KX665019-21<br>(as <i>F. candida</i> ) [7]          | -   |
| 34  | Qinglong, Hebei                  | KX665028<br>(as <i>F. candida</i> ) [7]             | -   |
| 35  | China: Hebei                     | HQ619704,<br>HQ619710 (as <i>F. candida</i> ) [9]   | HQ651081, HQ651074<br>(as <i>F. candida</i> ) [9] |
| 36  | Xiaowutai Mountain, Hebei        | KX665029-30<br>(as <i>F. candida</i> ) [7]          | -   |
| 37  | Zhong-Tiao Mountains, Shanxi     | KX665033<br>(as <i>F. candida</i> ) [7]             | -   |
| 38  | Liupan Mountains, Ningxia        | KX665034<br>(as <i>F. candida</i> ) [7]             | -   |
| <b>Outgroups</b>  |                                  |   |   |
| <b><i>F. lemani</i> Belarus: Vitebsk oblast, Braslav district, bank of Uklya lake, N55.60001 E27.333918</b> |                                  | <b>ON220877</b>                                     | <b>ON228284</b>                                   |
| <b><i>F. lemani</i> Magadan, Arman town high river terrace, N59.867 E150.567</b>                            |                                  | <b>ON220872</b>                                     | <b>ON228283</b>                                   |
| <i>F. lemani</i> China  |                                  | -   | HQ651082, HQ651086 [9]                            |
| <i>F. lemani</i> Magadan  |                                  | -   | JX170882 [10]                                     |
| <i>F. gagatoides</i> China: Ningxia   |                                  | -   | HQ651073 [9]                                      |
| <i>F. gagatoides</i> China  |                                  | KX665069,<br>KX665071,<br>KX665072 [7],<br>HQ619703 | -   |
| <i>F. gagatoides</i> Canada   |                                  | JN291934  | -   |
| <i>F. gagatoides</i> Norway   |                                  | LT977396,<br>LT977398 [4]                           | -   |
| <b><i>F. gagatoides</i> Russia: Magadan oblast, Arman town, N59.667 E150.117</b>                            |                                  | <b>ON220874</b>                                     | <b>ON228282</b>                                   |

Locations refer to Figure 1. Sequences obtained in this study shown in bold; \*, *terra typica*; \*\*, type specimen. If an accession was deposited under another species name, this is acknowledged in parentheses; references to published articles are given whenever possible.

Morphological variations within the complex were studied by Dlussky [11] based on extensive collections. Dlussky [11] concluded that there is no ground to split it into several species. Later on, Bolton [12] suggested that the name *F. candida* has the priority over *F. picea*; this was picked up by some—but not all—of the scientists. Seifert [13] suggested that *F. picea* and *F. candida* are two separate species, based on the features of pubescence and some morphometric parameters. According to Seifert [13], the distribution of *F. picea* includes Europe, the Caucasus, and the West Siberian Lowlands, and that of *F. candida* is in Central Asia, the Altai Mountains, and East Siberia. The habitats of *F. picea* include the bogs and European mountains above the forest belt, while *F. candida* lives in diverse habitats, including river floodplains and dry steppes of Asia.

However, Zakharov et al. [14] refuted Seifert's [13] conclusion, stating that the variation of pubescence within the European populations of *F. picea* is higher than the proposed

differences between the species. They also stated that there is a smooth gradient of morphological variation from Europe to the Far East. Based on this, the authors returned to treating *F. picea* and *F. candida* as synonyms.

Molecular genetic analysis is increasingly used to resolve problematic questions in ant taxonomy, due to its high resolution and its potential to detect cryptic taxa. [15] There were several molecular genetic attempts to resolve the relationships in the «*F. picea*—*F. candida*». Goropashnaya [3] performed a phylogenetic analysis based on the mitochondrial *cytb* gene. She demonstrated that the complex is split into two clades: one restricted to Europe and West Siberia and the other, to Central Asia. Later Goropashnaya et al. referred to these clades as a pair of species [5]. Antonov and Bukin [16] studied a sample of *cytb* sequences of specimens from several regions of the Palearctic identified as *F. candida* and *F. picea*. They concluded that genetic distances within *F. candida* are higher than those between the two species. Important datasets were also obtained by Chen et al. [7] and Schär et al. [4], based on the mitochondrial *cox1* gene.

