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Formica gagatoides Ruzsky, 1904, and Siberian *F. kozlovi* Dlussky, 1965 (Hymenoptera: Formicidae); Two or One Species?

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Abstract: Ants of the genus *Formica* play an important role in biogenesis by participating in various processes, including the formation of complex trophic networks. The role of ants in an ecosystem depends on their species and geographic population, which can be difficult to identify. *Formica gagatoides* with a wide range and *F. kozlovi* are among some examples. The question is whether the Siberian populations of *F. kozlovi* really belong to this species or are local populations of *F. gagatoides*. Based on the materials collected in Russia (Murmansk Region, the north of the Krasnoyarsk Territory, Altai, Far East), a morphological analysis (key diagnostic features) and molecular genetic analysis (COI, ITS1, D2 28S) were carried out. In all localities, there were individuals with pure (*gagatoides*, *kozlovi*) and mixed (*gagatoides/kozlovi*) morphotypes, with the exception of the Magadan Region, where the *kozlovi* morphotype was absent. According to the phylogenetic trees, *F. gagatoides* formed separate geographical branches, with the Siberian *F. kozlovi* being close and clearly conspecific to the Asian branch of *F. gagatoides*. A relatively high COI divergence, along with some differences in the ITS1 sequences, between the Asian and European *F. gagatoides* raises the question about the conspecificity of the Asian and European branches of this species.

Keywords: habitat area; diagnostic features; COI gene; ribosomal RNA genes sequences; phylogeny

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1. Introduction

Formica kozlovi Dlussky, 1965 (Hymenoptera: Formicidae), is a species of the Serviformica group (earlier defined as the subgenus *Serviformica* Forel, 1913) that inhabits a relatively wide range of habitats in Mongolia and the mountains of southern Siberia [1–5]. In terms of its morphology, *F. kozlovi* is very close to the polar ant, *Formica gagatoides* Ruzsky, 1904, which was long thought to exclusively inhabit the northern part of the Palaearctic [6]. According to the description of *F. kozlovi*, the main distinguishing features of this species from *F. gagatoides* are: (i) a greater number of setae (erect hairs) on the femora of the midlegs, and (ii) a less dense pubescence of tergite I (similar to that of tergites II and III) in workers and females, and also greater number of setae (erect hairs) along the posterior margin of the pronotum and their arrangement in two rows in females [5].

In addition, an important reason for distinguishing *F. kozlovi* as an independent species is the fact that, by the time of its description, *F. gagatoides* had not been found south of 60° N, and there was a significant gap between the range of *F. gagatoides* and the collection sites, as well as the proposed range of *F. kozlovi* [6]. However, isolated populations of *F. gagatoides* were found in the mountains of central Japan [7]. Studies conducted in middle Siberia (northern and central Yakutia, the Podkamennaya Tunguska River—the right tributary of the Yenisei River) and the southern part of the Russian Far East (southern Sikhote-Alin Ridge) have shown that *F. gagatoides* inhabits areas of sphagnum bogs and various types of forests with conifers in these regions [8,9]. Moreover, a study of the

cold tolerance and biotopic distribution of *F. gagatoides* in northeast Asia showed that, according to its physiological characteristics, the polar ant had no strict limitations in its distribution and could be found throughout the middle mountains, from the Kolyma and Indigirka Rivers to the mountains framing the left bank of the Amur River [10]. The indications of findings of *F. gagatoides* in the territory of North America [11,12] significantly changed the understanding of the range of *F. gagatoides* and gave it the status of a Holarctic species, which was recently confirmed using methods of molecular genetic analysis [13]. Furthermore, there are some notes about the discovery of the polar ant near the southern borders of Tibet and in the Himalayas that can expand its range to the northern part of the Oriental Region [14–16]. However, this information needs to be confirmed.

Thus, according to the currently available data, there is no gap between the ranges of these two species (Figure 1). All this has suggested that *F. kozlovi*, described as an independent species, is, in fact, a morphological form of *F. gagatoides*. With the expansion of ideas about the geographical distribution and biotopic distribution of *F. gagatoides*, G.M. Dlussky (the author of the description of *F. kozlovi*) also began to doubt the species independence of *F. kozlovi*. In 2013, in a methodological manual on the ants of the genus *Formica*, *F. kozlovi* was excluded from the keys [17]. In addition, in the species sketches, a high morphological similarity and the probability of the synonymization of *F. kozlovi* with *F. gagatoides* were noted [17].

In one of his papers, Seifert [18] compared representatives of *F. kozlovi* and *F. gagatoides* using 15 morphological characteristics. According to this paper, the discriminant function offered a clear separation between the two taxa [18]. However, very few samples of both species were used by the author for the analysis (*F. kozlovi*: 18 individuals from 7 nests; *F. gagatoides*: 36 individuals from 14 nests). This means that no more than 2–3 individuals were examined from each ant colony. Moreover, the geographical origin of the analyzed material also remained unclear. Since both intra-colony and geographical variations were not taken into account, the results of the study are supposed to be far from final, and the problem of the species status of *F. kozlovi* is still unclear.

To test the species status (validity) of *F. kozlovi*, a study of the species' materials from the type localities specimens (Mongolia and China) is required. Unfortunately, due to the difficult epidemiological situation in the world in recent years, the authors of the present study were unable to obtain topotype materials for the study from Mongolia. Therefore, at the first stage, it was decided to clarify the species status of Siberian *F. kozlovi* (hereafter *F.* cf. *kozlovi*, because of the unclear situation with the species status). To answer the question of whether the Siberian populations of *F.* cf. *kozlovi* belong to this species or are local populations of *F. gagatoides*, a morphological analysis (using key diagnostic features) and molecular genetic analysis (COI, ITS1, D2 28S) on samples of both species from different parts of their ranges were carried out. The authors of the present study tried to assess the degree of the morphological differences between the representatives of the Siberian *F.* cf. *kozlovi* and *F. gagatoides* from the different parts of the range and find out whether there are molecular–genetic differences between the studied specimens of these species, in terms of the mitochondrial COI gene fragment, ITS1 spacer, and D2 variable region of the 28S related to nuclear rRNA genes.

