

## **Integrative taxonomy of the freshwater harpacticoid *Attheyella crassa* G.O. Sars, 1863 (Crustacea: Copepoda: Canthocamptidae) in the Palearctic region**

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**ABSTRACT:** The phylogenetic structure of the freshwater harpacticoid copepod *Attheyella crassa* G.O. Sars, 1863 was studied. This species is wide-spread in Europe and European Russia. Genetic and morphological divergence of populations from distant localities were analyzed. The most variable morphological characters, as well as genetic diversity among ten Western Palearctic populations, were revealed.

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**KEY WORDS:** morphology, systematics, phylogenetics, phylogeography, meiofauna.

## **Интегративная таксономия пресноводной гарпактициды *Attheyella crassa* G.O. Sars, 1863 (Crustacea: Copepoda: Canthocamptidae) в Палеарктике**

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**РЕЗЮМЕ:** В данной работе проведены филогенетические исследования широко распространенного в Европе и европейской части России вида пресноводной гарпактициды *Attheyella crassa* G.O. Sars, 1863. Выявлены морфологические и молекулярно-генетические различия между географически изолированными популяциями. В результате представлены наиболее изменчивые морфологические признаки и уровень генетической дивергенции среди десяти популяций вида в Западной Палеарктике.

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КЛЮЧЕВЫЕ СЛОВА: морфология, систематика, филогенетика, филогеография, мейофауна.

## Introduction

Harpacticoids are a group of copepods with high morphological diversity and species richness (Borutzky, 1952, 1964). Some studies, focused on harpacticoid taxonomy and phylogeny, have revealed a significant heterogeneity among the populations of different species (depending on geographical location and environmental conditions) and have demonstrated the presence of several complexes of cryptic species and forms (Wells, 2007; Garlitzka *et al.*, 2012; Karanovic, McRae, 2013; Huys *et al.*, 2016; Kochanova *et al.*, 2018). However, the systematics and ecology of freshwater harpacticoid copepods are not well studied yet and are based mainly on morphological descriptions (Dussart, 1967; Wells, 2007) and on investigations published in the middle of the last century (Lang, 1948; Borutzky, 1952, 1964). The integrative approach including morphological and genetic studies, allows to investigate the population structure of a species, its biogeography and possible patterns of distribution (Dayrat, 2005). Moreover, the study of morphological and genetic variation helps to elucidate the processes that create and maintain diversity (Masta, Maddison, 2002; Garcia-Paris *et al.*, 2003).

The copepod *Attheyella crassa* Sars, 1863 belongs to the family Canthocamptidae, the most species-rich family of the order Harpacticoida (Gaviria, Defaye, 2015; Walter, Boxshall, 2018). The species shows a Palearctic distribution and is widespread in Europe and Russia (from Pskov region to Carelian Isthmus) in different types of water bodies (Enckell, 1980; Defaye, Dussart, 2011). Due to the resis-

tance of *A. crassa* to various organic and inorganic compounds, as well as its relatively short period of development (4–6 weeks), this species was used in toxicological studies (Turesson *et al.*, 2007; Gardeström *et al.*, 2008). The latter studies showed that due to the exposure to toxic substances, body length and individual fertility may change, as well as the genetic diversity, which decreases significantly in affected populations.

In order to study systematics (taxonomy and phylogeny) of the freshwater harpacticoid copepod *A. crassa*, morphological and genetic variability among different European and Russian populations of the species were analysed.

## Material and Methods

Samples were collected from ten different freshwater habitats (Fig. 1) using a 100- $\mu$ m mesh size hand net. Living (non-fixed) samples were sorted under a stereomicroscope Micromed (China), harpacticoids were individually preserved in 96% ethanol. In order to analyze morphological characters, 98 adult females of *A. crassa* from eight different populations were measured (Table 1). The identification and measurements of the species were done under a microscope Leica DM 4000B (Germany). Body length of these specimens was measured from the tip of prosome to the distal end of the caudal rami in dorsal position. More than 20 quantitative morphological and morphometric characters, important for diagnostic identification, were considered. The statistical variability of characters was assessed with help of the Kruskal-Wallis test (each character was compared among all populations) and of the Kolmogorov-Smirnov

