

A new species of the genus *Protracheoniscus* Verhoeff, 1917 (Isopoda: Oniscidea: Agnaridae) from the northwestern Caucasus

Новый вид рода *Protracheoniscus* Verhoeff, 1917 (Isopoda: Oniscidea: Agnaridae) с северо-западного Кавказа

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KEY WORDS: Isopoda, COI, phylogenetic analysis, Oniscidea, Abrau Peninsula, woodlouse, *Protracheoniscus*.

КЛЮЧЕВЫЕ СЛОВА: Isopoda, COI, филогенетический анализ, Oniscidea, полуостров Абрау, мокрица, *Protracheoniscus*.

ABSTRACT. *Protracheoniscus krivolutskyi* sp.n. (Agnaridae) is described from the Utrish State Nature Reserve at the Black Sea coast of Russia. Analysis of COI gene and Scanning Electron Microscopy support the validity of this species. Affinities of the species within the genus are discussed.

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РЕЗЮМЕ. *Protracheoniscus krivolutskyi* sp.n. (Agnaridae) описан из заповедника «Утриш» на черноморском побережье России. Валидность нового вида подтверждена анализом гена COI и сканирующей электронной микроскопией. Обсуждается родство вида в пределах рода.

Introduction

Among the family Agnaridae Schmidt, 2003, the genus *Protracheoniscus* Verhoeff, 1917 is the most species-rich in Europe [Schmalfuss, 2003]. Some species have vast ranges spreading for thousands of kilometers. One of these species, *Protracheoniscus fossuliger* (Verhoeff, 1901) spreads from Spain and southern France to the south of European Russia. Since the original description, a number of similar species were described (*P. babori* Frankenberger, 1938; *P. occidentalis* Vandel, 1939; *P. albanicus* Arcangeli, 1952), which were later synonymized with Verhoeff's species [Karaman, 1974; Schmalfuss, 1983; Taiti, Ferrara, 1996]. At the easternmost end of the species range, *P. fossuliger* was documented in two regions: (i) in the Rostov Region by D.D. Khisametdinova [2007, 2014] and (ii) in the south of the Krasnodar Region by the first author of this paper [Gongalsky, Kuznetsova,

2011; Kuznetsova, Gongalsky, 2012]. Another species, *P. giljarovi* Borutzky, 1957, was described from the steppes located very close to the Rostov Region [Borutzky, 1957]. In his World catalogue of woodlice, H. Schmalfuss [2003] noted that *P. giljarovi* is “probably identical with *P. fossuliger*”. In the present study we are not able to argue about the synonymy of *P. fossuliger* and *P. giljarovi* since fresh material from topotypes is not yet available for molecular analysis. We are preparing a special publication about this issue. However, a thorough study of *Protracheoniscus* from the north-western Caucasus resulted in a conclusion that the ecosystems of Black Sea coast are populated by a species different from both *P. fossuliger* and *P. giljarovi*. This new species is described below.

Materials and Methods

Morphological analysis

Sampling in the Utrish State Nature Reserve was done in 2004–2021 by K.B. Gongalsky and D.M. Kuznetsova and students. Woodlice were collected by hand and fixed in 96% ethanol. Terminology used in the species description is mainly based on Vandel [1960]. Processing and dissections were done by using a Leica MZ8 binocular microscope. Micro preparations of diagnostic body appendages were done in euparal (Carl Roth GmbH). Line drawings were executed with the help of an Olympus BX41 microscope supplied with an Olympus U-DA camera lucida. Some individuals were attached to stubs, coated with gold in a S150A Sputter Coater, and studied under a Tescan Vega TS5130MM scanning electron microscope.

Position of noduli laterales in each pereonite was expressed as the distance to posterior margin (b/c) and the distance to lateral margin (d/c) related to the length of pereonite according to Vandel [1960].

The material is deposited in the collection of the Zoological Museum of Moscow University, Russia (ZMMU), and partly retained in private collection of K. Gongalsky at A.N. Severtsov Institute of Ecology and Evolution of Russian Academy of Sciences, Moscow, Russia, as indicated below.

Table 1. List of specimens, sampling sites and accession numbers of the sequences for COI mt DNA included in this study. References are given for sequences obtained from GenBank.

Таблица 1. Список видов, мест сбора и номеров последовательностей гена COI мтДНК в GenBank, включенных в анализ.

Specimen	Locality	Reference / or year of sampling	
		COI mtDNA	Reference
<i>Protracheoniscus krivolutskyi</i> sp.n.	Russia, Krasnodar Region, Utrish, 1 Topolnaya Valley, broadleaf forest. (44.725° N, 37.490° E). VI.2021, K. Gongalsky leg.		This study
<i>Protracheoniscus fossuliger</i> (Verhoeff, 1901)	Russia, Krasnodar Region, 20 km NE from Temryuk. 100 m from the Sea of Azov. (45.398° N, 37.559° E). 23.IX.2020. D. Korobushkin leg.		This study
<i>Protracheoniscus kryzhanovskii</i> Borutzky, 1957	Russia, Republic of Kalmykia, 1 km W of Tsagan Nur, bank of lake Sarpa (47.362° N, 45.201° E), 27.IV.2017 K. Gongalsky leg.	MH400727	Gongalsky <i>et al.</i> , 2018
<i>Protracheoniscus major</i> (Dollfus, 1903)	Russia, Republic of Kalmykia, 1 km N of Bolshoy Tsaryn (47.904° N, 45.393° E) 29.IV.2017, K. Gongalsky leg.	MH400726	Gongalsky <i>et al.</i> , 2018
<i>Protracheoniscus nogaicus</i> Demianowicz, 1932	Russia, Republic of Kalmykia, 1 km N of Bolshoy Tsaryn (47.904° N, 45.393° E) 29.IV.2017, K. Gongalsky leg.	MH400724	Gongalsky <i>et al.</i> , 2018
<i>Protracheoniscus pokarzhevskii</i> Gongalsky et Turbanov, 2018	Russia, Republic of Kalmykia, 1 km N of Bolshoy Tsaryn, (47.904° N, 45.393° E) 29.IV.2017, K. Gongalsky leg.	MG696253, MH400723	Gongalsky <i>et al.</i> , 2018
<i>Protracheoniscus politus</i> (C. Koch, 1841)	Hungary, Budapest, Janos Hegy Mt. (47.516° N, 18.960° E) 29.VIII.2017, K. Gongalsky leg.	MG696252	Gongalsky <i>et al.</i> , 2018
<i>Desertoniscus zaitsevi</i> Gongalsky, 2017	Russia, Republic of Kalmykia, 1 km N of Bolshoy Tsaryn (47.904° N, 45.393° E) 29.IV.2017, K. Gongalsky leg.	MH400725	Gongalsky <i>et al.</i> , 2018
<i>Burmoniscus kathmandius</i> (Schmalfuss, 1983)	Nepal	LC075192	Karasawa, 2016
<i>Ligia baudiniana</i> Milne Edwards, 1840	Colombia	KF555872	Santamaria <i>et al.</i> , 2014