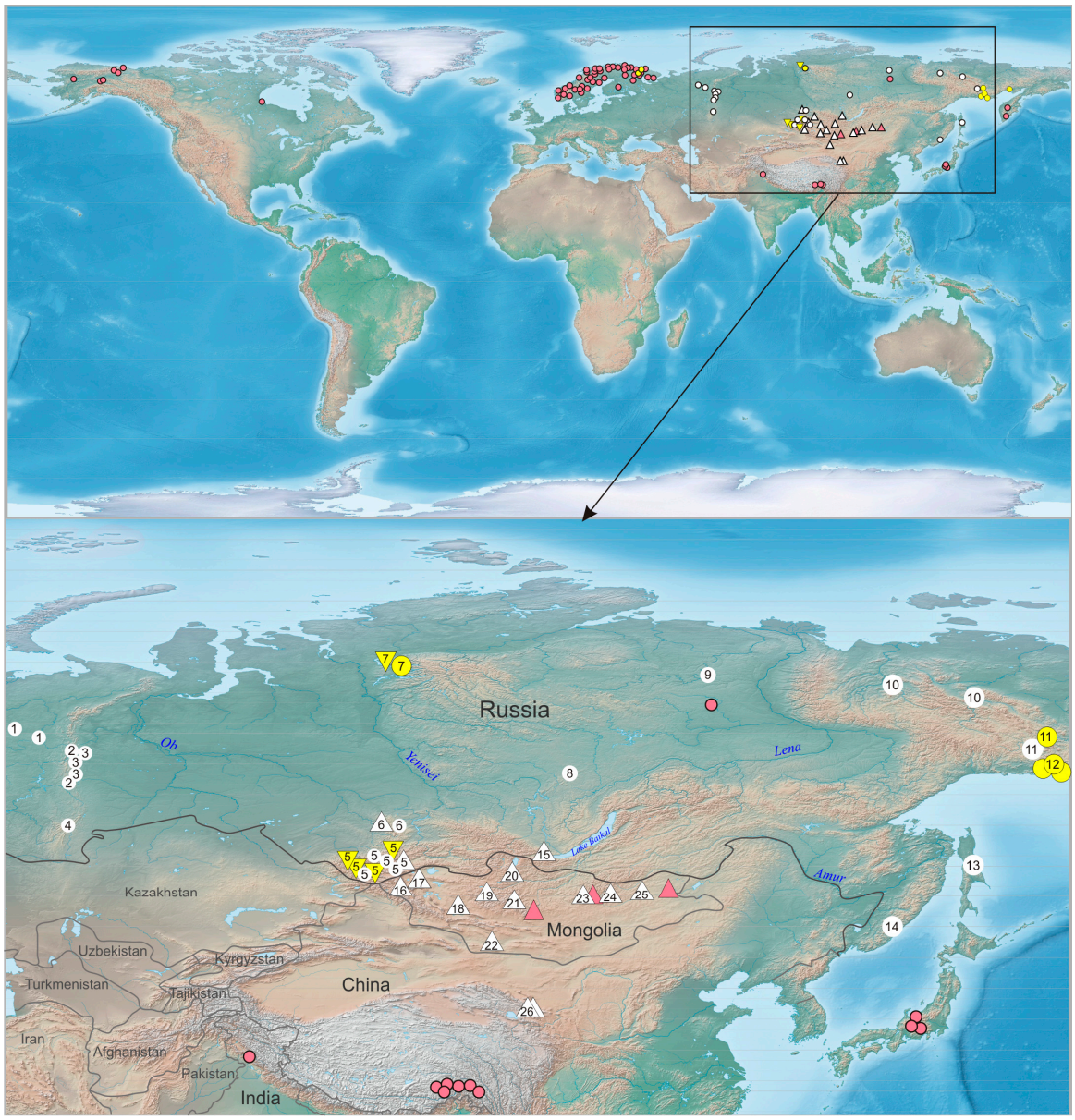

Figure 1. Geographical distribution of *Formica kozlovi* and *F. gagatoides*. Different icons correspond to the data of different species: circle—*F. gagatoides*, triangle with the top upwards—*F. kozlovi*, triangle with the top downwards—*F.* cf. *kozlovi* in Siberia (Altai Republic, the north of the Krasnoyarsk Territory). The color reflects the source of the data: pink—data of Antmaps [12]; yellow—data of the authors, white—literature data. The numbers inside the figures indicate the region given in the literature: 1—the East European Plain (Komi Republic, Russia [19]); 2–4—Ural mountains (2—Perm Region, Russia [17]; 3—Sverdlovsk Region, Russia [6,19]; and 4—Republic of Bashkortostan, Russia [19]); 5—Altai Mountains (Altai Republic, Russia [1]); 6—Kuznetsk Alatau (Kemerovo Region, Russia [20]); 7–9—Middle Siberia (7—Norilsk, Krasnoyarsk Territory, Russia [8]; 8—Podkamennaya Tunguska River, Irkutsk Region, Russia [8]; 9—central Yakutia, Saha Republic, Russia [6,8]); 10—northeastern Yakutia (Saha Republic, Russia [6,8]); 11–14—the Russian Far East (11—Kolyma River [10], 12—Magadan, Magadan Region, Russia; 13—Sakhalin island [21]; 14—Southern Sikhote-Alin Ridge, Primorye Territory, Russia [9]); 15—Baikal Region (the Republic of Buryatia, Russia [2]); 16–25—Mongolia (16—Khovd, 17—Depression of Great Lakes, 18—Gobi Altai, 19—Khangai, 20—Khuvsgul, 21—Central Mongolia, 22—Trans-Altai Gobi, 23—Ulaanbaatar, 24—Khentii, 25—Eastern Mongolia) [3–5]; and 26—China (Nayan Shan Ridge) [5,6].

2. Materials and Methods

In this work, the authors used their own materials on the species *Formica* cf. *kozlovi*, *F. gagatoides*, *F. lemani* (Bondroit), and *F.* aff. *candida* (Smith), collected by the authors in different years (2005–2006, 2008, 2019, and 2020–2022) in Russia: in Altai (within the Altai Republic), in the plain part of the south of western Siberia (Novosibirsk Region), in the Far East (Magadan Region), in the north of the European part of Russia (Murmansk Region), and in the Krasnoyarsk Territory (vicinity of Norilsk) (Figure 1, Supplementary Material Table S1). According to the COI sequences, the studied specimens of *F.* aff. *Candida* were identical to one of the haplotypes of the new species form within the *F. candida*/*F. picea* group [22].

The materials determined as *F. kozlovi* were specified as *F.* cf. *kozlovi*, because of the unclear situation with the species status of the collected material. Some materials of *F. gagatoides*, *F. lemani*, and *F.* aff. *candida* from the northern part of Europe (Murmansk Region), the Krasnoyarsk Territory (vicinity of Norilsk), and the Far East (Magadan Region) were kindly provided for research by N.N. Tridrikh, V.S. Sorokina, N.A. Bulakhova, Z.M. Yusupov, E.V. Vaulin, and I.K. Iakovlev. The collection sites are presented in Table S1.

2.1. Morphological Features

Several sources were used to determine the taxonomic affiliation of the collected material [5,6,17]. In each specimen of the *F.* cf. *kozlovi* and *F. gagatoides*, the main distinguishing features were studied in detail: (i) the number and arrangement of the setae on the inner edge of the femora of the middle legs (Figure S1); (ii) the type of pubescence of the gaster tergites I–III based on an assessment of the ratio of the length of the pubescence hairs and the distance between them: very dense ($\geq$1.5), dense (1.1–1.4), slightly sparse (0.7–1.0), and sparse (0.5–0.6); and (iii) additionally, the character of the chaetotaxy of the hind margin of the pronotum in females (the number of setae and their arrangement, i.e., the number of rows (Figure S2)). To estimate the type of pubescence, the measurements were taken in two main parts of the central longitudinal zone of the gaster tergites I–III. A preliminary investigation showed that the nature of the pubescence of the tergites was clearly, visually distinguishable and could be qualitatively assessed in the form of several main types (Figure S3). This approach was used since it significantly increases the speed of processing the material and, as a result, allows for increasing the sample size, which is essential when assessing the geographical variability of traits.

Using the main types of the chaetotaxy of the mid-leg femora and the pubescence of the first gaster tergites, the morphotype characteristics of the workers studied were identified. For females, a combination of the chaetotaxy of the mid-leg femora and pronotum was used.

The degree of homogeneity of the samples from the nests was assessed by the ratio of the proportions of different morphotypes among workers. A comparison of the proportions of groups of "pure" (*F. gagatoides* and *F. kozlovi*) and "mixed" (*F. kozlovi*/*gagatoides*) morphotypes in Altai was performed using a Yates corrected Chi-square with the Bonferroni correction ($p < 0.017$). To compare the proportions of the "pure" variants of the morphotypes, unambiguously determined as *F. gagatoides* and *F. kozlovi*, as well as intermediate forms in mixed ant colonies, the Kruskal–Wallis test was used, with the Mann–Whitney test with the Bonferroni correction ($p < 0.017$) as a post hoc test.

In total, 593 specimens of workers and 24 females of the *F. gagatoides*/*F. kozlovi* species complex were analyzed (Table S1). The studied material was stored at the Institute of Systematics and Ecology of Animals, Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia).

2.2. Molecular Genetics Study

For the molecular genetic analysis, the samples were fixed in 96% ethanol. The materials from Siberia (Altai Republic, Novosibirsk Region, the north of the Krasnoyarsk Territory, vicinity of Norilsk, Russia), the Far East (Magadan Region, Russia), and northern

European Russia (Murmansk Region, Russia) were studied (Table S1). The isolation of DNA was performed according to the protocol described earlier [23].

The standard marker DNA regions were used for a PCR and subsequent sequencing: COI, ITS1, and the D2 variable region of the 28S rDNA. These markers were chosen to compare the obtained results with the data obtained for *Formica gagatoides*, using the same DNA regions [13,24]. The sequences of the used primers and their annealing temperatures are presented in Table 1. The composition of the reaction mixture (volume 20 µL) for COI and ITS1 was as follows: 1xPCR-buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.1% Tween-20); 4 mM $MgCl_2$; 0.4 mM of each dNTP; 0.5 µM of each primer; and a 0.8 unit of Taq-polymerase produced by IMCB SB RAS. In the case of the D2 variable region of the 28S rDNA, a $MgCl_2$ concentration of 1.5 mM was used to decrease the possibility of production from the rDNA pseudogenes.

Table 1. Sequences of the used primers.