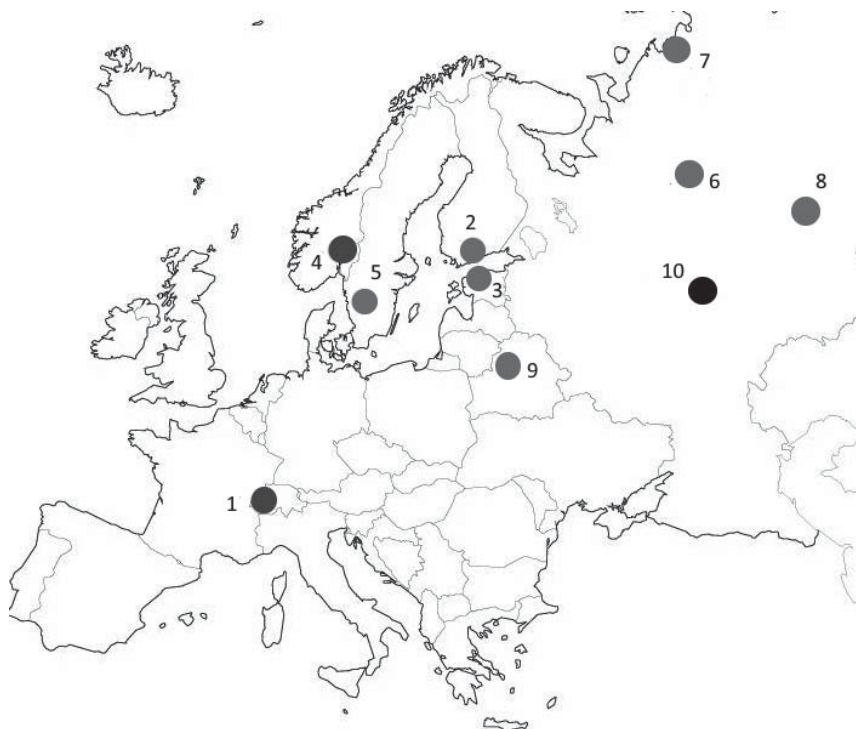


Fig. 1. Map of sample locations of examined specimens of *A. crassa*. Locality names are listed in Table 2. Number 1 and 4, group I; 10, Rybinsk reservoir; other numbers, group II.

Рис. 1. Карта мест сбора исследованных экземпляров *A. crassa*. Названия местообитаний перечислены в таблице 2. Номер 1 и 4 относятся к группе I, номер 10 — Рыбинское водохранилище, остальные номера относятся к группе II.

test (each character was compared between two populations, and then all populations were gathered in one matrix to point out the differences) (Kruskal, Wallis, 1952; Stephens, 1974). Measurements were performed with the help of an ocular micrometer under the microscope. Statistical analysis were performed with Statistica 7.0 and Past 3.20 softwares. Principal Component Analysis (PCA) plot was built in Past 3.20. All values were transformed to a standard normal distribution before plotting.

To carry out the phylogenetic analysis, 35 females of *A. crassa* from ten different populations were used (Table 1). Genomic DNA was extracted from animals preserved in 96% ethanol, following the protocols outlined in Walsh *et al.* (1991). Specifically, the bodies of each specimen were introduced into to 6% Chelex-100 solution (Sigma-Aldrich, St Louis, MO, USA)

in bi-distilled water. Samples were spun for 30 s at 12.000 rpm. The mixture was incubated at 90 °C for 30 min, then again spun for 30 s at 12.000 rpm. Samples were incubated at 90 °C for 15 min and spun for 15 s at 12.000 rpm. Afterwards samples were stored at 20 °C and amplified through PCR. In order to amplify the COI mtDNA gene, direct and reverse universal primers designed for invertebrates were used: COIH 2198(5'-TAAACTTCAGGGTGAC-CAAAAATCA-3') and COIL 1490 (5'-GGT-CAACAAATCATAAAGATATTGG-3') (Folmer, 1994). Additionally, special primers for zooplankton ZplankF1\_t1(5'-TGTA AAC-GACGGCCAGTTCTASWAATCAT-AARGATATTGG-3') and ZplankR1\_t1 (5'-CAGGAAACAGCTATGACTTCAGGRT-GRCCRAARAATCA3-') (Prosser, 2013) were used. The reaction mix consisted of 1 µL (10

Table 1. Sampling localities, number of specimens studied for phylogenetic and morphological analysis, and accession numbers in the GenBank of *Attheyella crassa*.Таблица 1. Места сбора материала, количество изученных морфологически и филогенетически образцов и номера последовательностей *Attheyella crassa* в базе данных GenBank.

