

***Pseudocalanus* species (Calanoida: Clausocalanidae) in the Baltic and Black Seas: morphological and genetic differences**

Виды рода *Pseudocalanus* (Calanoida: Clausocalanidae) в Балтийском и Черном морях: морфология и генетические отличия

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КЛЮЧЕВЫЕ СЛОВА: *Pseudocalanus acuspes*, *Pseudocalanus elongatus*, морфологические и молекулярно-генетические исследования, сравнение видов, Юго-Восточная Балтика, Черное море.

ABSTRACT. A comparison of morphological and genetic variation between *Pseudocalanus acuspes* (Giesbrecht, 1881) from the Baltic and *Pseudocalanus elongatus* (Brady, 1865) from the Black Seas was conducted to find specific visible characteristics that could be used for their taxonomic identification in routine zooplankton studies. We made drawings of 72 females of *Pseudocalanus*, randomly selected from fixed zooplankton samples collected in waters of the southeastern Baltic Sea and the northwestern Black Sea, to measure their bodies in detail. We revealed that *P. acuspes* is distinguished by a rounder anterior cephalosome in a lateral view, shorter antennae (A1L), and a shorter prosome (PrL) as well as a larger genital segment (GL) of urosome (UrL). Several statistically significant differences were found in morphometric ratios between the body measurements of *P. acuspes* from the Baltic Sea and *P. elongatus* from the Black Sea. Specifically, for *P. acuspes*, the ratios are PrL/UrL < 2.2, A1L/L (%) < 87; GL/PrL > 16.8. For *P. elongatus*, these ratios are PrL/UrL > 2.2; A1L/L (%) > 92; GL/PrL < 16.5. The species identity of *Pseudocalanus* was confirmed using partial COI mtDNA gene analysis. Both regional populations form well-supported subgroups within species clusters on the COI phylogenetic tree. Individuals

of *P. elongatus* from the Black Sea differ from all other haplotypes of this species, and no other geographically distinct sub-groups have been identified within the clade of *P. elongatus*. The *P. acuspes* cluster also consists of two well-supported subclusters, with Baltic Sea samples grouped with those from Balsfjorden (Norwegian Sea). Thus, the studied populations of both *Pseudocalanus* species are genetically distinct from those living in other parts of their geographical range. Therefore, morphological differences noted above need to be confirmed through additional investigations of these species from different parts of the range.

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РЕЗЮМЕ. Сравнение морфологических и генетических различий между *Pseudocalanus acuspes* (Giesbrecht, 1881) из Балтийского моря и *Pseudocalanus elongatus* (Brady, 1865) из Черного моря

было проведено с целью выявления специфических признаков, которые можно было бы использовать для их таксономической идентификации при рутинных исследованиях зоопланктона. Для морфологического и морфометрического анализа были выполнены рисунки 72 самок *Pseudocalanus*, отобранных случайным образом из проб зоопланктона, собранных в водах юго-восточной части Балтийского моря и северо-западной части Черного моря. Было обнаружено, что *P. acuspes* отличается более округлой передней частью цефалосомы, более короткими антеннулами (A1L) и просомой (PrL), а также более крупным генитальным сегментом (GL) уросомы (UrL). Было обнаружено несколько статистически значимых различий в морфометрических соотношениях между размерами отдельных частей тела *P. acuspes* из Балтийского моря и *P. elongatus* из Черного моря. В частности, для *P. acuspes* эти соотношения составляют $PrL/UrL < 2,2$, $A1L/L (\%) < 87$; $GL/PrL > 16,8$, а у *P. elongatus* — $PrL/UrL > 2,2$; $A1L/L (\%) > 92$; $GL/PrL < 16,5$. Видовая принадлежность *Pseudocalanus* была подтверждена генетически на основе анализа фрагментов гена COI мтДНК. Обе региональные популяции с высокой поддержкой образуют отдельные подгруппы в пределах видовых кластеров на филогенетическом древе COI. Особи *P. elongatus* из Черного моря отличаются от всех других гаплотипов этого вида, и никаких других географически обособленных подгрупп в пределах клады *P. elongatus* не выявлено. Кластер *P. acuspes* также состоит из двух подгрупп, где образцы из Балтийского моря группируются с образцами из Балльсфьорда (Норвежское море). Таким образом, изученные популяции обоих видов *Pseudocalanus* генетически отличаются от популяций, обитающих в других частях их географического ареала. Отмеченные выше морфологические различия должны быть подтверждены дополнительными исследованиями этих видов из разных частей их ареала.