Amplified Region	Forward Primer	Reverse Primer	Annealing Temperature	Reference
COI	GGTCA-ACAAA-TCATA-AAGAT-ATTGG	TAAAC-TTCAG-GGTGA-CCAAA-AAATC-A	53 °C	[25]
ITS1	TCAC-ACCGC-CCGTC-GCTAC-TA	ATGTG-CGTTC-RAAAT-GTCGA-TGTTC-A	58 °C	[26]
D2 28S	5′-GGAGT-CGTGT-TGCTT-GATAG-TGCAG-3′	5′-TTCTT-GGTCC-GTGTT-TCAAG-ACGGG-3′	60 °C	[27]

The products of the PCR were purified from the not-specific PCR products using electrophoresis in 1% agarose gel. These PCR products were sequenced using resources from the Center for Collective Use Genomics (Siberian Branch, Russian Academy of Sciences; http://www.niboch.nsc.ru/doku.php/sequest) (accessed on 6 March 2023). For a further analysis of the full-size sequences of the COI gene "standard" fragment (658 bp), the most reliable part of ITS1 (697–701 bp long, positions from 66 to 762 of the *F*. aff. *candida* sequence with the NCBI number KX609260) and the D2 variable region of the 28S rDNA (fragment 630–631 bp long) were used. In addition, another part of ITS1 (positions from 763 to 956 relative to the *F*. aff. *candida* sequence with the NCBI number KX609260) was used to compare most of the *F. gagatoides* and *F*. cf. *kozlovi*. This region was not used for the single specimen of *F*. cf. *kozlovi* (F3-2) and the specimens of *F*. aff. *candida* and *F. lemani*, due to the variable quality of the sequences.

The sequences were submitted into the NCBI nucleotide database (Genbank) with the numbers: OM722019-OM722036 and OQ629842-OQ629848 (COI), OM722038-OM722055 and OQ641670-OQ641676 (the D2 variable region of the 28S rDNA), and OM728488-OM728505 and OQ641663-OQ641669 (ITS1).

2.3. Phylogenetic Analysis

In addition to the sequences obtained, the DNA sequences provided in the NCBI DNA database and described as belonging to *F. gagatoides* were used for phylogenetic constructions and a construction of the distance matrix. No sequences described as belonging to *F. kozlovi* were found in the database. The origin of the sequences from the DNA database is shown in Table S2. The sequence JN291934 (Canada), which is related to the standard COI gene region, was excluded from the analysis, because it was shown to be very different from the other sequences assigned to *F. gagatoides* by different authors. The Kimura genetic distances [28] to the other specimens assigned to *F. gagatoides* were 0.039–0.051, which rules out their conspecificity [29]. However, three sequences from Canada (FJ413256, HQ569283, and JX829423), similar to the COI of *F. gagatoides* but not assigned to this species by the authors, were included in the analysis. Two of them (FJ413256 and HQ569283) had previously been attributed to *F. gagatoides* by other authors [13], and the third one (JX829423) is identical to one of these sequences (HQ569283).

For the D2 variable region of the nuclear rDNA, a homology search (in the sense of the identity of the alignment nucleotide sequences) was performed with the sequences from the DNA database (BLAST algorithm). It was performed to clarify the possible existence of several phylogenetic branches within the studied taxa, as well as to detect if there were any differences with the species identification provided by other authors. The alignment of the sequences was performed using the Clustal Omega software [30].

The software MEGA11 [31] was used both to construct the distance matrix for the COI sequences and search for the best-fit DNA substitution model. The COI sequence matrix was constructed using Kimura-2-parameter distances [28], which is a standard approach for such studies. The search for the best-fit model for the phylogenetic trees performed by MEGA11 found the Hasegawa–Kishino–Yano model [32] for the COI data and the Tamura-3-parameter model [33] for the COI and 28S concatenated sequences. Due to the known information about the phylogeny of the genus *Formica* and the distant relation between *Formica lemani* and the *F. candida/picea* group [34], all the trees were rooted on their longest branches, i.e., the branch between the specimens close to these two species. The maximum likelihood phylogenetic reconstructions with the best-fit DNA substitution model and bootstrap and Bayesian analyses were performed using the IQ-TREE software [35]. The trees were visualized using the FigTree 1.4.4. software (http://tree.bio.ed.ac.uk/software/figtree/) (accessed on 25 April 2023).

The molecular–genetic criteria of the species affiliation and the possibility of finding hybrid specimens: according to the information on the ratio of the intra- to interspecific variation at the COI marker site, the degree of intraspecific variation tended to be less than 1% and rarely exceeded 2%, while the interspecific diversity usually exceeded 2% [29]. Therefore, it can be assumed that the specimens with a divergence of less than 1% were conspecific, more than 2% were non-conspecific, and between 1 and 2% were probably conspecific.

Due to the mechanisms of the concerted evolution of repetitive DNA, the interspecific variability of the rRNA gene regions (in this study: ITS1 and the D2 variable region of 28S) disappears [36]. Therefore, any difference in the rRNA gene regions may indicate the reproductive isolation of the compared sample groups. However, in parasitic wasps of the genus of *Encarsia*, an interspecific variability of the ITS1 region was shown [37]. The additional problems of rRNA genes are pseudogenes and the possibility of the presence of several rDNA blocks, which makes concerted evolution incomplete.

The features of the mtDNA and nuclear rDNA inheritance shown for different groups of animals, including social insects, allow for a detection of the possible consequences of interspecific hybridization in the studied groups of ants [38,39]. The hybrids of F_1 are expected to have superpositions of the sequencing patterns of the rDNA of the parent species. The descendants of introgressive hybridization may have nuclear markers from one species, but mtDNA from another.

Some authors use other genes to clarify the relations between ant species or near-species forms [13,22]—mitochondrial *cytB*, nuclear unique genes *wingless,* and *topoisomerase 1*. These markers are not used in the present study for the following reasons. First, the considered sequences have to be compared mainly to the specimens assigned to *F. gagatoides* in the studies by Schär et al. [13] and Chen and Zhou [24], where the authors did not use *cytB*. As for the unique nuclear genes, the threshold of species differentiation ("one or two species?") is highly unclear for these markers, making them inappropriate for solving the discussed problem. It is also unclear how to interpret the patterns of the heterozygous sequences for these genes and whether this is an intra-species polymorphism or a consequence of hybridization.

3. Results

3.1. Degree of Morphological Differences between F. cf. kozlovi and F. gagatoides

During the morphometric analysis of the material, the chaetotaxy of the mid-leg femora of the workers was found to vary greatly: more than 30 variants were identified that

differed in the number and arrangement of their setae (Figure S1). For a further analysis of the material, the approach of Dlussky [5,6] to the differentiation of the species studied was used: *F. gagatoides*, type 1—< 3 setae on the middle femora (chaetotaxy variants according to Figure S1: 1–12); and *F. kozlovi*, type 2—≥ 3 setae (Figure S1: 13–36). Based on the ratio of the length of the pubescence hairs and the distance between them, four types of pubescence of the gaster tergites I–III of the workers were identified (Table S3). Type 1 (similar to *F. gagatoides*): tergite I—a very dense pubescence (the length of hair was much more than the distance between the hairs, the ratio was 1.6–1.7); tergite II—dense in the first half (1.1–1.3), slightly sparse in the posterior part (the length of hair was approximately equal to the distance between them, the ratio was about 0.8–0.9); and III—sparse (the ratio was about 0.5–0.6). Type 2 (typical of *F. gagatoides*): tergite I—dense in its first half (1.3–1.4), slightly sparse in the posterior part (0.9–1.0), usually due to the clearly visible wedge-shaped area of sparse pubescence; tergite II—slightly sparse (0.7–0.8); and tergite III—sparse (0.5–0.6). Type 3 (similar to *F. kozlovi*): tergite I—slightly sparse in the first part (0.9–1.0), evenly and slightly sparse pubescence in the posterior part (0.7–0.8), without a clearly visible wedge-shaped area of sparse pubescence; and tergites II and III—sparse (0.5–0.6). Type 4 (typical of *F. kozlovi*): tergites I, II, and III—distinctly sparse uniform pubescence (0.5–0.6).

According to the combination of the chaetotaxy types of the mid-leg femora and variants of pubescence of the first gaster tergites, eight morphotypes were distinguished among the studied material of the workers, the number of which is a combination of the variants of the chaetotaxy of the mid-leg femora and the pubescence of the first gaster tergites (Table 2). These morphotypes included both "pure" variants, uniquely identified as *F. gagatoides* (1-1, 1-2) and *F. kozlovi* (2-3, 2-4), and "mixed" ones—intermediate forms with features of both species—*F. kozlovi/gagatoides* (1-3, 1-4, 2-1, 2-2).

Table 2. The occurrence of different morphotypes of workers within the species complex *F. gagatoides*/*F. kozlovi*. The number of morphotypes reflects the combination of the chaetotaxy type of mid-femora (the number of setae: 1 < 3, 2 ≥ 3) and pubescence of the first gaster tergites (1—dense in tergite I and almost to the end of tergite II, sparse on tergite III; 2—dense to the end of tergite I or to the middle of II; 3—dense to the middle of I, and then sparse, a sparse area in tergite I often wedge-shaped; and 4—evenly sparse in I–III, but denser at the beginning of tergites I and II).