No.	Country, area	Locality (water body)	Coordinates	Number of specimens for		GenBank
				genetics	morphology	
1	Geneva, Switzerland	Lake Geneva	46°27'N, 06°31'E	3	6	MH477669- MH477671
2	Hämeenlinna, Finland	Lake Pääjärvi	61°04'N, 25°08'E	3	44	MH477643- MH477645
3	Tallinn, Estonia	Creek Hiiu	59°38'N, 24°67'E	1	3	MH477651
4	Oslo, Norway	Lake Sognsvann	59°58'N, 10°43'E	6	20	MH477663- MH477668
5	Sweden	Lake Vänern	58°55'N, 13°30'E	2	2	MH477652, MH477653
6	Komi Republic, Russia	Nyuvchim reservoir	61°39'N, 50°74'E	3	5	MH477638- MH477639
7	Naryan-Mar, Russia	Pechora River	68°18'N, 54°22'E	1	1	MH477650
8	Udmurtia, Russia	Creek Karkalai	56°98'N, 52°40'E	2	3	MH477654, MH477655
9	Belarus	Lake Narach	54°87'N, 26°69'E	4	4	MH477646- MH477649 MH477640- MH477642,
10	Borok, Russia	Rybinsk reservoir	58°08'N, 38°28'E	10	10	MH477656- MH477662

µM) of direct and reverse primers each, 12 µL of DreamTaq Hot Start PCR Master Mix (ThermoFisher Scientific), 9 µL of water without nucleases (Ambition, United States) and 1 µL of isolated DNA. Thermocycler conditions for PCR started with DNA denaturing at 95°C (60 s), followed by 35 cycles of 30 s denaturing at 95°C, 30 s annealing at 50°C, and 50 s extension at 72°C, and then a final extension at 72°C for 7 min (Lee, 1999). PCR products were visualized by electrophoresis in 2% agarose gel in 1x triacetate buffer solution. A 100 bp Ladder DNA marker (100 bp – 3000 bp) (ThermoFisher Scientific) was used as a marker for evaluation of the length of DNA fragments. To purify the obtained product, a set of ExoSap-IT PCR Product Clean-Up (Applied Biosystems) was applied. PCR products amplified with Folmer primers were sequenced with Folmer primers,

while those amplified by zooplankton-specific primers were sequenced with M13F and M13R. Sequencing was carried out using the BigDye Terminator v3.1 (Life Technology) reagent kit on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). In order to avoid errors in sequences, the bi-directional sequencing was used.

Nucleotide sequences were aligned with the algorithm CrustalW using the program package Geneious (version 7.0.6.) (Kearse *et al.*, 2012). After comparing sequences obtained from the two above mentioned sets of primers, the length of the nucleotide sequences was 550 bp. Phylogenetic trees were also constructed in this program, using a Maximum Likelihood method with a high level of the bootstrap coefficient (1000 replications). Genetic distances were calculated using Tamura-Nei model in Mega 7.0.