1. Introduction

Planktonic copepods *Pseudocalanus* Boeck, 1873 are widely distributed in the Northern Hemisphere, often dominating zooplankton in the Boreal Arctic [Corkett, McLaren, 1979; Questel *et al.*, 2016; Aarbakke *et al.*, 2017]. They play an important role in marine food webs as a major prey for fish larvae in Arctic and temperate waters of the North Atlantic and Pacific Oceans [Mollmann *et al.*, 2003; Aarbakke, 2013; Ershova, 2020; Aleksandrov, Semenova, 2023]. The genus is known for its subtle morphological differences between species and at the same time demonstrates a wide range of biological and ecological diversity. Several species often co-exist in the same area, and their identification, especially for immature specimens, is usually only possible through molecular genetic methods. However, these techniques may not always be available for the routine processing of zooplankton samples [Frost, 1989; Holmborn *et al.*, 2011; Ershova, 2020]. Therefore, these copepods are often recorded as *Pseudocalanus* sp. or spp. [McGillicuddy *et*

al., 1998; Isla *et al.*, 2008; Schulz *et al.*, 2012; Musialik-Koszarowska *et al.*, 2019].

Throughout the study of species within the genus, misidentification has occurred due to the complicated nomenclatural history and synonymy, as well as the unclear status of certain species [Brodsky *et al.*, 1983; Frost, 1989; Razouls *et al.*, 2024]. Seven species were included in the genus *Pseudocalanus* by Frost [1989], who conducted a comprehensive taxonomic analysis of the genus, revised it, and described three new species. This taxonomic analysis confirmed the extremely weak interspecific divergence of morphological and morphometric features of *Pseudocalanus*. Molecular genetic data obtained from six of the seven species of *Pseudocalanus* supported previous taxonomic analyses [Seigny *et al.*, 1989; Bucklin *et al.*, 2003]. *Pseudocalanus acuspes* (Giesbrecht, 1881) is distributed in the Northeast Atlantic, “occurs from the Baltic and North Sea, all along the Scandinavian coast and throughout the continental shelves in the Arctic region” [Aarbakke *et al.*, 2017: 52]. *Pseudocalanus elongatus* (Brady, 1865) is a boreal species with the most southern records from the Mediterranean and Black Seas and is registered to the North in the English Channel, the North Sea, Icelandic waters and along the coast of Norway [Frost, 1989; Aarbakke *et al.*, 2014, 2017] up to about 70 °N (Ramfjord) [Ershova, 2020]. In the North Sea, bordering the Baltic, both species co-occur. Many researchers identified *Pseudocalanus* specimens from the Baltic Sea as *P. elongatus* [Apstein, 1905; Pesta, 1928; Niermann *et al.*, 1998; Mollmann *et al.*, 2003; Aleksandrov *et al.*, 2009; Polunina *et al.*, 2021; Aleksandrov, Semenova, 2023], or sometimes as *P. minutus elongatus* [Kankaala, Johansson, 1986; Dzierzbicka-Głowacka *et al.*, 2006; Schuka, 2012; Dzierzbicka-Głowacka *et al.*, 2013]. The reason for the uncertainty in identifying *P. acuspes* and *P. elongatus* in the Baltic Sea is their weak morphological differences and a complicated nomenclatural history, as we noted above for this genus.

Nowadays, *P. acuspes* has been confirmed to be the only ecologically significant species of *Pseudocalanus* in the Baltic Sea using various molecular genetic methods [Holmborn *et al.*, 2011; Grabbert *et al.*, 2010]. However, *P. elongatus* can also be found in single records in this region. Periodic influxes of cold saline water from the North Sea, where both species co-exist, can bring zooplankton to the southern part of the Baltic Sea [Grabbert *et al.*, 2010; Holmborn *et al.*, 2011; Aleksandrov, Semenova, 2023].

In the Black Sea, *P. elongatus* was first recorded in 1895 [Karavaev, 1895]. It is considered the only *Pseudocalanus* species inhabiting the Black Sea and is one of the most abundant representatives of cold-water copepods [Greze, 1979; Gubanov *et al.*, 2014]. Its species status has been confirmed through genetic analysis [Unal *et al.*, 2006].