Phylogenetic analysis

To confirm the difference between *P. fossuliger* and the new species, a phylogenetic analysis was undertaken. For molecular genetic analysis, 2 specimens of each species were taken (Table 1). In phylogenetic construction, a number of species from the GenBank (Table 1) were used as outgroup taxa. To isolate total DNA, pereopods 3 to 5 were used from individuals fixed alive in 96% ethanol. Total cellular DNA was isolated using the Diatom DNA Prep 100 kit (Isogen, Russia). For the analysis of genetic variability, fragments of the mtDNA COI locus were used. mtDNA was amplified with primers HCO2198/LCO1490 [Folmer *et al.*, 1994]. The polymerase chain reaction was carried out on a Bio-Rad T 100 thermocycler (Bio-Rad, USA) in a specially selected temperature regime: the initial denaturation of 95° C was 5 min; annealing of 93° C for 35 seconds, 45° C for 40 seconds, 72° C for 40 seconds (35 cycles), the final elongation of 72° C lasted for 7 minutes. For the PCR, a set of reagents for the amplification of “5x Mas Mix-2025” manufactured by Dialat Ltd (Moscow, Russia) was used. The 10 µl reaction mixture contained 2 µl total DNA, 2 µl mix and 1 µl of each primer. The amplification products were separated by electrophoresis in 1.5% agarose gel in 1x TBE and visualized

with ethidium bromide. The DNA sequence was determined with a forward primer on an ABI 3500 genetic analyzer (Applied Biosystems, USA).

The resulting sequences were aligned using BioEdit v. 5.0.9 [Hall, 1999]. The obtained mtDNA COI gene fragments were used in the phylogenetic analysis. Genetic distances (p-distance) between species were calculated using the MEGA 6.0 [Tamura *et al.*, 2013] software package (Table 2). Genetic relationships were reconstructed based on the obtained sequences and GenBank (NCBI) data on the basis of the GTR+G+I (+G = 0.4875) molecular evolution model [Nei, Kumar, 2000] chosen by the Maximum Likelihood (ML) method, with the calculation of bootstrap support for branch nodes (1000 repetitions) [Felsenstein, 1985] in the software package MEGA 6.0. The construction of a phylogenetic tree based on the Bayes principle and the evaluation of branching supports (10,000 repetitions) were carried out in the MrBayes program [Huelsenbeck, Ronquist, 2001; Chevenet *et al.*, 2006]. The loci of COI mtDNA of *Burmoniscus kathmandius* [Karasawa, 2016] and *Ligia baudiniana* [Santamaria *et al.*, 2014] were used as outgroup taxa.

Table 2. Estimates of pairwise sequence divergence (uncorrected p-distances) of gene COI mtDNA haplotypes.
Таблица 2. Оценки попарных расстояний между гаплотипами на основании гена COI мтДНК.

GenBank number/ Species	1	2	3	4	5	6	7	8	9	10
<i>P. krivolutskyi</i>										
<i>P. fossuliger</i>	0,17									
MG696252 <i>P. politus</i>	0,17	0,17								
MH400723 <i>P. pokarzhevskii</i>	0,18	0,17	0,19							
MG696253 <i>P. pokarzhevskii</i>	0,18	0,17	0,19	0,002						
MH400724 <i>P. nogaicus</i>	0,21	0,19	0,20	0,21	0,21					
MH400727 <i>P. kryszanovskii</i>	0,18	0,17	0,19	0,19	0,19	0,20				
MH400726 <i>P. major</i>	0,21	0,18	0,22	0,20	0,20	0,18	0,15			
MH400725 <i>D. zaitsevi</i>	0,19	0,18	0,20	0,18	0,18	0,19	0,16	0,16		
LC075192 <i>B. kathmandius</i>	0,23	0,23	0,23	0,23	0,23	0,25	0,23	0,26	0,25	
KF555872 <i>L. baudiniana</i>	0,23	0,22	0,24	0,24	0,24	0,26	0,21	0,24	0,23	0,25

Results

TAXONOMY

Class Malacostraca Latreille, 1802

Order Isopoda Latreille, 1817

Family Agnaridae Schmidt, 2003

Genus *Protracheoniscus* Verhoeff, 1917

Protracheoniscus krivolutskyi Gongalsky **sp.n.**

Figs 1–7.

Protracheoniscus fossuliger: Gongalsky, Kuznetsova, 2011; Kuznetsova, Gongalsky, 2012 (pro parte); Korobushkin, 2014.