Therefore, the status of the «*F. picea*—*F. candida*» species complex remains uncertain. The problem with molecular data is that very similar sequences can be deposited under different species names depending on the authors' viewpoint on this matter. Moreover, some authors sequenced the *cytb* gene, and the others sequenced *cox1*, so the resulting samples cannot be compared directly.

We collected a sample of the «*F. picea*—*F. candida*» complex specimens from the Northern Palearctic. Moreover, we studied the diversity, geographic and habitat distribution, and ecology of its population from the north of the Russian Far East [17]. The most important specimens were identified by Dlussky and Radchenko, who are specialists in the genus *Formica*; both consider this complex a single species (Radchenko: [18], personal communication). Here, we attempt to resolve the issue on the «*F. picea*—*F. candida*» complex using molecular analysis, based on the mitochondrial *cox1* and *cytb* genes, as well as three nuclear loci, with the integration of the available GenBank sequences obtained by other authors.

## 2. Materials and Methods

We collected a set of *Formica* specimens from Eurasia (Figure 1, Table 1). DNA was isolated from single ethanol-fixed worker ants using the commercial silica columns (BioSilica, Novosibirsk, Russia), as described in [4]. A fragment of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene was amplified using the universal primers LCO1490m (5'-TACTC-AACAA-ATCAC-AAAGA-TATTG-G-3'; modified from [10]) and HCO2198 (5'-TAAAC-TTCAG-GGTGA-CCAAA-AAATC-A-3'; [19]). A part of the cytochrome b (*cytb*) was amplified using the primers Fcbl-F (5'-ACCCT-CACCT-GTAAA-TATTT-CTT-3') and Fcbl-R (GGAAT-AGATC-GTAAA-ATTGC-AT-3') designed in this study. PCR was performed using the Biomaster HS-Taq PCR Mix (Biolabmix, Novosibirsk, Russia).

Three nuclear loci were amplified using the primers designed in this study: a fragment of the *wingless* (*wg*) gene, with FWg-F (5'-CGTGG-TCGGC-GATAA-TCTA-3') and FWg-R (5'-CACCA-CCACC-TCCTG-AGTCT-3'); *topoisomerase 1* (*Top1*), with FTPO-F (5'-GAACC-ATTGC-CACCC-ATAGT-3') and FTPO-R (5'-AGCGC-CAGCT-TGTCA-ATAAA-3'); the ribosomal internal transcribed spacer (ITS2), with FITS2-Fw (5'-TCATT-AACGT-TCCGG-AGGTC-3') and FITS2-Rv (5'-TAAAA-TCGTT-GGCCC-TTACG-3').

The obtained DNA fragments were visualized by agarose gel electrophoresis; unincorporated primers and nucleotide phosphates were removed using the shrimp alkaline phosphatase/*E. coli* exonuclease I mix (New England Biolabs, Ipswich, MA, USA). Sanger sequencing was performed on a 3130xl DNA Analyzer (Applied Biosystems, Framingham, MA, USA) in the SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia) using both forward and reverse primers. The obtained sequences were deposited in GenBank under accession numbers ON228270-ON228284, ON220872-ON220887, and ON862896-ON862909. Sequences obtained by other authors were also used in this study (see Table 1 for GenBank accessions). This dataset includes the *cytb* sequence for the *F. candida* neotype (AY786154).