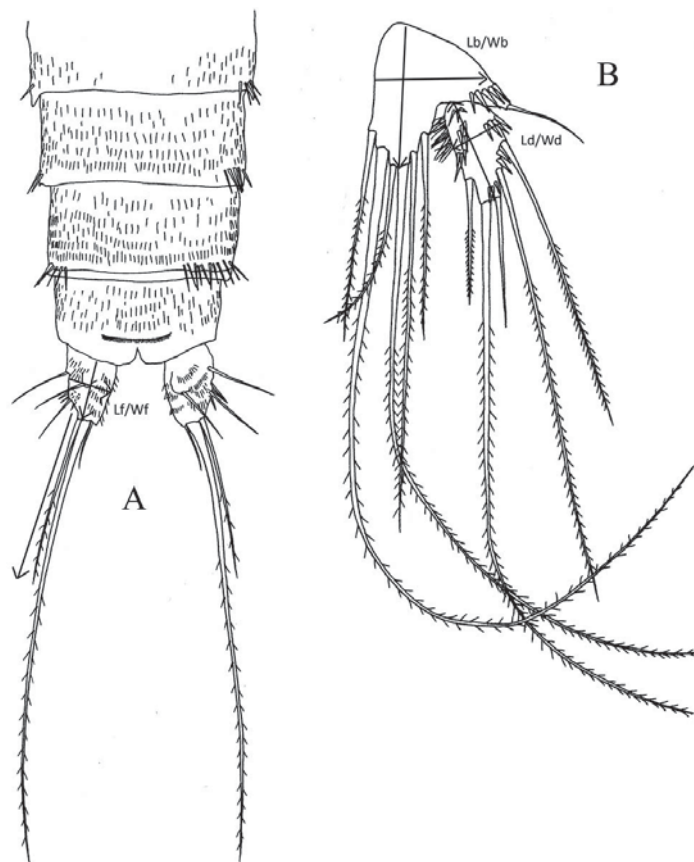


Fig. 2. Abdomen and caudal rami (A) and fifth pair of legs (B) of *A. crassa*. Arrows show measured morphological characters and indices. Drawings made by E.B. Fefilova.

Рис. 2. Абдомен и каудальные нити (A) и пятая пара ножек (B) гарпактициды *A. crassa*. Стрелки указывают на изученные морфологические признаки и индексы. Рисунки выполнены Е.Б. Фефиловой.

All sequences of *A. crassa* were registered in the GenBank under the accession numbers MH477638–MH477669. In addition, a sequence of *Canthocamptus staphylinus* (Jurine, 1820) (accession number KP974713) was included in the phylogenetic analysis. A sequence of *Elaphoidella humphreysi* Karanovic, 2006 (accession number JN039173) was used as the out-group.

## Results

### Morphological variability

According to the main diagnostic features — segmentation and structure of antennules and

antennae, structure of thoracic legs and form of caudal rami and anal operculum — all studied specimens correspond to the description of the species *A. crassa*, following Borutzky (1952, 1964) and Lang (1948). Among all measured morphological structures, the most significant variable characters ( $p < 0.05$ ) were as follows: total body length, length of the seta V (= longest apical armament) of the caudal rami, length to width ratio of caudal rami (Lf/Wf), length to width ratio of exopod (distal segment) of the fifth pair of legs (Ld/Wd), and length to width ratio of basoendopod of the fifth pair of legs (Lb/Wb) (Fig. 2).

The mean values of measured morphological traits among populations are listed in Table

Table 2. Mean values and standard deviation of variable morphological characters among the eight populations of *A. crassa*.Таблица 2. Средние значения и стандартное отклонение переменных морфологических признаков среди восьми популяций *A. crassa*.

Morphological characters					
Waterbody	Body length, mm	Caudal rami: seta V, length, mm	Lf/Wf	Ld/Wd	Lb/Wb
Pääjärvi	0.671±0.066	0.372±0.039	1.925±0.205	2.014±0.074	2.308±0.075
Geneva	0.766±0.028	0.403±0.071	1.733±0.292	2.510±0.790	2.282±0.405
Vänern	0.630±0.127	0.440±0.014	1.994±0.001	2.181±0.001	2.153±0.001
Sogsvann	0.700±0.082	0.416±0.052	1.533±0.144	2.470±0.241	2.023±0.047
Hiiu	0.810±0.018	0.443±0.038	2.159±0.155	2.303±0.052	2.153±0.001
Narach	0.720±0.050	0.415±0.035	1.866±0.001	1.923±0.001	2.307±0.001
Karkolai	0.738±0.047	0.393±0.041	2.236±0.205	2.666±0.135	2.000±0.001
Rybinsk reservoir	0.635±0.035	0.385±0.021	2.029±0.041	2.588±0.001	2.153±0.001

Lf and Wf — length and wide of caudal rami respectively; Ld and Wd — length and wide of exopod of fifth leg, respectively; Lb and Wb — length and wide of basipod of fifth leg, respectively.