Material. Holotype: ♂ (ZMMU, Mc-1460), Russia, Krasnodar Region, Utrish, 1 Topolnaya Valley, broadleaf forest. (44.725200° N, 37.489844° E). VI.2021, K. Gongalsky leg. Paratypes: 2 ♂♂, 2 ♀♀ (ZMMU, Mc-1461), 3 ♂♂, 4 ♀♀ (private collection of K. Gongalsky), Russia, Krasnodar Region, Utrish, 2 Topolnaya Valley, broadleaf forest. (44.729834° N, 37.486861° E). VI.2013, K. Gongalsky leg.; 10 ♂♂, 10 ♀♀ (ZMMU, Mc-1462), same as the holotype.

DIAGNOSIS. The species of *Protracheoniscus* is characterized by the antennal flagellum with the articles of a ratio 1:2; noduli laterales are located anteriorly of the middle line of pereonite 1.

TYPE LOCALITY. The Utrish State Nature Reserve (44.729834° N, 37.486861° E), Krasnodar Region, Russia.

DESCRIPTION. *Somatic characters*. Maximum body length: male 6 mm; female 10 mm. Body colour dark brown (Fig. 1). First two articles of antennae paler than other articles. Uropods (Fig. 2C) coloured as dorsal surface of body. Dorsal surface of tergites smooth. Posterior edges of coxal plates of pereonites straight (Fig. 2A). Dorsal surface covered with scattered sharp triangular scale setae (Fig. 6). Noduli laterales on pereonites located close to coxal plates edge (Fig. 2A). Noduli laterales located anteriorly of middle line of pereonite 1 (Fig. 2A). Cephalic lateral lobes poorly developed, much smaller than eyes, median lobe rounded (Fig. 3B). Eyes with 13 ommatidia each (Fig. 2). Telson with triangular distal part and slightly concave margins (Fig. 3C).

Appendages. Antennula of three articles (Fig. 2D): second article slightly shorter than first; third article almost as long as first and narrow, bearing tuft of aesthetascs at apex. Antenna medium long, reaching pereonite 3 (Fig. 2E); flagellum of 2 articles, proximal one shorter than distal one, ratio of flagellar articles 1:2; flagellum as long as 5th article (Fig. 2E).

Left mandible with 2+1 penicils and dichotomized molar penicil; right mandible with 1+1 penicils and dichotomized molar penicil (Fig. 3A, B). Maxillula (Fig. 3C): medial corner of inner endite with 2 strong penicils. Apical edge of outer endite bearing 8 teeth divided into two groups: 4 medial spines slenderer with split tips and 4 lateral spines stouter with simple tips. Tip of endite covered with dense brush of setae. Maxilla with bilobate edge, medial half of apical edge of inner lobe



Fig. 1. Alive specimen of *Protracheoniscus krivolutskyi* sp.n. (female), from the Urtish State Nature Reserve, NE Caucasus, Russia, kept in culture.

Рис. 1. Общий вид *Protracheoniscus krivolutskyi* sp.n. (самка), содержащегося в культуре, из Государственного природного заповедника «Утриш».

with dense brush of short hairs (Fig. 3D). Maxilliped with outer corner of endite with 2 acute tips and large seta near the inner corner (Fig. 3E).

Pereopods of typical shape of genus (Fig. 4A–C). Pleopods: all exopods on lateral margin are with monospiracular lungs.

Male: Carpopodite 7 in proximal part with dorsal crest. Genital papilla slightly extended at tip (Fig. 5H). Exopod of pleopod 1 (Fig. 5A) with slightly concave tip and 1–2 setae at apex. Endopod of pleopod 1 with sharp triangular tip inclined laterally bearing setae (Fig. 5B). Pleopod 2: exopod triangular bearing 4 setae (Fig. 5C); endopod much longer than exopod, narrow, with parallel sides (Fig. 5D). Pleopods 3–5: exopods (Fig. 4E–G) triangular, slightly decreasing in size from 3 to 5.

REMARKS. This species is the closest to *P. fossuliger* (Verhoeff, 1901) due to a similar shape of body and a potential overlap in the ranges. However, these two differ in the following: (i) tip of endopod of the male pleopod 1 is not inclined laterally in *P. krivolutskyi*; (ii) carpus of pereopod 7 in *P. krivolutskyi* has a distinct bump which is much smaller in *P. fossuliger* (Fig. 6); (iii) noduli laterales are located anteriorly of the middle line of pereonite 1 in *P. krivolutskyi*, and posteriorly of the middle line in *P. fossuliger*; (iv) proximal segment of the flagellum is much shorter than the distal one (ratio 1:2), while in *P. fossuliger* it is only slightly shorter (ratio 1:1.25).

PHYLOGENETIC ANALYSIS. As a result, 4 sequences of the mtDNA COI locus fragment, 565 bp long, two of each species, were obtained. The two species, *P. fossuliger* (2 specimens) and *P. krivolutskyi* (2 specimens), belong to different haplotypes. As much as 94 variable sites were identified between the COI sequences of the two species, the genetic distance (p-distance)

was 0.166 (Table 2). Reconstruction of phylogenetic relationships, including related species showed common features in the topology of phylogenetic trees. Species of the genus *Protracheoniscus* were part of the same clade together with *Desertioniscus zaitsevi*. A high level of support was found only for branch nodes of individuals of the same species in cases where a species was represented by several specimens. This pattern is obvious because there were very few or no differences between individuals of each of these species. Regardless of the method of reconstructing phylogenetic trees, the supports for the formation of most branches were low (less than 0.5), the values of the remaining supports do not exceed 0.8 (Figs 8, 9). It is impossible to describe the nature of phylogenetic relationships at this level of reliability. The high variability of the selected marker and the small number of representatives of the genus *Protracheoniscus* (7 species out of more than 60) can be the main reason for the observed pattern. Differences in the relative position the branches of *P. fossuliger* and *P. krivolutskyi* on phylogenetic trees built by the Maximum Likelihood method (paraphyletic position) (Fig. 8) and based on the Bayesian principle (monophyletic position) (Fig. 9) are associated with this. The significant genetic distance between *P. fossuliger* and *P. krivolutskyi* revealed in this study, is comparable with the genetic distances between other *Protracheoniscus* species (Table 2). This indicates the genetic isolation of *P. fossuliger* and *P. krivolutskyi* despite the above difficulties in the phylogenetic structure of the genus and quite similar morphology.