Region	Morphotypes of Workers, %								Number of Individuals
	1-1	1-2	1-3	1-4	2-1	2-2	2-3	2-4	
Murmansk Region	2.08	42.71	23.96	2.08	5.21	14.58	9.38	0.00	96
Krasnoyarsk Territory (Norilsk)	3.45	16.09	11.49	1.15	17.24	43.68	4.60	2.30	87
Altai Republic	0.00	20.29	10.87	1.81	0.36	50.36	13.04	3.26	276
Magadan Region	54.96	34.35	2.29	0.00	6.11	2.29	0.00	0.00	131

Workers of *F. gagatoides/F.* cf. *kozlovi* complex from different regions included representatives of 5–8 morphotypes (Table 2). The whole range of morphotypes (8) among workers was registered in the north of the Krasnoyarsk Territory (vicinity of Norilsk). The smallest number of morphotypes (5) was noted in the Far East (Magadan Region), where both "pure" morphotypes of *F. kozlovi* (2-3, 2-4) and one "mixed" form (1-4) were absent. In Altai and the Murmansk Region, representatives of 7 morphotypes were noted (Table 2). In Altai, the only exception was individuals with morphotype 1-1 (mid femora with < 3 setae and dense pubescence up to the middle of tergite II). Representatives of this morphotype were also quite rare in the Murmansk Region and the vicinity of Norilsk (less 4 %) and prevailed only in the Magadan Region (about 55%). In the Murmansk Region, one of the "pure" morphotypes of *F. kozlovi* (2-4) was not registered (Table 2).

In the Far East (Magadan Region), the morphotypes of the *F. gagatoides* group (1-1, 1-2) prevailed (89.3%) and the "mixed" variants of the *F. kozlovi/gagatoides* group made up 10.7% (Figure 2, Table 2). In the other regions (Altai, the north of the Krasnoyarsk Territory, and the Murmansk Region), the proportion of individuals with "mixed" morphotypes (*F. kozlovi/gagatoides*), in most cases, was significantly higher than the proportion of individuals

with "pure" morphotypes, for both *F. kozlovi* and *F. gagatoides* (Figure 2). The only exception was the Murmansk Region, where the percentage of individuals with *F. gagatoides* morphotypes was almost as high as *F. kozlovi/gagatoides*, with no significant differences between these groups (Figure 2).

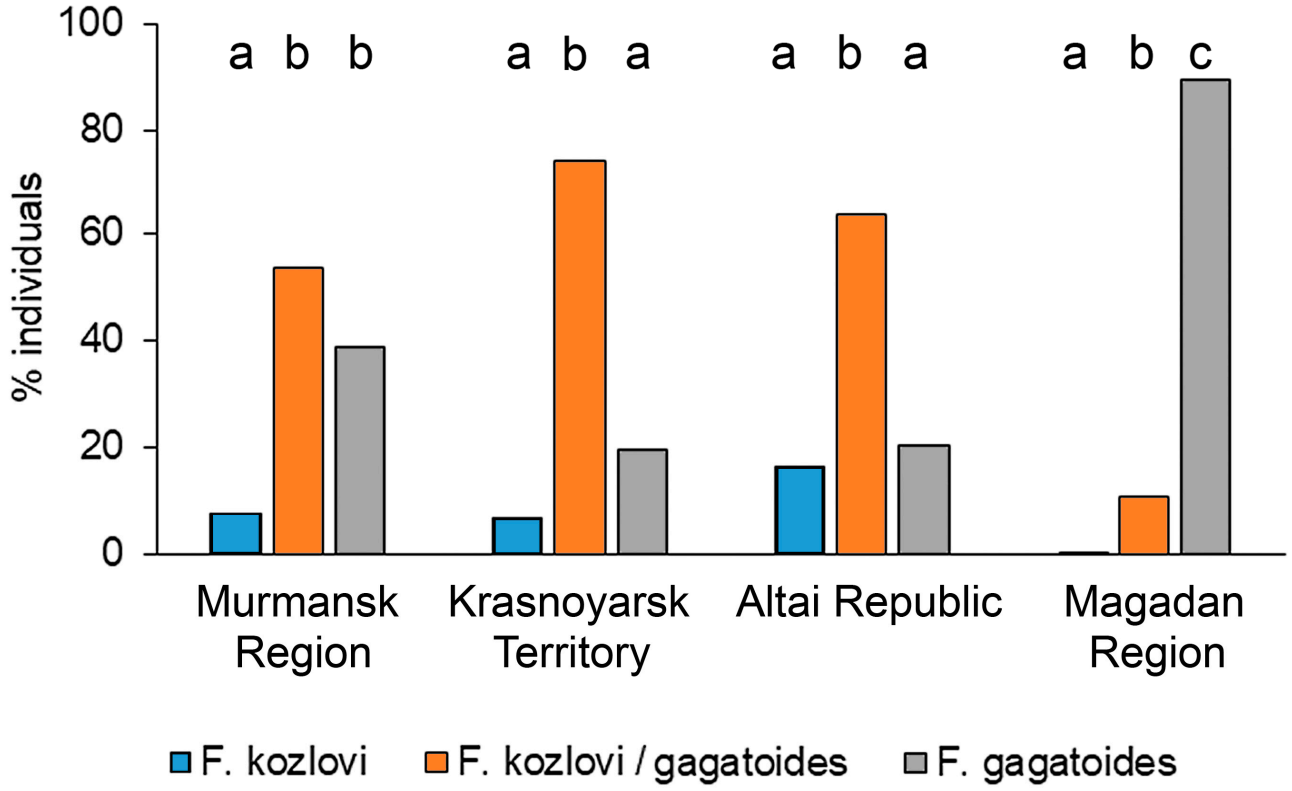

Figure 2. Proportion of morphotypes among workers clearly defined as *F. kozlovi* and *F. gagatoides* and "mixed" variant *F. kozlovi/gagatoides* in various regions of Russia from Europe to Far East. Different letters indicate significant differences between groups within each region (Yates corrected Chi-square with the Bonferroni correction, $p < 0.017$).

To analyze the intra-colony variation of the morphotypes, the material of 49 ant colonies (from 5 to 35 individuals, on average 11.4 individuals per nest) was used: the Murmansk Region—6 ant colonies, Altai—26, the Krasnoyarsk Territory—10, and the Magadan Region—7 colonies. Most of the studied ant colonies were found to contain "mixed" morphotypes (86.7%): the Murmansk Region—83.3 %, Altai—88.5%, the Krasnoyarsk Territory—90%, and the Magadan Region—71.4%. Among the "monomorphic" colonies (the studied workers belonged to the same morphotype group (*F. kozlovi*, *F. gagatoides*, or *F. kozlovi/gagatoides*)), the colonies with the "mixed" morphotypes of *F. kozlovi/gagatoides* prevailed in Siberia (Altai—66.7%, the Krasnoyarsk Territory—100%), and in the north of the European part of Russia (the Murmansk Region), the only "monomorphic" colony found had the *F. gagatoides* morphotype. In the ant colonies, the proportion of the "pure" morphotypes identified as *F. gagatoides* and *F. kozlovi* also turned out to be approximately the same and, in both cases, significantly lower than the proportion of the group with the "mixed" variants *F. kozlovi/gagatoides* (Figure 3).