2. The body length of adult females varied from 0.6 to 0.828 mm. The smallest specimen was found in Lake Pääjärvi, the largest one in the creek Hiiu. The length of seta V of the caudal rami varied from 0.3 (Lake Pääjärvi) to 0.52 mm (Lake Sogsvann). The ratio Lf/Wf changed from 1.375 (Lake Sogsvann) to 2.365 (Lake Pääjärvi). The ratio Ld/Wd varied from 1.69 (Lake Geneva) to 2.72 (Lake Pääjärvi). The ratio Lb/Wb changed from 1.92 (Lake Narach) to 3.35 (Lake Geneva).

As a result of clustering of females of *A. crassa* on the PCA plot (Fig. 3, Table 3), two main groups of populations were found. The first group (I) includes populations from the lakes Pääjärvi (Finland), Vänern (Sweden), Narach (Belarus), Rybinsk reservoir (Borok, Russia) and creeks Karkalai (Udmurtia, Russia) and Hiiu (Estonia). The second group (II) includes specimens from lakes Sogsvann (Norway) and Geneva (Switzerland).

### Phylogenetic variability

Thirty-five original COI sequences of *A. crassa* contained 67 polymorphic sites, where 65 showed informative parsimony. Thirteen

haplotypes were identified, the total haplotype diversity was 0.882. The total number of mutation was 67.

All sequences formed two main clades in the phylogenetic tree constructed with the Maximum Likelihood Method (Fig. 4). The first clade (I) includes sequences of specimens collected from the lakes Pääjärvi (Finland), Vänern (Sweden), Narach (Belarus), Nyuvchim (Komi Republic, Russia) and Rybinsk reservoirs (Borok), the creeks Hiiu (Estonia) and Karkalai (Udmurtia, Russia), and the river Pechora (Naryan-Mar, Russia). The second clade (II) includes sequences of specimens from the type locality of the species Lake Sogsvann (Norway), Lake Geneva (Switzerland) and Rybinsk reservoirs (Borok, Russia). The genetic distances between these two clades were 19.6% and between populations of *A. crassa* and *C. staphylinus* (both belonging to the family Canthocamptidae) — 27.2%. The genetic distances between populations inside the clades ranged from 0.5 to 3%.

### Discussion

The order Harpacticoida and particularly the family Canthocamptidae show significant

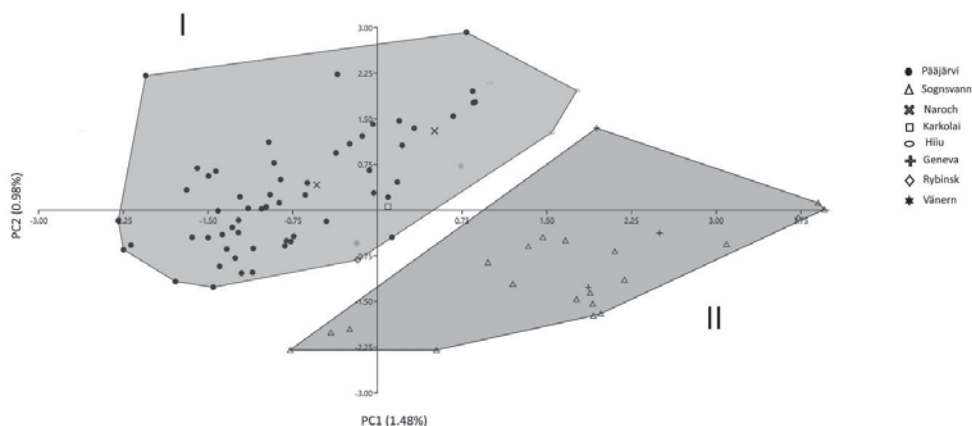


Fig. 3. Principal component analysis of morphological traits. I, II — groups of populations of *A. crassa*, similar to the clades in the phylogenetic tree (Fig. 4). PC1 and PC2 are plotted on x- and y-axes. The percentage of variation explained by the plotted principal coordinates is indicated on the axes.