DISTRIBUTION. The species has been found in the Urtish State Nature Reserve and its surroundings at the Black Sea coast of Abrau Peninsula so far. It occupies various habitats, from broadleaf forests (*Carpinus betulus*, *Fagus orientalis*)

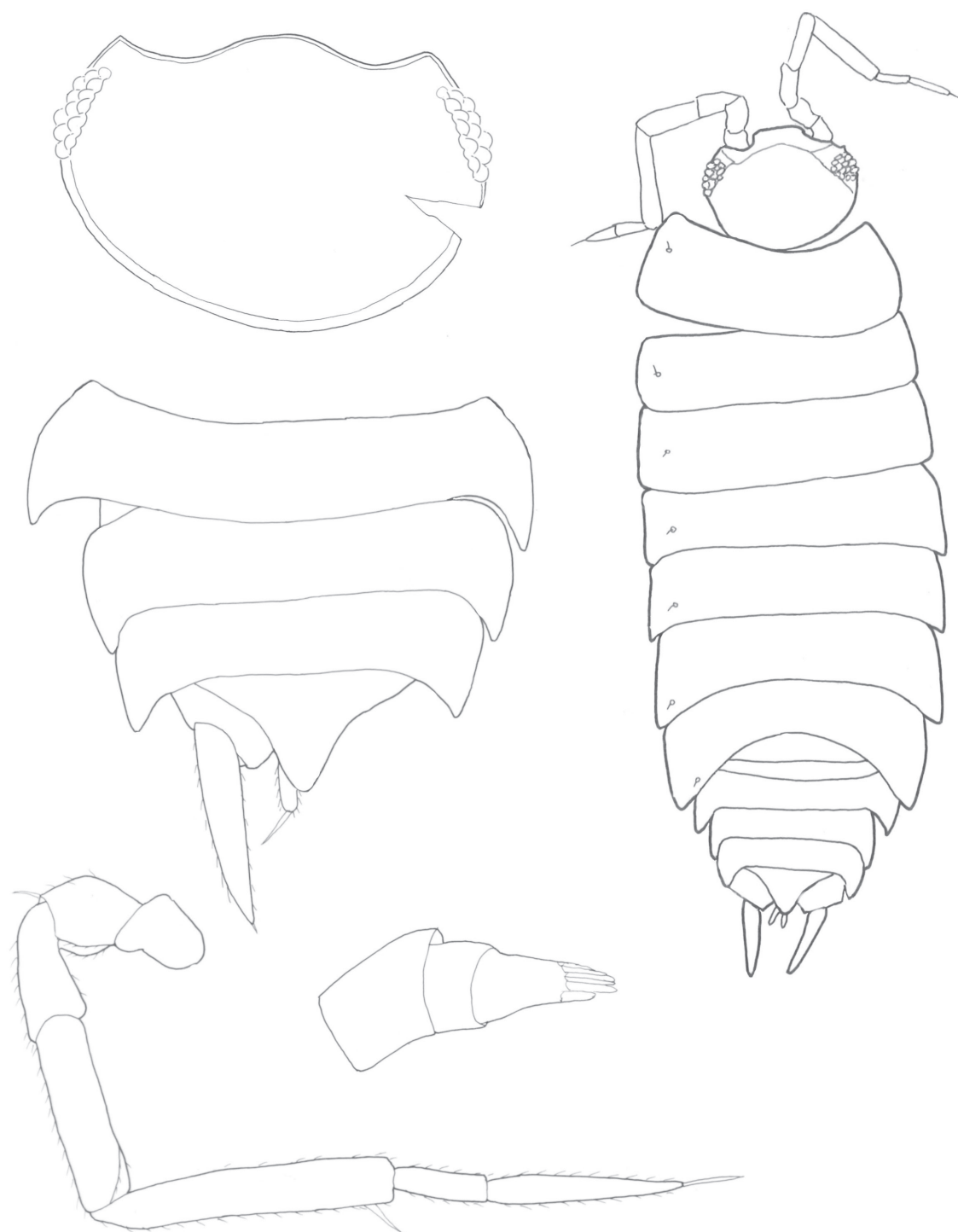


Fig. 2. *Protracheoniscus krivolutskyi* sp.n. (male): A — body outline; B — head; C — pleonites 3–5, telson and left uropod; D — antennula; E — antenna.

Рис. 2. *Protracheoniscus krivolutskyi* sp.n. (самец): А — тело; В — голова; С — плеониты 3–5, тельсон и левый уropод; D — антеннула; E — антенна.