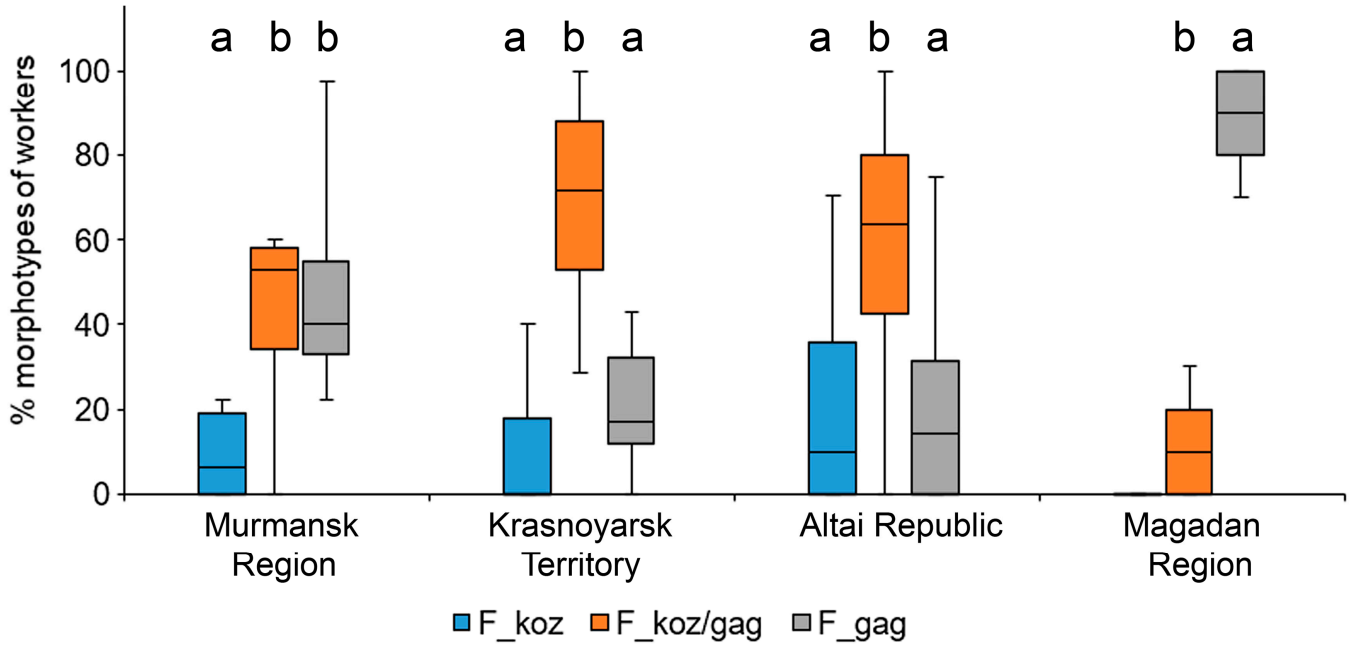

Figure 3. Proportion of morphotypes among workers clearly defined as *F. kozlovi* (F_koz) and *F. gagatoides* (F_gag) and "mixed" variants *F. kozlovi/gagatoides* (F_koz/gag) in the examined ant colonies in various regions of Russia, from Europe to the Far East. Different letters indicate significant differences between groups (Mann–Whitney test with the Bonferroni correction, $p < 0.017$).

On the whole, the results of the analysis on the intra-colony variability of the key morphological characteristics of the collected material were similar to the results of the general analysis. In the Far East (Magadan Region), the proportion of representatives with *F. gagatoides* morphotypes was significantly higher than that of others; moreover, morphotypes of *F. kozlovi* were not registered in the explored territory (Figure 3). In Altai and the north of the Krasnoyarsk Territory, the proportion of individuals with "mixed" morphotypes (*F. kozlovi/gagatoides*) prevailed. In the Murmansk Region, the proportions of individuals with *F. gagatoides* and *F. kozlovi/gagatoides* morphotypes were similar and significantly higher than those of *F. kozlovi* (Figure 3).

As for females, the pubescence of their first gaster tergites was not taken into account in this case, since this feature was not used as a key one for distinguishing between the females of *F. kozlovi* and *F. gagatoides* [6]. At the same time, two types of pronotal chaetotaxy were additionally distinguished: 1—one row of setae along the posterior margin, 2—one row of setae along the posterior margin and 1–2 additional setae nearby (the second row is not clear), and 3—two rows of setae (an additional row consisting of more than two setae).

According to the combination of the types of chaetotaxy of the mid-leg femora and pronotum, five of six possible morphotypes were distinguished among the studied material of the females (Table 3). The identification number of the female morphotypes was a combination of the variants of the first and second characters, respectively. These morphotypes included both "pure" variants, uniquely identified as *F. gagatoides* (F1-1) and *F. kozlovi* (F2-3), and "mixed" ones—intermediate forms—*F. kozlovi/gagatoides* (F1-2, F1-3, F2-1, and F2-2). Representatives of the F1-3 morphotype were not found.

Table 3. The occurrence of different morphotypes of females (F) within the species complex *F. gagatoides*/*F. kozlovi*. The identification number of morphotypes reflects the combination of the chaetotaxy type of mid-leg femora (the number of setae: $1 \leq 3$, $2 > 3$) and pronotal chaetotaxy (1—one row of setae along the posterior margin, 2—one row of setae along the posterior margin and 1–2 additional setae nearby, and 3—two rows of setae (an additional row consisting of more than two setae)).

Region	Proportion of Females with Different Morphotypes, %					Number of Individuals
	F1-1	F1-2	F2-1	F2-2	F2-3	
Murmansk Region	0	0	0	25.0	75.0	4
Krasnoyarsk Territory (Norilsk)	28.6	0	57.1	0	14.3	7
Altai Republic	7.7	7.7	7.7	46.2	30.8	13
Magadan Region	50	50	0	0	0	2

The highest number of females (20 individuals) was collected in Siberia: Altai Republic—13, and Krasnoyarsk Territory (Norilsk)—7 individuals. The majority of them had "mixed" *F. kozlovi/gagatoides* morphotypes: the Altai Republic—61.6, and Krasnoyarsk Territory (Norilsk)—57.1%. In the north of Siberia (Norilsk), the proportions of individuals with the "pure" morphotype of *F. gagatoides* made up less than 28%. In the north of the European part of Russia (Murmansk Region), females with "pure" morphotypes of *F. kozlovi* were also noted.

To clarify the reasons for the current situation (hybridization in the zone of the overlapping ranges of these species, or the conspecifity of the specimens studied), a molecular genetic analysis of the samples from the different regions was carried out using representatives of both the "pure" and "mixed" morphotypes (Table S1).

3.2. Molecular Genetic Assay

Diversity of the COI Gene

The maximum likelihood phylogenetic tree for the specimens studied and the COI sequences from the DNA database is shown in Figure 4. The genetic distances between all the COI gene sequences are shown in Table S4. In the dendrogram, the *Formica* aff. *candida* specimens form an inner branch within the *F. gagatoides* cluster; the *F.* cf. *kozlovi* from the Altai Republic occupies a basal position in a branch, including *F. gagatoides*, *F.* aff. *Candida*, and the North American specimens close to *F. gagatoides*.

The diversity of the ITS1 of the rRNA genes: According to the nucleotide substitutions within the main studied fragment of ITS1 (positions from 66 to 762 of the *F*. aff. *candida* sequence with the NCBI number KX609260), the studied ants were divided into two monomorphic groups of specimens. One of them included *F*. cf. *kozlovi*, *F. gagatoides*, and *F.* aff. *candida*, and another was formed by *F. lemani* and *F. gagatoides* (China) (Figure S5). The genetic Kimura-2-parameter distance between these groups was 0.004. A part of the variability was related to insertions and deletions. The species *F.* aff. *candida* differed from the studied specimens of *F.* cf. *kozlovi* and *F. gagatoides* with a single deletion of 3 bp. The specimens of *F. lemani* had a variability in the numbers of monomers in a microsatellite block within their ITS1 (5-7 GC monomers), and variability in the single nucleotide repeat of their 3–4 G nucleotides. The specimens of *F. gagatoides* (China) were identical to specimens of *F. lemani* (with 5 GC monomers) from both the Far East and north Europe.