Рис. 3. График, построенный на основе Метода Главных Компонент. I, II — группы популяций *A. crassa*, соответствующие кладам на филогенетическом древе (рис. 4). ГК1 и ГК2 обозначены вдоль осей абсцисс и ординат. Коэффициент вариации (%) построенного графика указан рядом с осями.

Table 3. Factor loadings on components based on PCA plot (Fig. 3).

Таблица 3. Факторные нагрузки к графику, построенному методом главных компонент (рис. 3).

Character	PC 1	PC 2	PC 3	PC 4	PC 5
Body length	0.09515	0.016687	0.0087835	0.89285	0.43973
Apical spine	-0.0047163	-0.0041321	-0.04123	0.4415	0.89625
Lf/Wf	0.40324	0.90113	0.14316	-0.048947	0.024227
Ld/Wd	0.90901	-0.3985	0.062073	-0.06987	0.039558
Lb/Wb	0.0034898	0.15338	0.98629	0.023923	0.034741

morphological diversity (Wells, 2007). The canthocamptid copepod *A. crassa* is considered to be a morphologically stable species across its distributional range (Borutzky, 1952, 1964). However, its subspecies *Attheyella crassa thracica* Michailova-Neikova, 1966 described from Bulgaria has been accepted (Walter, Boxshall, 2018) based on the different shape of the caudal rami and the particular bend of the longest apical armament (seta V). A second subspecies *Attheyella crassa schmeili* Gagern, 1938 collected in Germany (Harz region), with minor morphological differences on the caudal rami and the size of setae on the legs (compared with

Scandinavian populations of *A. crassa*), caused Lang (1948) to consider it as not valid.

The present study revealed variability of several morphological traits among populations of *A. crassa*. The morphology of the caudal rami and length of its longest seta (seta V), the body length as well as the morphology of basoendopod and exopod of the fifth pair of legs, showed significant fluctuations among the examined populations. For example, the caudal rami of specimens from group I, which includes populations from the lakes Geneva and Sogsvann, were smaller compared with populations of the localities of group II (Table 2).