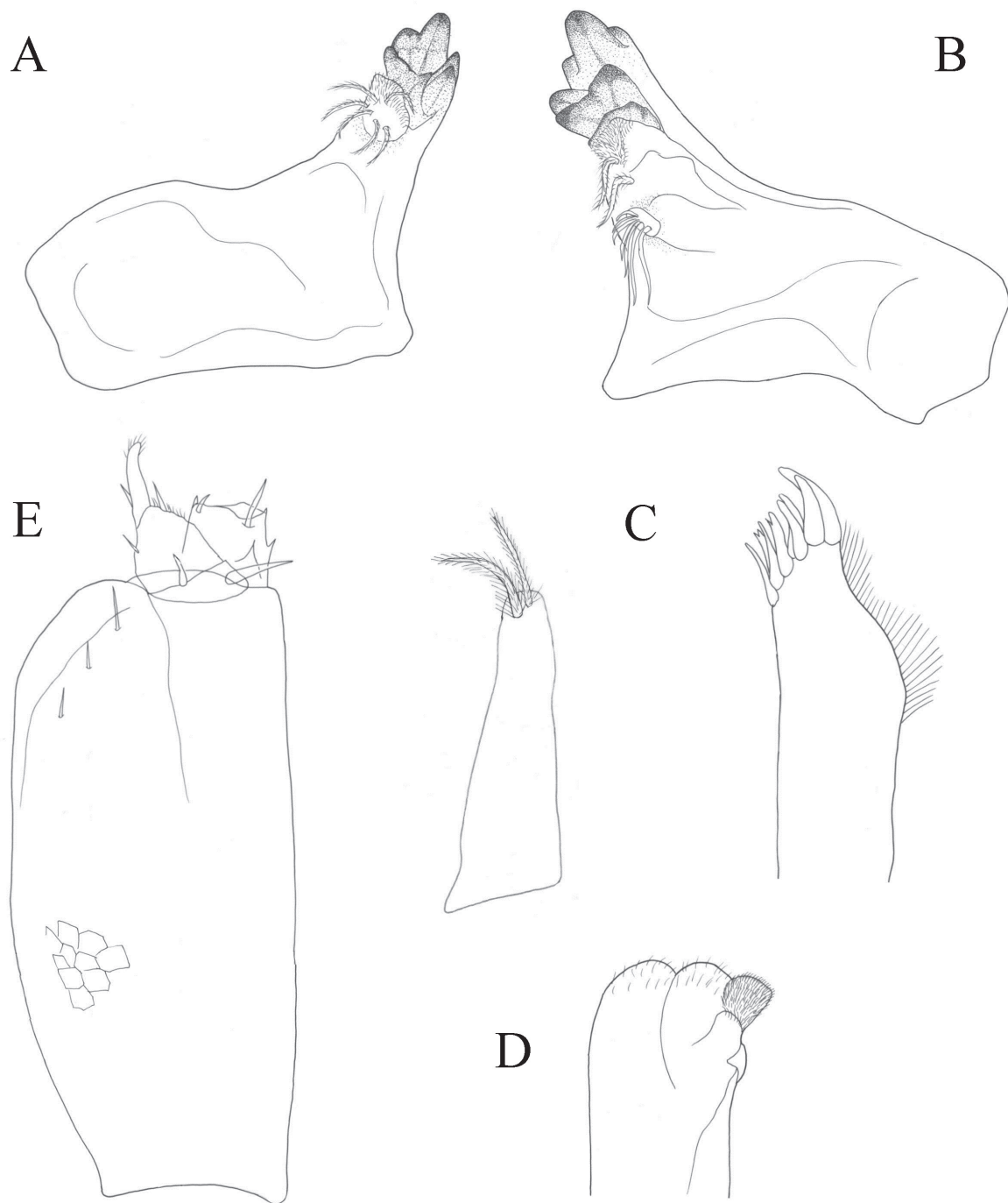


Fig. 3. *Protracheoniscus krivolutskyi* sp.n. (male): A — left mandible; B — right mandible; C — maxillula; D — maxilla; E — maxilliped.

Рис. 3. *Protracheoniscus krivolutskyi* sp.n. (самец): А — левая мандибула; В — правая мандибула; С — максиллула; D — максилла; Е — максиллипод.