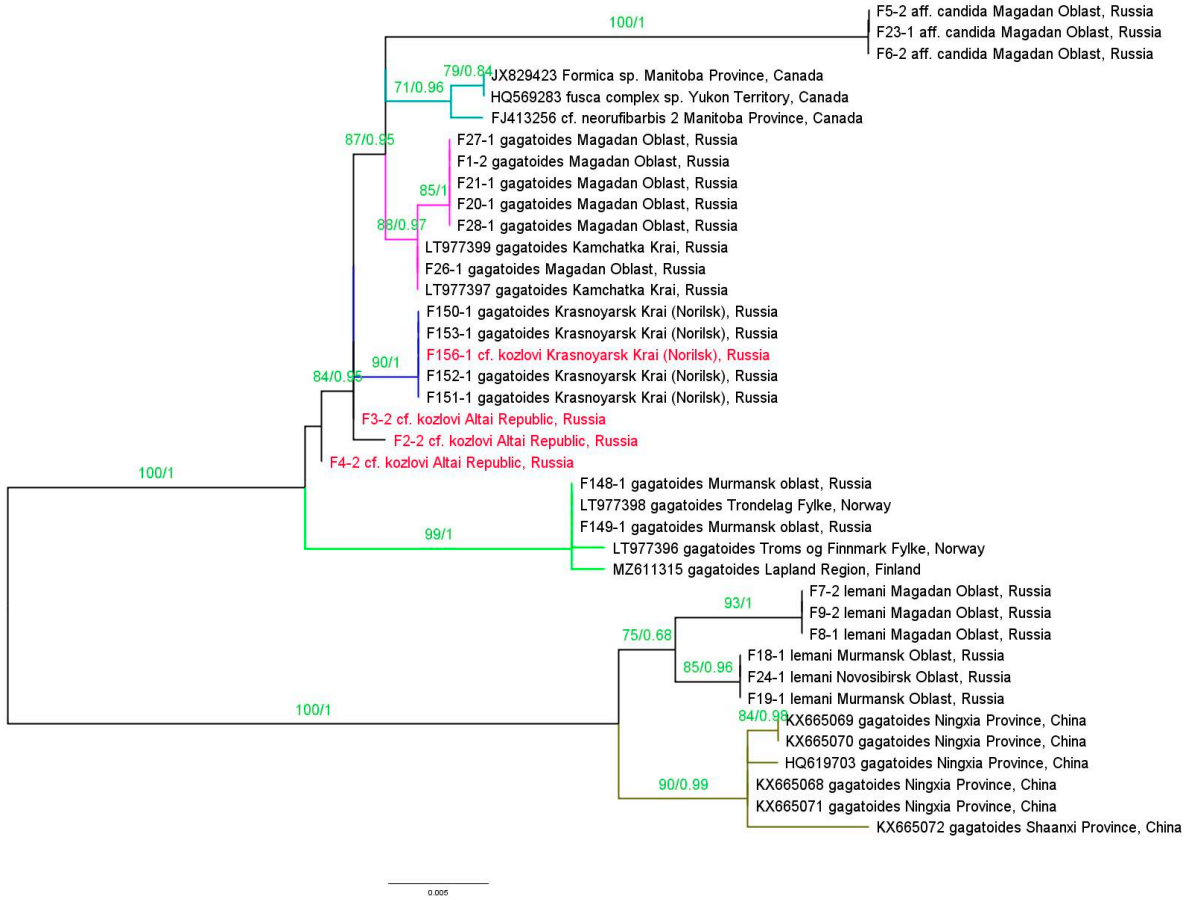

Figure 4. The maximum likelihood phylogenetic tree based on the COI gene sequences of the specimens sequenced by the authors and also described as *F. gagatoides* in the DNA database. The evaluations of branch support are shown: the bootstrap values in percent at 1000 iterations and Bayesian posterior probabilities. These evaluations are hidden when the Bayesian posterior probabilities are less than 0.5 or the bootstrap values are less than 50%. Branches that can be assigned to *F. gagatoides* according to their phylogenetic place, and also the branch including specimens identified as *F. gagatoides* by other authors are colored; specimens of *F*. cf. *kozlovi* are highlighted in red color.

However, in another fragment of ITS1 (positions from 763 to 956 of the *F.* aff. *candida* sequence with the NCBI number KX609260), there were two variable sites within the *F.* cf. *kozlovi* and *F. gagatoides* (Figure S6). The first variable site was in an in/del of a single nucleotide and the second one was a substitution. The Asian specimens demonstrated a superposition of two sequence variants in the first site (Figure S7).

They mostly had a slight but discernible or undetected admixture of the variant with deletion. However, two specimens (F151-1 with the *gagatoides* morphotype and F-156-1 with the *kozlovi* morphotype) had both variants in similar proportions. The other *F*. cf. *kozlovi* specimens (F2-2 and F4-2) had a common pattern with the other Asian *F. gagatoides* in this site.

The difference in the second variable position looked to be as discrete. In this way, no differences in the ITS1, which could differentiate the *F*. cf. *kozlovi* from the Asian *F. gagatoides*, were found. However, there were two positions in the sequence by which the European *F. gagatoides* could be distinguished from the Asian ones, as well as the *F.* cf. *kozlovi*, but one of these differences was not discrete.

The diversity of the D2 variable region of the 28S rDNA: A fragment of the gene with a length of 631 bp was studied for the *F*. cf. *kozlovi*, *F. gagatoides*, and *F.* aff. *candida*. In *F. lemani*, this sequence was 630 bp long because of a single nucleotide deletion (Figure S8). Due

to the sequence features that terminated the sequencing reaction, there were difficulties in obtaining complete reads: the D2 28S of the F150-1 specimen was not fully read in the most conservative part. According to the D2 28S sequences, no signs of intraspecific polymorphism were found: *F*. cf. *kozlovi* was identical to *F. gagatoides*.

A comparison of the studied D2 28S sequences with the sequences of D2 28S from the DNA database (BLAST algorithm) showed that the sequences obtained for the *F. gagatoides* and *F*. cf. *kozlovi* were identical to the sequences of the *F*. *gagatoides* from Kamchatka (LT977164) and Norway (LT977165). Similarly, the sequences obtained for *F*. aff. *candida* were identical to the sequences of this species from Genbank (LT977166, Russia, Kamchatka Territory); the specimens of *F*. *lemani* were identical to the sequence of this species from the north-east of Spain (LT977114) and *F*. *podzolica* (LT977127, USA, Lopez Island). The identity of *F. lemani* and *F*. *podzolica* in the D2 28S can be explained by the low evolution rate of this sequence for the studied ant group. The dendrogram based on the combined data of the COI and D2 28S sequences of the specimens studied and the *F*. *gagatoides* from the DNA database is shown in Figure 5.

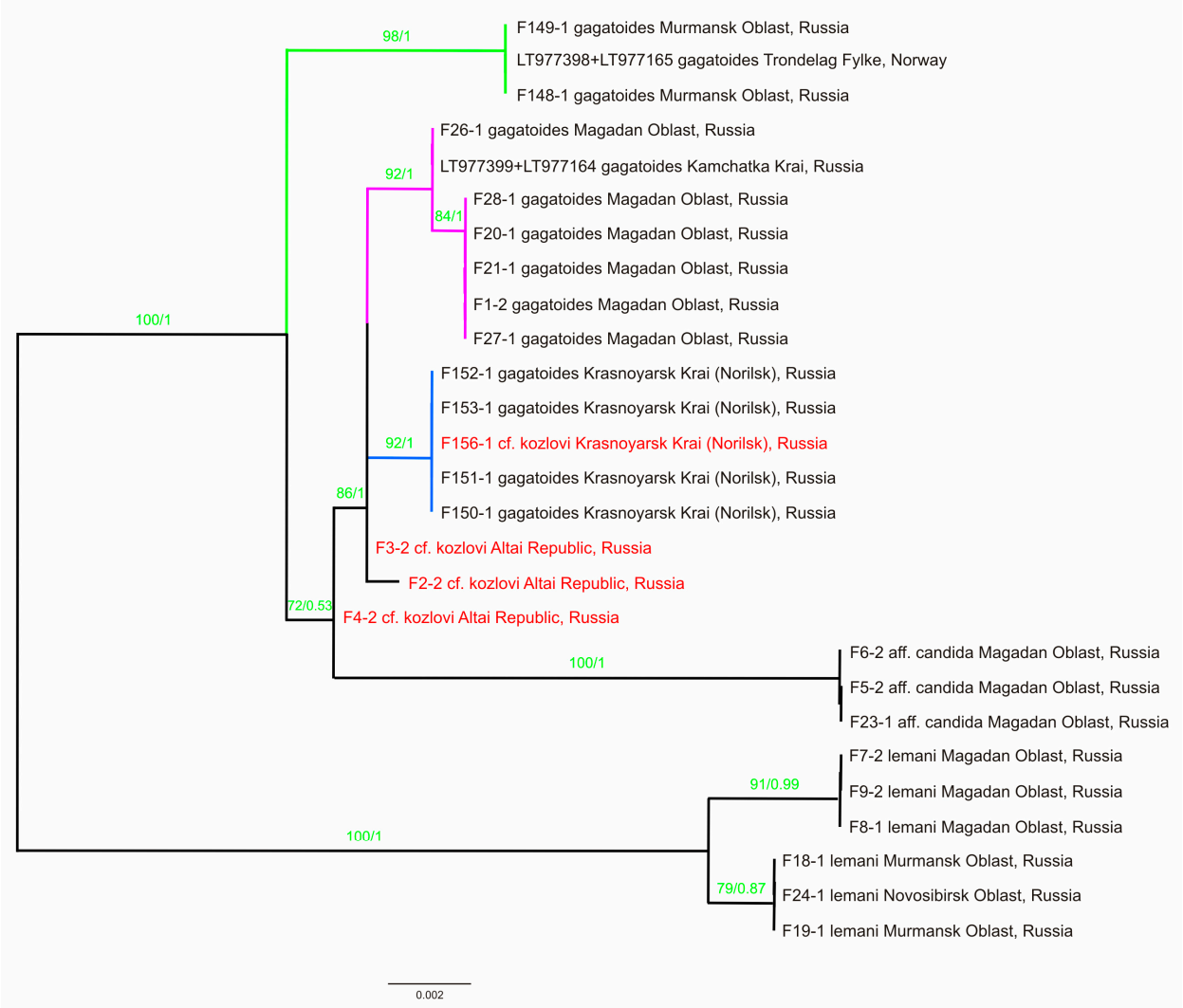

Figure 5. The maximum likelihood phylogenetic tree based on the COI and D2 28S combined sequences. The evaluations of branch support are shown: the bootstrap values in percent at 1000 iterations and Bayesian posterior probabilities. These evaluations are hidden when the Bayesian posterior probabilities are less than 0.5 or the bootstrap values are less than 50%. Branches of *F. gagatoides* are colored; specimens of *F*. cf. *kozlovi* are highlighted in red color.