The cytochrome-c oxidase subunit-I (COI) fragment is commonly used to DNA barcode zooplankton, including copepods [Bucklin *et al.*, 1998, 2003]. For most species, the variation of mtCOI within a species is significantly less than the variation between species, making this gene a valuable tool for molecular taxonomy, including

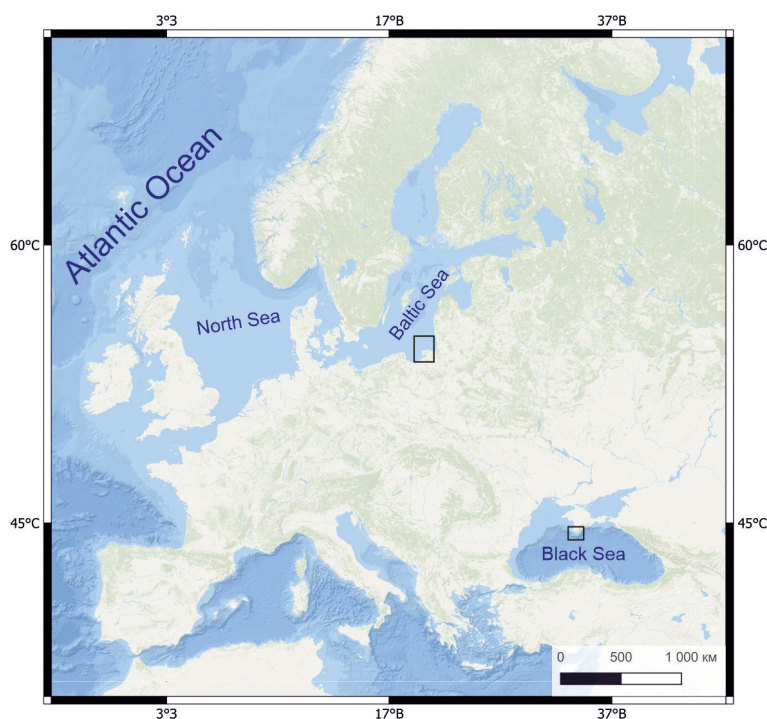


Fig. 1. Sampling regions in the Baltic and Black Seas.
Рис. 1. Районы отбора проб в Балтийском и Черном морях.

distinguishing between different *Pseudocalanus* species [Bucklin *et al.*, 2003; Aarbakke *et al.*, 2011]. A previous study using mtCOI data on five closely related *Pseudocalanus* species from the North Atlantic revealed two distinct evolutionary lineages that differ significantly in their population genetic structure and demographic history. This divergence is presumably due to species' ecological peculiarities [Aarbakke *et al.*, 2014]. The species we are examining in this study belong to the different lineages. The Baltic and Black Seas are geographically isolated from the Atlantic Ocean, making zooplankton in these seas particularly interesting for phylogeographic research.

The present study aims to compare morphological and genetic variation between two *Pseudocalanus* species found in geographically isolated areas of the Atlantic Ocean — the Baltic and Black Seas and includes: (i) description of morphological differences between *P. acuspes* and *P. elongatus*, inhabiting the Baltic and Black Seas, respectively; and (ii) assessment of the extent of genetic diversity within and between populations of *Pseudocalanus* from two seas, using the cytochrome-c oxidase subunit-I fragments obtained in this study and available in the GenBank.

2. Materials and methods

2.1. Sampling data

Zooplankton samples for morphological study were collected in the Exclusive Economic Zone of the Russian Federation in the southeastern Baltic Sea during cruises by the Shirshov Institute of Oceanology, Russian Academy of Sciences (IO RAS) aboard the research vessels “Professor Shtokman” in April

2017 and “Akademik Boris Petrov” in November 2021. Samples were taken during daytime using a WP-2 plankton net ($\Phi = 56$ cm, mesh size 100 μm) and preserved in 4% formalin. Mature females of *Pseudocalanus* were selected from these samples: 32 individuals in 2017 and 18 in 2021. *Pseudocalanus elongatus* females (33 specimens) were sorted out from zooplankton samples taken using a Juday net ($\Phi = 37$ cm, mesh size 150 μm) aboard a small vessel near the southern Crimean coast in February 2024 (Fig. 1, Table 1).