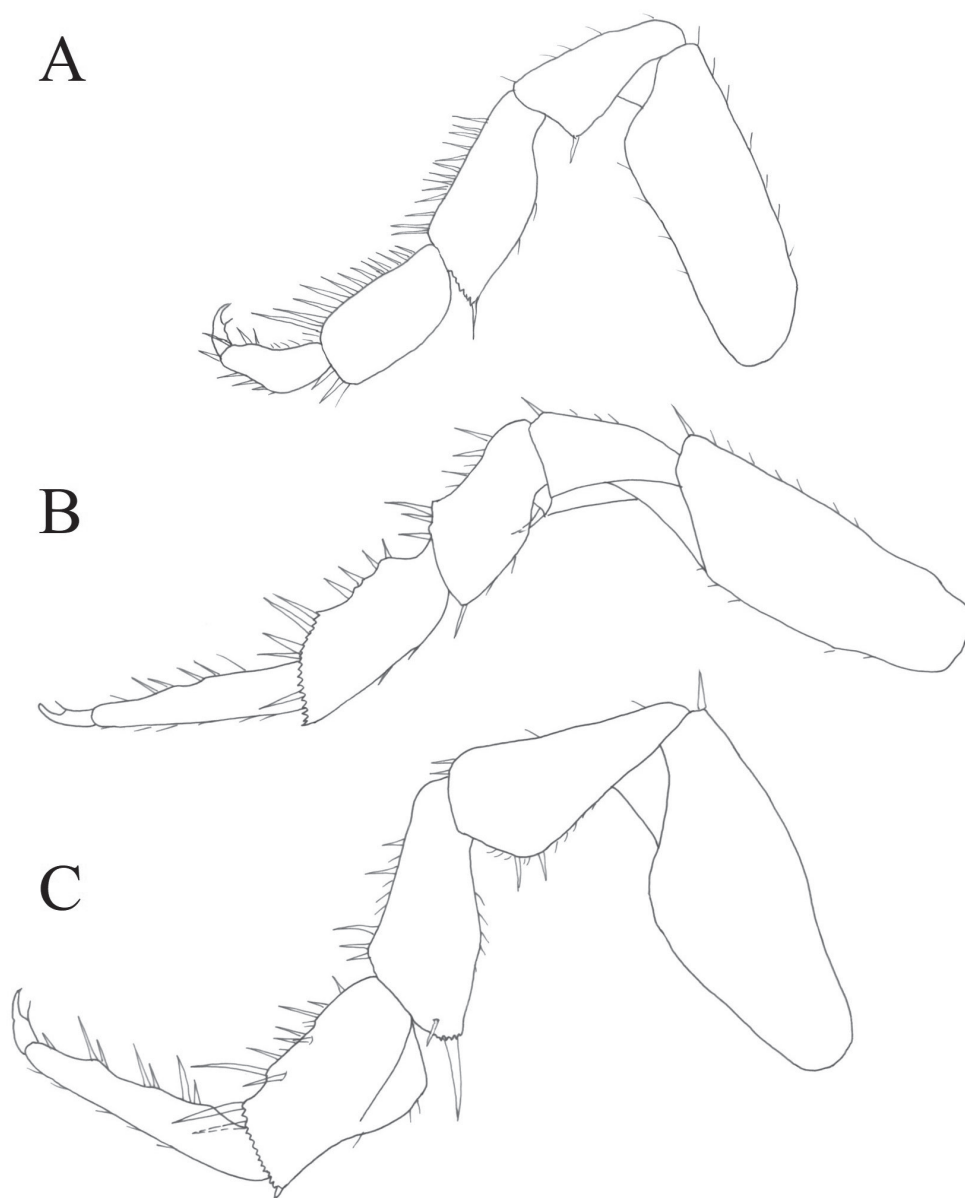


Fig. 4. *Protracheoniscus krivolutskyi* sp.n. (male): A — pereopod 1; B — pereopod 6; C — pereopod 7.

Рис. 4. *Protracheoniscus krivolutskyi* sp.n. (самец): A — переопод 1; B — переопод 6; C — переопод 7.

to semiarid forests (*Quercus petraea*, *Carpinus orientalis*) and shrublands (*Juniperus* spp., *Paliurus spina-christi*). This is one of the most common species among woodlice in the area [Gongalsky, Kuznetsova, 2011; Korobushkin, 2014] repeatedly reported before as *P. fossuliger*.

ETYMOLOGY. The species name *krivolutskyi* originates from Prof. Dr. Dmitri A. Krivolutsky (1939–2004), a prominent Russian soil zoologist who encouraged the first author to study soil biology.

Compliance with ethical standards

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

Ethical approval: No ethical issues were raised during our research.

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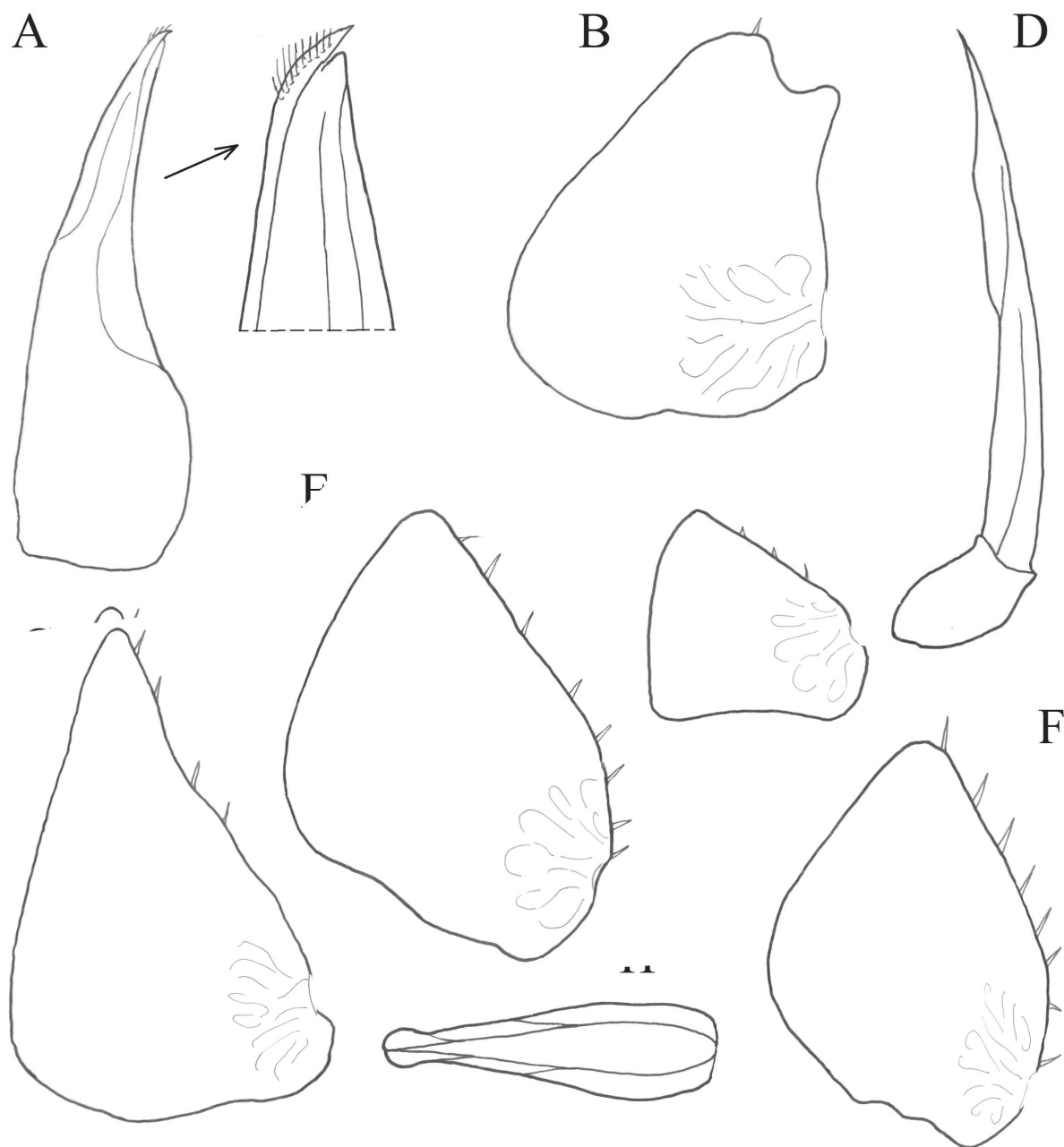


Fig. 5. *Protracheoniscus krivolutskiy* sp.n. (male): A — exopod of pleopod 1; B — endopod of pleopod 1; C — exopod of pleopod 2; D — endopod of pleopod 2; E — exopod of pleopod 3; F — exopod of pleopod 4; G — exopod of pleopod 5; H — genital papilla.