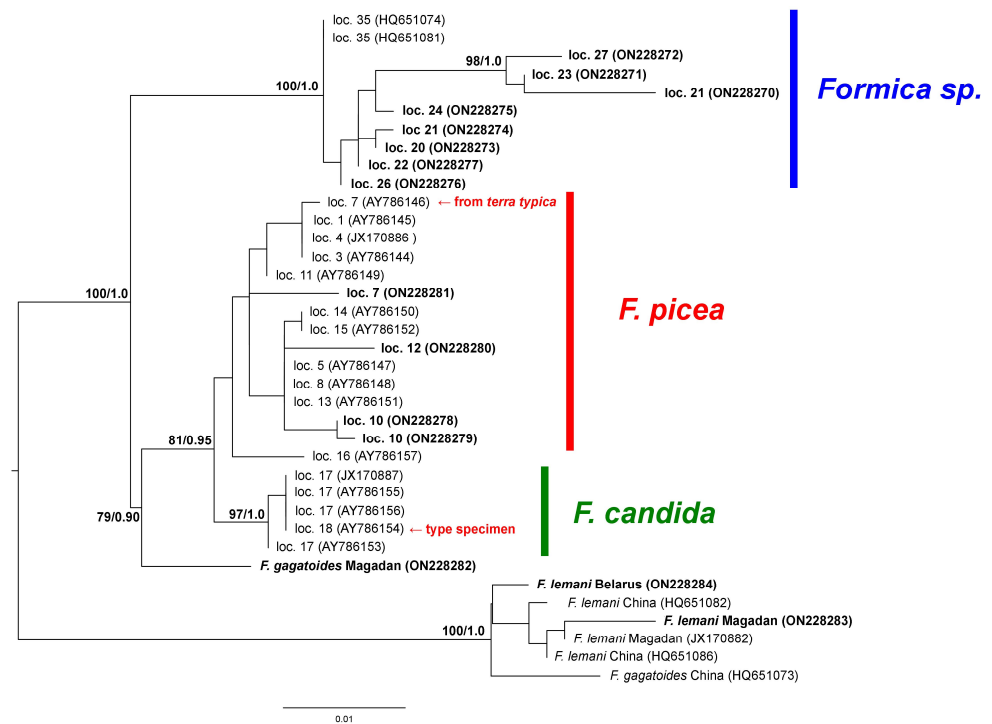
Phylogenetic trees were constructed using the Maximum Likelihood (ML) algorithm and Bayesian Inference, as described in [20]. ML trees were built in RAxML v. 8.2.12 (A. Stamatakis, Hedelberg, Germany) [21], using the GTR + I + G model, as suggested by MrModeltest v.2.0 (J. Nylander, Uppsala, Sweden) [22]. Bayesian analysis was made in MrBayes v. 3.4 (F. Ronquist et al., Stockholm, Sweden) [23] as two simultaneous independent analyses from different random starting trees. There were 20 million generations performed; 25% of the generations were discarded as burn-in.