No signs of a hybrid origin of the studied *F*. cf. *kozlovi* specimens were found in the studied DNA regions, using both relatively variable ITS1 and conservative D2 28S. There were no superpositions in the rDNA that could differentiate the *F*. cf. *kozlovi* from the Asian *F. gagatoides* in both the ITS1 and D2 28S sequences (Figures S5, S6 and S8). Additionally, these groups were inseparable by the strict species threshold (a 1% divergence level) in the COI (Table S4). In all cases, the *F*. cf. *kozlovi* specimens were univocally placed into the Asian *F. gagatoides* branch.

4. Discussion

A detailed analysis of the key morphological diagnostic characters for material belonging to the *F. gagatoides*/*F*. cf. *kozlovi* species complex showed a high degree of variability among the studied specimens. Furthermore, there was a great problem in the species identification of the material from the examined ant nests, because of the high percentage of individuals with "mixed" morphotypes. In Altai, individuals with a large number of setae on the femora of their middle legs (character of *F. kozlovi*), but with a dense pubescence of the first gaster tergite (character of *F. gagatoides*) were most frequently encountered (67%). Up to nine setae were observed on the mid-leg femora of the ants from the Altai population, with individuals with three–five setae predominating (about 53% of the examined specimens). Similar results were obtained for the northern part of the Krasnoyarsk Territory (near Norilsk): the maximum number of setae on the femora of their middle legs was seven, and individuals with three–four setae prevailed (57%). A different situation was observed in the north of European Russia (the Murmansk Region) and the Far East. The maximum number of setae on the mid-leg femora was no more than four and individuals with one–two setae made up over 70% (north of Europe) and 90% (Far East). As for the pubescence of the first gaster tergites, in the north European population, it was usually sparse (42%), while in the Far Eastern population, individuals with a dense pubescence of the first tergite and half part of the second one predominated (95%).

Generally, the diversity of the types of chaetotaxy of the mid-leg femora and pubescence of the first gaster tergites (I–III) in the workers of this complex was presented with eight morphotypes. In Siberia, the diversity of morphotypes was the highest: the whole range of the variants (eight) was noted in the Krasnoyarsk Territory and seven morphotypes were found in Altai. In this territory, the proportion of individuals with "mixed" morphotypes was over 70% (Krasnoyarsk Territory) and 60% (Altai), which significantly exceeded the proportion of individuals with "pure" morphotypes of both the *F. kozlovi* and *F. gagatoides*. It is known that the morphology of F_1 hybrids is usually a combination of their parent species' features [38,39]. The predominance of intermediate forms with traits of both species in Altai could indicate both the conspecifity of the studied specimens and the presence of hybridization in the overlapping ranges of these species. However, unexpectedly, a high proportion of "mixed" morphotypes (about 54%) was registered in the European part of Russia (the Murmansk Region).

A molecular genetics analysis of the specimens with "pure" and "mixed" morphotypes from the different regions unequivocally refuted the assumption of a possible hybridization of the Asian specimens of *F*. cf. *kozlovi* and *F. gagatoides* and clearly showed their conspecificity. The rDNA sequence patterns of the *F*. cf. *kozlovi* excluded any possibility of the origination of these specimens from a hybridization of the *F. gagatoides* with any other form. The variability of the COI sequences of the *F*. cf. *kozlovi* does not show signs of a mitochondrial DNA introgression from any forms distant from *F. gagatoides*.

The results of the analysis based on the COI sequences clearly showed the formation of geographic clusters within *F. gagatoides* and *F. lemani*. *F. gagatoides* had the northern European branch, the Far Eastern branch (Kamchatka and Magadan Region), and the Siberian branch, with an intermediate position of Altai *F*. cf. *kozlovi*. The North American specimens formed a separate branch within *F. gagatoides*, which was most similar to the Far East one. The cluster of *F. lemani* included three branches: Europe–Siberian (the Murmansk Region and Novosibirsk Region), Far East (Magadan Region), and also a group

of *F. gagatoides* from central China. However, these branches mostly diverged only slightly and the 1% threshold (COI divergence) was only crossed by two pairs—European vs. Asian *F. gagatoides* (including *F.* cf. *kozlovi*) and the "*F. gagatoides*" from central China vs. other branches of *F. lemani*.

The level of the COI sequence differences between the *F.* cf. *kozlovi* from Altai and *F. gagatoides* from both the Far East and the north of the Krasnoyarsk Territory was within intraspecific variation (0.003–0.006). In the case of the hybrid origin of *F.* cf. *kozlovi*, one would expect to find mitochondrial DNA genetically distinct from *F. gagatoides* in some of the *F.* cf. *kozlovi*. However, the studied *F.* cf. *kozlovi* specimens had no signs of an introgression of their mitochondrial DNA distant from the variants of the *F. gagatoides*. According to the sequences of the ITS1 and D2 28S, the Asian *F. gagatoides* and *F. kozlovi* appeared to be identical. Therefore, there was no molecular genetic evidence to differentiate the Siberian *F.* cf. *kozlovi* and Asian *F. gagatoides*, as they appeared to be conspecific.

The specimens registered in GenBank as *F. gagatoides* from central China were found to be identical to *F. lemani*. According to the COI sequences, the Kimura-2-parameter distances between these groups were found to be only 0.011–0.015. The similarity or identity between these groups in the ITS1 sequences of the rDNA and the similarity between them in their COI seemed to be due to a mistake in the species identity of the material from central China and its belonging to *F. lemani*. The variability within the ITS1 of *F. lemani* and "*F. gagatoides*" from central China was represented by the variability in the number of identical nucleotide blocks. This kind of variability appears easily and the mechanism of the isogenisation of rRNA genes can work ineffectively within a species. A dendrogram based on the COI sequences, including samples of both the *F. gagatoides* from central China and *F. lemani* from different regions (including eastern China, Hebei province), clearly demonstrated a high similarity degree between these groups (Figure S4). It is worth noting that, among the studied *F. lemani* specimens from the Far East, some individuals morphologically similar to *F. gagatoides* with a slightly sparse pubescence, starting from the posterior margin of tergite II, were also found. This trend may be more pronounced in more southern populations of *F. lemani*, which may make the identification of the species more difficult.

As for the specimens of *F. gagatoides*, according to the COI sequences, the genetic distances between the specimens of the north European and Asian branches of this species were within an interval of 0.012–0.019. This is close to the conventional limit of intraspecific variations [29]. The sequences of the COI gene fragment originating from North America were highly similar to the Asian *F. gagatoides* and *F.* cf. *kozlovi* (distances were less than 1%), but were significantly different from the *F. gagatoides* from Europe (distances were about 2–2.2%). It is most likely that the sequences from Canada, named in the DNA database as *F. fusca*-complex, "*F. neorufibarbis* 2", and *Formica* sp., represent a part of the geographical population of the polymorphic *F. gagatoides* species. In this case, the variability within *F. gagatoides* was quite similar to the differences between the species [29]. It can be noted that the habitats of origin of the two specimens from Canada (NCBI numbers FJ413256 and JX829423, [40,41]) and Finland (MZ611315, [42]) were similar. This type of habitat (a borderland between the boreal forest and tundra) is common for the specimens considered in this study. The differences in the ITS1 sequences between the European and Asian *F. gagatoides* raise the question of the conspecifity of these branches. Unlike the differences in the ITS1 within *F. lemani*, the differences within *F. gagatoides* (with *F.* cf. *kozlovi*) included a nucleotide substitution, the phylogenetic weight of which was much higher than the variability in the number of nucleotide repeats. The pattern of variability in the ITS1 sequences showed the absence of mixing between the north Europe and Asian parts of *F. gagatoides*. Therefore, from the point of view of the molecular genetic criteria for species differentiation [29], which were justified in the Material and Methods section, there is a reason for giving a different species status to the European and Asian branches of *F. gagatoides* (the Asian branch also includes the Siberian *F.* cf. *kozlovi*). The north American specimens seemed to be conspecific with the Asian branch of *F. gagatoides*, but further research is required to clarify this point. To clarify the question about the conspecifity of

the Asian and European branches of *F. gagatoides*, additional samples from the intermediate habitats from the Murmansk Region to the north of the Krasnoyarsk Territory should be examined.