Рис. 5. *Protracheoniscus krivolutskiy* sp.n. (самец): А — экзопод плеопода 1; В — эндопод плеопода 1; С — экзопод плеопода 2; D — эндопод плеопода 2; E — экзопод плеопода 3; F — экзопод плеопода 4; G — экзопод плеопода 5; H — генитальная папилла.

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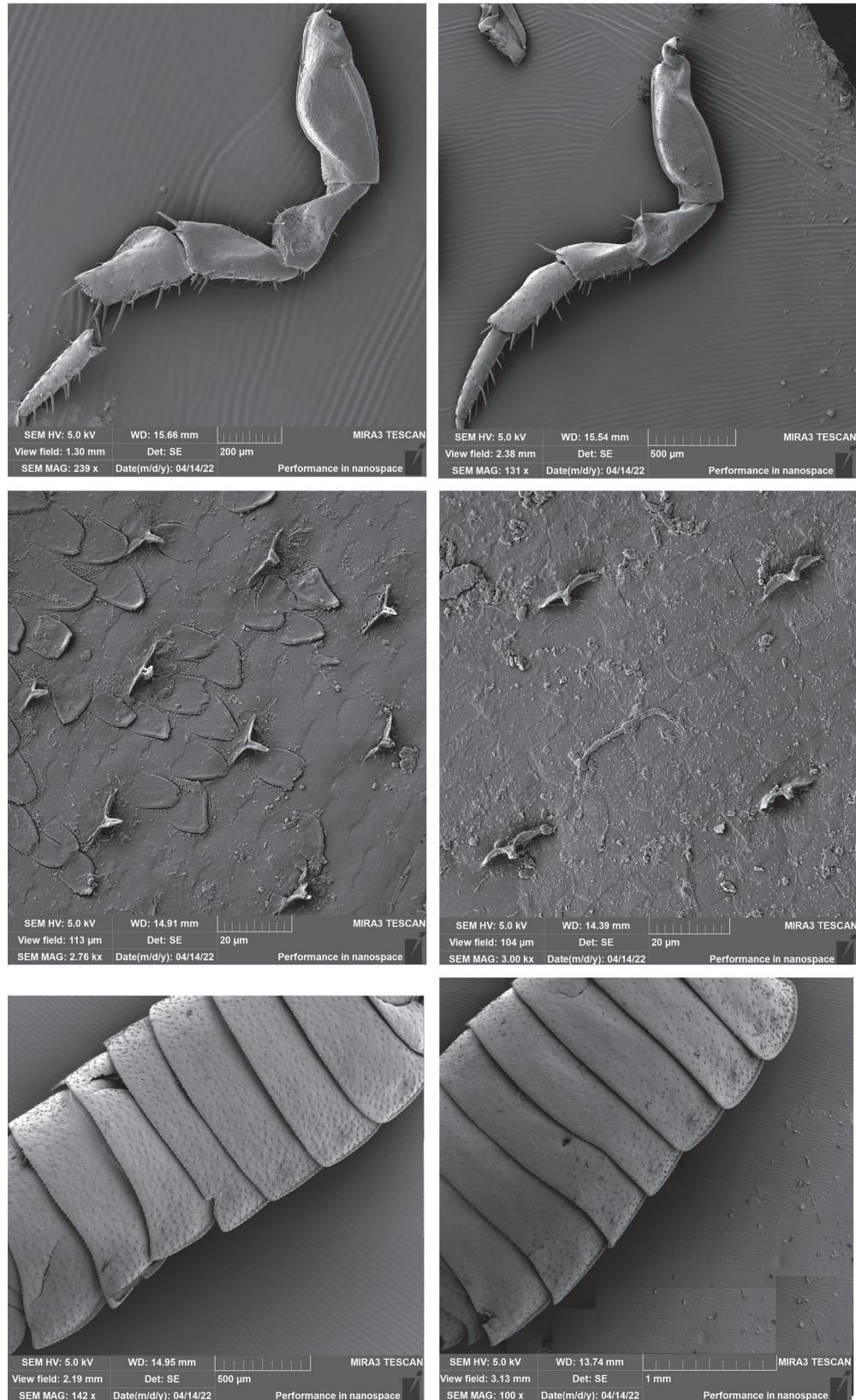


Fig. 6. Morphological characters of *Protracheoniscus krivolutskyi* sp.n. (left) and *P. fossuliger* (right): pereopod 7 (above), dorsal setae on pereonite 2 (middle) and *noduli laterales* of pereonites (below).

Рис. 6. Морфологические признаки *Protracheoniscus krivolutskyi* sp.n. (слева) и *P. fossuliger* (справа): переопод 7 (сверху), дорсальные сеты (посередине) и *noduli laterales* переонитов (снизу).