### 3. Results

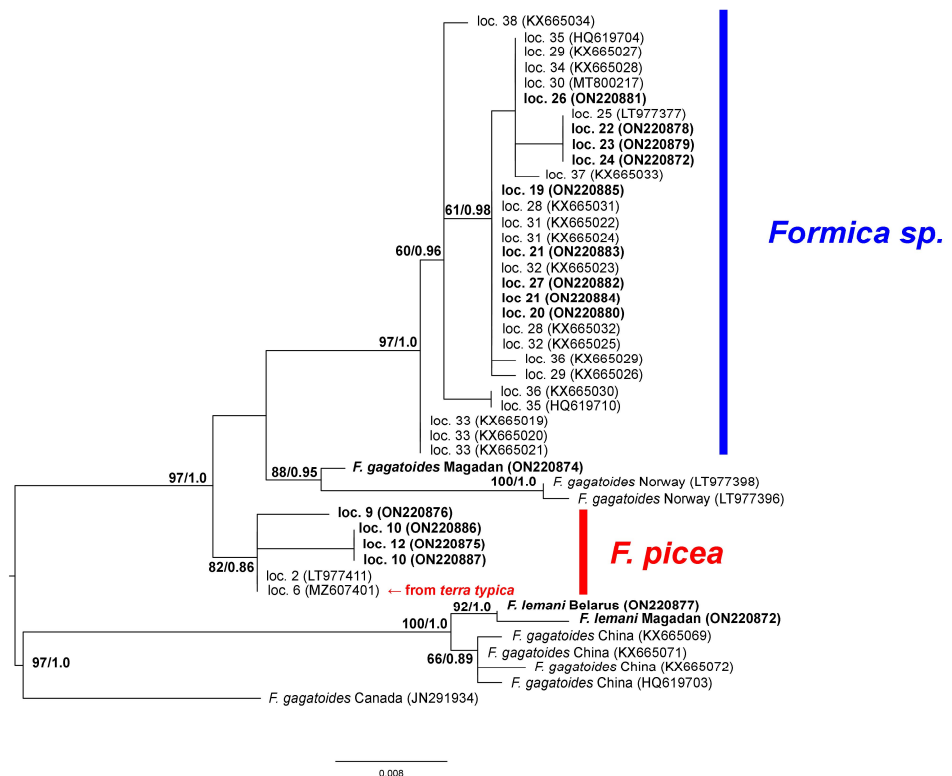
We sequenced fragments of the mitochondrial *cox1* and *cytb* loci for our sample (Table 1) and constructed phylogenetic trees. The final alignment for *cox1* contained 593 bp; for *cytb*, 693 bp. On the *cytb* tree (Figure 2) the «*F. picea*—*F. candida*» complex was split into two big clades. Nucleotide distances between them were as large as those among other *Formica* species. One of the clades included the specimens from Kyrgyzstan, Europe, the Urals, the Caucasus, and Tibet. The sequences from Kyrgyzstan that included the *F. candida* neotype fell into a well-supported subclade. The rest of the specimens (shown on Figure 2 as *F. picea*) formed a group with no statistical support. This group included *F. picea* (including a specimen from southern Finland, the *terra typica* of *F. picea*), as well as those submitted to GenBank as *F. candida*. The second clade (shown on Figure 2 as *Formica* sp.) contained the specimens from West and East Siberia, the Russian Far East, China, and Korea. For *cytb*, the average p-distances between *F. candida* and *F. picea* were 1.34%; between *F. picea* and *Formica* sp., 3.25%; between *F. candida* and *Formica* sp., 3.35%. The mean p-distance within *F. picea* was 0.81%; within *F. candida*, 0.06%; within *Formica* sp., 1.05%.

The tree constructed using the *cox1* gene (Figure 3) was built on more specimens than the *cytb* tree. However, there were no data for Kyrgyzstan, where the neotype of *F. candida* was described, or other regions of Central Asia, and there were few *F. picea* specimens. On the *cox1* tree, the «*F. picea*—*F. candida*» complex was also found to contain two clades: one corresponding to *F. picea* (including *terra typica*, i.e., the region where the original type specimens were collected) and the other to *Formica* sp. The average p-distances between *F. picea* and *Formica* sp. were 2.52%; within the species, they were 0.57 and 0.38%, respectively. We should also note that the *cox1* tree contained as many as three clades, with specimens identified as *F. gagatoides*.

The final alignment for the *wg* locus included 312 bp; for *Tpo1*, 717 bp; for ITS2, 678 bp. Sequences of *wg* and TPO of *F. picea*, *Formica* sp., and *F. gagatoides* were identical, except for some degenerate positions. In the ITS sequence, *F. picea* and *Formica* sp. could be distinguished by one A<>G substitution in position 216 of the alignment, while these two taxa differed from *F. gagatoides* by two substitutions and one short indel.



**Figure 2.** Phylogenetic tree constructed using *cytb* sequences. Numbers near branches indicate ML bootstrap support/Bayesian posterior probabilities. GenBank accessions given in parentheses; sequences obtained in this study are shown in bold.



**Figure 3.** Phylogenetic tree constructed using *cox1* sequences. Numbers near branches indicate ML bootstrap support/Bayesian posterior probabilities. GenBank accessions given in parentheses; sequences obtained in this study are shown in bold.