On the dendrograms for the DNA fragment of the COI gene and COI + D2 28S combination, *F*. aff. *candida* was combined into one branch with the Asian *F*. cf. *kozlovi* and *F*. *gagatoides*. At the same time, *F*. aff. *candida* demonstrated some minimal but discrete differences in its rRNA gene fragments (ITS1 and D2 28S). It can be assumed that the age of the mitochondrial DNA polymorphism in the *F*. *gagatoides* (including *F*. *kozlovi*) approximately corresponded to the time of the divergence from *F*. aff. *candida*.

As for the branches of *F. gagatoides* revealed in this study, these results significantly expand the knowledge and understanding of the information obtained earlier. A molecular study of *F. gagatoides* from different parts of its habitat was previously carried out by Schär et al. [13]. The authors also found a relatively high divergence in the COI sequences between specimens from north Europe and the Far East, but were unable to confirm this result with rDNA, due to limited length and variability of the studied sequences. Thus, the 28S rDNA sequence used by the authors was quite conservative, due to the selective limitations of its structure. The sequences of the spacers ITS1 and ITS2 have no selective limitation (except for the possibility of an excision from rRNA) and are more variable. It is necessary to mention that the fragments of the rDNA, regardless of their selective limitations, were combined in unified blocks, and these blocks were subjected to the concerted evolution mechanism [36]. A DNA sequence can be considered to be an ordered sample, and possible differences between sequences are the differences between these samples. Thus, the length and variability of the studied sequences may limit the possibility of finding differences between specimens. Therefore, in addition to the region of the 28S (631 bp) studied by Schär et al. [13], the ITS1 sequence (893–894 bp) was examined. The total length of the rDNA sequences examined in this study made up 1524–1525 bp, which was more than twice as much as that in the study by Schär et al. [13]. This made the applied approach more effective and made it possible to confirm the divergence of the north European and Asian branches using rDNA.

The possibility of subdividing *F. gagatoides* into separate branches was also noted in a study of the *F. picea*/*F. candida* species complex, based on the preliminary results of a COI sequence analysis [22]. This corresponded to the data obtained in the present study, but the conclusion of the existence of three cryptic species made by the authors of this study seems to be somewhat premature. According to the obtained results and the analysis of the literature data, both the number and character (cryptic or not) of the proposed species need further investigation and discussion.

Therefore, the definite divergence of the European and Asian representatives of the *F. gagatoides*/*F. kozlovi* species complex was also noted by Seifert ([18]; pers. comm.) using a complex morphological analysis. It can be assumed that this result was not about the differences between *F. gagatoides* and *F. kozlovi*, but about the differences between the European and Asian (or even wider) branches of *F. gagatoides*, which seems to be different species. According to the data of Seifert [18], Asian *F.* cf. *kozlovi* differs from European *F. gagatoides*, with a significantly longer scape, a larger unilateral number of setae protruding more than 10 μm from the cuticular surface of the pronotum (nPn), and a smaller average distance of the transversal microripples on the dorsal plane of the first gaster tergite (RipD). Thus, in general, these data confirm the obtained results and seem to demonstrate the morphological features that can be used to distinguish European and Asian *F. gagatoides*. To verify this assumption, an extended morphological analysis is needed with an increase in sample size and the involvement of Asian *F. gagatoides* (pure morphotypes of this species) from different regions.

On the whole, the results indicating the conspecificity of the *F*. cf. *kozlovi* and Asian *F. gagatoides* explained the habitation of *F. kozlovi* and *F. gagatoides* in the same habitats in the territory bordering Mongolia, which was repeatedly noted throughout the course of the studies [1].

In the surveyed territory of Altai, representatives of the *F. gagatoides*/*F.* cf. *kozlovi* species complex were found at altitudes from 1740 to 2700 m a.s.l. These ants most densely inhabit mid-mountain woodlands of larch (*Larix sibirica* Ledeb.) and Siberian pine (*Pinus sibirica* Du Tour), combined with areas of low-grass subalpine meadows, as well as stony tundra located above woodlands along slopes of southern exposure (up to four nests per 25 m^2). These ants were not found in other tundra habitats. In addition to stony tundra and sparse woodlands, *F. gagatoides*/*F.* cf. *kozlovi* inhabit the upper part of the taiga middle mountains, but here, the density of their settlements is much lower—from 0.1 to 0.6 nests per 25 m^2 [43,44].

The example of the *F. gagatoides*/*F.* cf. *kozlovi* species complex clearly shows that using the number of setae on the mid femora of workers and females, as well as the pronotum of females, as a distinguishing feature may be unacceptable in some cases. The pattern of the chaetotaxy of the ant legs and pronotum and the gaster pubescence can vary greatly. In particular, this trend was observed in *F. gagatoides*, as it moved from north to south. Since only the species *F. kozlovi* has, so far, been recorded in Mongolia [3,45,46], it can be assumed that either mainly morphotypes with more than three femoral setae on their mid-legs (2-1, . . . , 2-4) are represented in this region, or only the regional literature was used when identifying the Mongolian material, without the polar ant, *F. gagatoides*, in the keys. Unfortunately, due to the difficult epidemiological situation in the world in recent years, it was not possible to obtain material for the study from Mongolia.

5. Conclusions

1. For species with a wide range (such as *F. gagatoides*), it is inappropriate to use their chaetotaxy type as a diagnostic feature, due to its strong variability. In particular, this concerns the number of setae on the mid-leg femora of workers and females, as well as on the pronotum of females.
2. In representatives of the *F. gagatoides*/*F.* cf. *kozlovi* complex in Siberia, a high diversity of variants was noted, according to the main morphological diagnostic characters for the workers and females—the number of setae on the mid-leg femora and pronotum, as well as the pubescence of the first gaster tergites. In Siberia, the diversity of the morphotypes among the workers was the highest (Krasnoyarsk Territory—eight, Altai—seven), with a predominance of intermediate forms (Krasnoyarsk Territory—73.6%, Altai—63.4%). In females, five morphotypes were noted, with a predominance of "mixed" forms (about 60%). As for "pure" morphotypes in females, rather high proportions of *F. kozlovi* (30.8%) and *F. gagatoides* (28.6%) were noted in Altai and the Krasnoyarsk Territory, respectively.
3. The results of the molecular genetic analysis of the representatives of the Siberian *F.* cf. *kozlovi* and Asian *F. gagatoides*, involving specimens with both "pure" and "mixed" morphotypes, revealed the conspecifity of the studied specimens and significantly expanded the knowledge on the distribution of *F. gagatoides.*
4. The data obtained raise the issue of the *F. kozlovi* species status, which requires an additional investigation into *F. kozlovi* materials from the areas of the description of its type specimens (Mongolia and China). In addition, the data from the molecular analysis also raise the issue of the concpecifity of the Asian and European branches of *F. gagatoides.*
5. Generally, the data obtained significantly expand the knowledge of the distribution, morphological variability, and ecotypes of *F. gagatoides*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15050686/s1. There are two files. Suppl 1, Table S1: The main characteristics of the material studied; Table S2: An origin of the sequences from DNA database. The sequences of DNA identified as *F. gagatoides* and Canadian sequences of *Formica* spp. similar to COI of *F. gagatoides* from NCBI nucleotide database; Table S3: The main types of pubescence of I-III gaster tergites of studied specimens of *F. gagatoides F.* cf. *kozlovi* complex according to the ratio of the length

of the pubescence hairs and the distance between them; Figure S1: The main types of chaetotaxy (1-36) reflecting the number, size and arrangement of setae on the mid-leg femora of workers of the *F. gagatoides*/*F.* cf. *kozlovi* complex collected in Altai; Figure S2: Morphological features of the pronotum of females *Formica gagatoides* and *F. kozlovi*; Figure S3: The main types of pubescence of I-III gaster tergites of studied specimens of *F. gagatoides*/*F.* cf. *kozlovi* species complex (photos were taken using Hitachi TM-1000 microscope); Figure S4. The maximum likelihood phylogenetic tree based on the standard region of COI sequences; Figure S5: Variable positions in the first studied region of ITS1 (A) and simple phylogenetic scheme based on nucleotide substitutions in the studied DNA region (B); Figure S6: Variabilities in two ClustalW alignment blocks of ITS1 of *F. gagatoides* and *F.* cf. *kozlovi*; Figure S7: Quantitative variability in the region of single in/del in ITS1 region of *F. gagatoides*; Figure S8: Variable positions in the studied 28S rDNA region (A) and simple phylogenetic scheme based on nucleotide substitutions in the studied DNA region (B). Suppl 2, Table S4: Kimura-2-parameter distances for pairs of COI sequences.

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