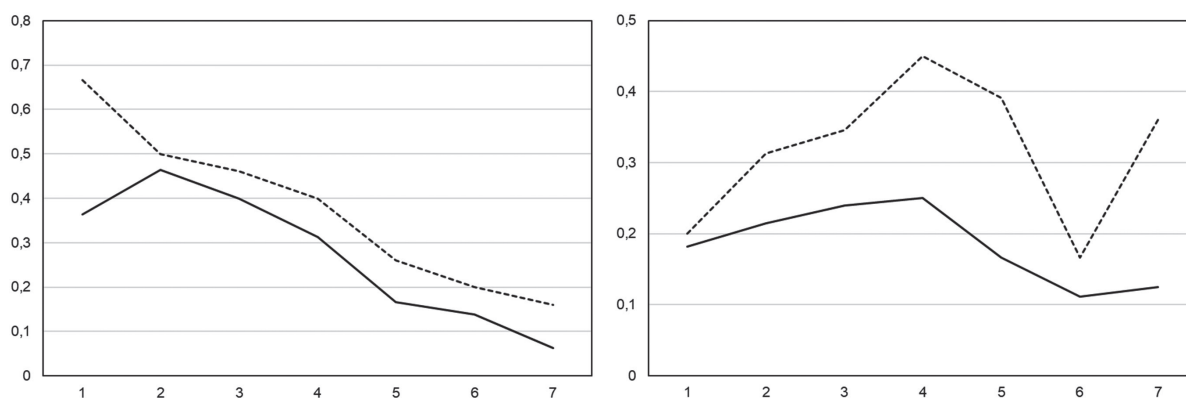


Fig. 7. Position of noduli laterales in *Protracheoniscus krivolutskyi* sp.n. (dashed line) and *P. fossuliger* (solid line): b/c (left) and d/c (right).

Рис. 7. Расположение noduli laterales у *Protracheoniscus krivolutskyi* sp.n. (пунктирная линия) и *P. fossuliger* (сплошная линия): b/c (левый график) и d/c (правый график).

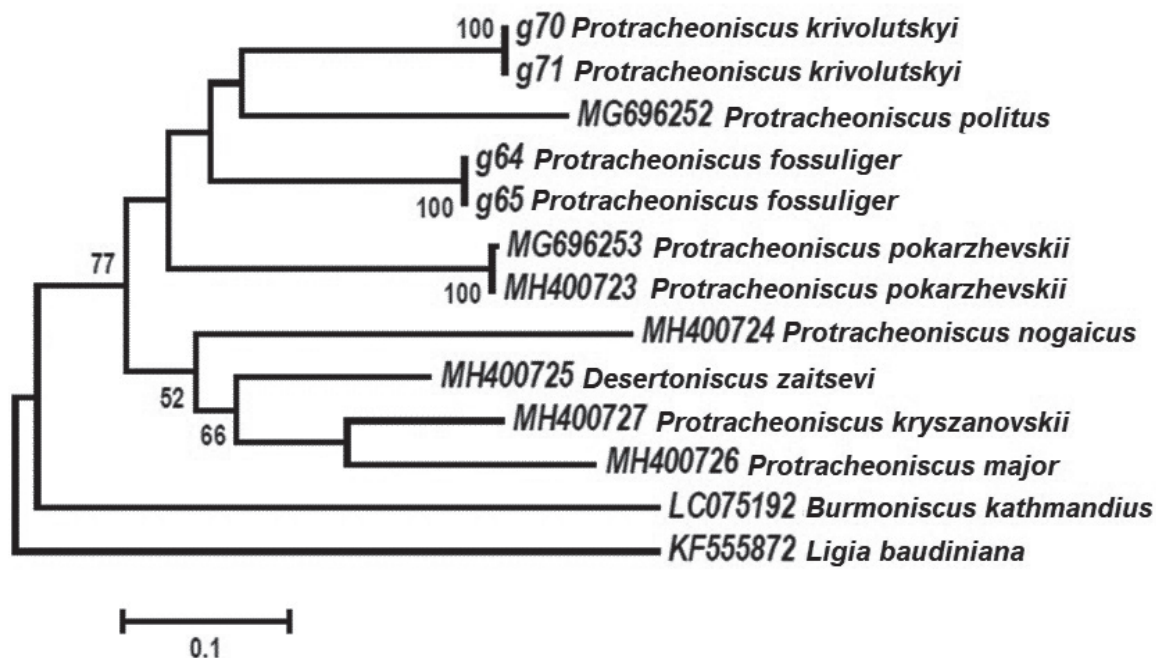


Fig. 8. Phylogeny of genus *Protracheoniscus* Verhoeff, 1917 with outgroup taxa based on the analysis of the mtDNA COI gene by the Maximum Likelihood (ML) method based on the General Time Reversible model with the calculation of bootstrap support of branch sites (1000 replications).

Рис. 8. Филогения рода *Protracheoniscus* Verhoeff, 1917 с таксонами внешней группы на основе анализа гена COI мтДНК методом максимального правдоподобия (ML) на основе модели General Time Reversible с расчетом поддержки бутстрапа точек ветвления (1000 повторов).

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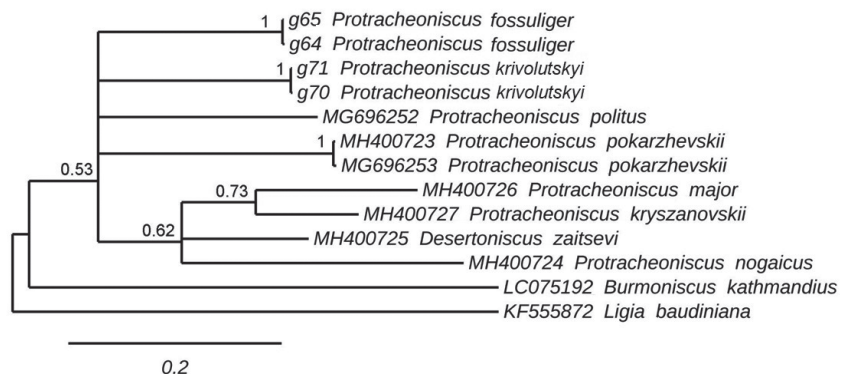


Fig. 9. Bayesian consensus tree of genus *Protracheoniscus* Verhoeff, 1917 with outgroup taxa based on the analysis of the mtDNA COI gene calculation of support of branch sites (10000 replications).

Рис. 9. Байесовское консенсусное дерево рода *Protracheoniscus* Verhoeff, 1917 с таксонами внешней группы на основе анализа гена COI мтДНК с расчетом поддержки сайтов ветвления (10000 повторов).

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