#### 4. Discussion

In this study, we analyzed the dataset of the «*F. picea*—*F. candida*» complex in order to find out if *F. picea* and *F. candida* are synonyms or two distinct species. The results were unexpected. The sample, indeed, contained two clades: one with *F. candida* and *F. picea* specimens and the other representing a previously unknown taxon (Figure 2). For mitochondrial sequences, genetic distances between these clades were as high as between the well-established species of the genus *Formica*. This is in line with earlier findings that recovered multiple cryptic species within the genus *Formica* [24,25], as well as other well-studied genera, such as *Pheidole* [26,27], *Myrmica* [28,29], *Lasius* and *Cardiocondyla* [24], etc. For the nuclear sequences, however, the differences were not as high: *wg* and *Tpo1* gene fragments were identical, and only one substitution distinguished *F. picea* from *Formica* sp. On the other hand, *F. gagatoidea* was also very close to these taxa, differing from *F. picea* by two substitutions and one indel in ITS2, with two other genes being identical. It is well known that nuclear DNA has a lower substitution rate compared to the mitochondrial genome, and closely related ant species may have few distinctive positions or none at all. From the data obtained in [4], one can see that there are very few differences in the nuclear sequences of the whole *F. picea*/*F. gagatoidea*/*F. lemani*/*F. fusca*/*F. neorufarbis* group. This is the usual situation that is found in ants, e.g., in the well-studied *Formica rufa* group, several species acknowledged by myrmecologists cannot be distinguished by nuclear markers and even show significant interspecific hybridization [30,31]. A similar case can also be observed in the genus *Proformica* [32]. Thus, we can conclude that the Asian *Formica* sp. is apparently distinct genetically from *F. picea* and *F. candida*, but it is unclear whether it should be regarded as a separate species or as a subspecies.

*F. candida* was initially described by Smith [33] based on a single specimen. This specimen was collected “On the road across the Pamir, from Sarikol to Panja”, which is probably somewhere near the easternmost part of China, on the border with Tajikistan. According to [13], this specimen was lost, so he fixed a neotype from Kyrgyzstan. This location is quite remote (about 315 km straight line distance in a mountainous area) from the original one. Our sample contained a *cytb* sequence of the neotype (AY786154). We can state that, so far, the *F. candida* clade is limited to the available specimens from Kyrgyzstan and was not found elsewhere. The type specimen of *F. picea* is old and highly damaged [13]. It was collected in the vicinity of Helsingfors (currently Helsinki, Finland) with no precise information on its whereabouts. Both locations available in our sample are several dozen km from Helsinki. Given the fact that no other group of the complex was found in Finland, we can suggest that these accessions represent the «real» *F. picea*.

Whether *F. picea* and *F. candida* are reciprocally monophyletic remains an open question. On our *cytb* tree *F. picea* was not supported as a clade (Figure 2), but more data are needed to verify that.

Based on the data on the type specimen origin, we cannot assign the *Formica* sp. clade to either *F. picea* or *F. candida* and have to conclude that it represents a new taxon that is still to be described. From the maps, we can see that all three ant taxa have distinct distributions: *Formica* sp. is found in Asian Russia, Siberia, and East Asia; *F. candida*, in Central Asia; *F. picea*, in Europe, with a single finding in Tibet. As suggested by multiple authors, morphological differences between these taxa are elusive [11,13,18]. There might be some differences in ecological preferences of these species: the European *F. picea* is found in swamps or in the alpine belt of mountains. The Asian *Formica* sp. is considered to be eurytopic in Siberia, while in the Russian Far East, it is found mostly in floodplains ([11] and our observations). However, it remains to be proved that these ecological differences coincide with the distribution boundaries of these species.

#### 5. Conclusions

By trying to resolve the relationships within the «*F. candida*—*F. picea*» complex, in order to separate these two species, we found a new third member of this complex. It is important to note that genetic distances between *F. candida* and *F. picea* are significantly

smaller than between them and the newly found taxon. The latter seems to not be the only cryptic taxon in this group: we obtained preliminary evidence that *F. gagatoides* might also represent as many as three cryptic taxa.

**Author Contributions:** Conceptualization, Z.A.Z. and D.I.B.; methodology, S.V.S. and T.V.P.; investigation, S.V.S. and T.V.P.; data curation, S.V.S.; writing—original draft preparation, D.I.B.; writing—review and editing, Z.A.Z. and S.V.S.; visualization, D.I.B. and S.V.S.; supervision, D.I.B.; project administration, D.I.B.; funding acquisition, D.I.B. All authors have read and agreed to the published version of the manuscript.

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