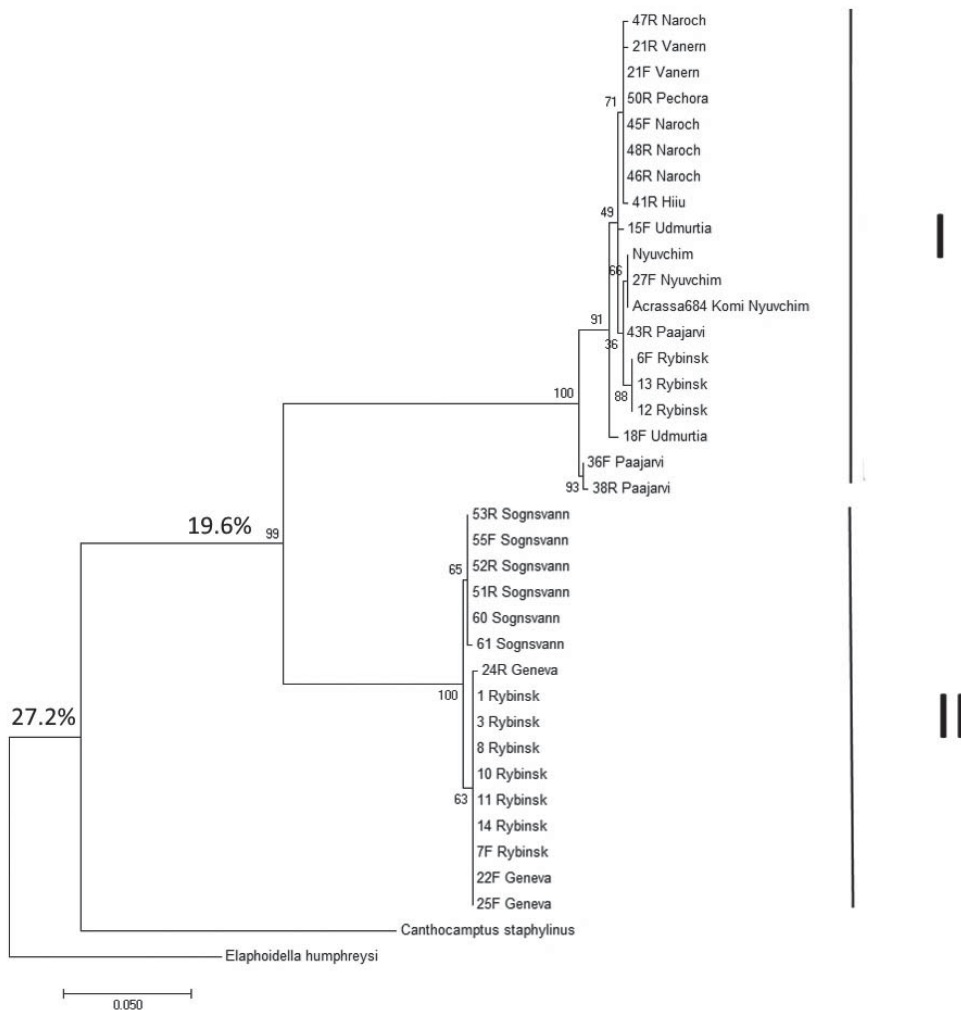


Fig. 4. Phylogenetic tree of the relationship between populations of *A. crassa* based on data from mitochondrial cytochrome oxidase subunit I (COI) region by the maximum likelihood method. Numbers beside nodes indicate bootstrap values and genetic distances (in %). A sequence of *Elaphoidella humphreysi* Karanovic, 2006 from NCBI was used as outgroup (accession number JN039173). I, II — clades.

Рис. 4. Филогенетическое дерево, построенное методом ML на основе сравнения нуклеотидных последовательностей участков гена COI гарпактикоид вида *Attheyella crassa*. В узлах указаны значения бутстреп-поддержки и генетические дистанции (%). В качестве внешней группы использована последовательность гена COI гарпактикоиды *Elaphoidella humphreysi* Karanovic, 2006 (JN039173). I, II — клады.

The genetic divergence (19.6%) between clades (Fig. 4), when compared to groups on the PCA plot (Fig. 3), confirmed heterogeneity among the populations. Similar genetic variability revealing the existence of several cryptic forms has been found in other studies of popula-

tion structure based on molecular-genetic analysis of harpacticoids. For instance, phylogenetic and phylogeographic structure among populations of the marine harpacticoid *Tigriopus californicus* (Baker, 1912) showed divergences of mtDNA that often exceeded 20% (Burton *et*



*al.*, 2007; Willet, Ladner, 2009). In another marine harpacticoid *Nannopus palustris* Brady, 1880 even higher values have been found: the genetic distances of mtDNA reached up to 78% between populations and allowed to separate several cryptic forms (Garlitzka *et al.*, 2012).

Our previous investigation of interpopulational diversity in *C. staphylinus* also showed polymorphism. It was expressed in genetic distances that reached up to 25% between geographically separated populations (Kochanova *et al.*, 2018). High levels of intra- and interspecific divergence seem to be a widespread phenomenon among members of the order Harpacticoida (Schizas *et al.*, 1999; Easton *et al.*, 2010). In freshwater copepod species, the observed molecular genetic divergence can be the result of founder and bottleneck effects accompanied by limited gene flow between populations, even in adjacent habitats (Bucklin *et al.*, 1998).

Therefore we can conclude that in the studied populations of *A. crassa* there are two morphologically and genetically distant forms, which should be considered as different taxonomical units at the subspecies level. The division of clades in the phylogenetic tree (Fig. 4) is identical to the separation of groups in the PCA plot (Fig. 3), except for the population from Rybinsk reservoir, which was present in both clades. We can assume that in this waterbody there are two genetic forms with similar morphology.

We recommend further study of additional material (particularly on populations of *A. crassa* from southern Europe, northern Africa and non-Russian Asia) together with the analysis of nuclear genes, in order to clarify the taxonomy of this species. In this way, the subspecies with predominantly northern distribution in the Palearctic Region could be described.

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