For genetic analysis, specimens of *Pseudocalanus* were obtained from zooplankton samples collected during Cruise 61 of the RV “Akademik Ioffe” in the southeastern Baltic Sea in July 2022, and in the Black Sea near the southern Crimean coast in February 2024 (see Fig. 1, Table 1). The samples were fixed in 96% ethanol and stored at 4 °C.

2.2. Morphological study

The formalin-fixed specimens were placed on glass slides in numbered droplets of glycerol and water (50:50), under a LOMO MBR-9 stereo microscope.

Thirty-nine females of *Pseudocalanus* from the Baltic Sea and thirty-three females from the Black Sea were drawn for measurements. All line drawings were made from specimens using a camera lucida on a Leica DM LS2 compound microscope at 100x and 200x magnification, and then used to make accurate measurements. The total body length was measured from the top of the cephalosome to the tip of the furcal ramus. Urosome measurements were taken to account for curvature following an approach similar to that of Aarbakke *et al.* [2013: fig. 2]. Photographs of the females were taken at a magnification of 40x.

The following measurements were taken: total body length (L), antennule length (A1L), prosome length (PrL), urosome length (UrL) and genital segment length (GL). Ratios between body details were calculated and then averaged. The morpho-

Table 1. Sampling information.
Таблица 1. Информация о собранном материале.

Station	Year/Month	Latitude (N)	Longitude (E)	Sampling depth (m)	Preservation solution	Number of specimens
Southeastern Baltic Sea	2017/Apr	54.750°	19.526°	0–90	4% formalin	32
Southeastern Baltic Sea	2021/Nov	55.069°	19.906°	0–65	4% formalin	18
Black Sea	2024/Feb	44.480°	33.595°	0–50	4% formalin	33
Southeastern Baltic Sea	2022/Jul	55.098°	19.196°	0–100	96% ethanol	2
Black Sea	2024/Feb	44.480°	33.595°	0–50	96% ethanol	3

logical measurements were compared with those published by Frost [1989] and Markhaseva *et al.* [2012].

To estimate the statistical significance of similarities and differences between datasets, a one-way analysis of variance (ANOVA) was used in IBM SPSS Statistics version 23.0.

2.3. Molecular genetic study

DNA was extracted from three individuals of *Pseudocalanus* from the Black Sea and two individuals from the Baltic Sea using the QIAmp DNA Mini Kit (QIAGEN GmbH, Germany) following the manufacturer's protocol. The concentration of DNA was measured using an Invitrogen Qubit fluorometer (Thermo Fisher Scientific, UK).

The partial fragment of mtCOI (length 500 bp) was amplified using specific primers designed based on published complete sequences of *Pseudocalanus* mitochondrial COI gene (GenBank total number records were 1100): forward CGAATAGARYTAGGTCARGC (Sense), reverse CCAGC-TAAYACTGGTAARGA (AntiSense). PCR amplification was carried out in a 15 µL reaction volume that included 6 µL template DNA, 0.6 µL of each primer, 3 µL 5X ScreenMix (Evrogen, Russia) and 4.8 µL RNase-free water using a C1000 Touch thermal cycler (Bio-Rad, USA). The PCR program consisted of an initial denaturing step at 94 °C for 3 min, 30 amplification cycles (94 °C for 30 s, 45 °C for 30 s, 72 °C for 60 s), and a final step at 72 °C for 5 min. PCR products were verified on 1% agarose/TBE electrophoretic gel. Amplicons were purified with the set of reagents for DNA purification from agarose gel and relative mixtures "ColGen" (Syntol, Russia).

Double-stranded sequencing of the PCR products was conducted by the dideoxy chain-termination method (Sanger Sequencing) using BrilliantDye™ Terminator (v3.1) Cycle Sequencing Kit (Numagen, Netherlands). After cleaning by the ethanol precipitation method, dye-labelled products were sequenced with PCR forward and reverse primers by the Genetic Analyzer Nanophore 05 (Institute for Analytical Instrumentation, RAS, Russia). Raw reads for each sequence were base-called using programs for analyzing and editing sequencing results (PAR2SEC, Institute of Analytical Instrumentation, RAS, Russia), assembled and checked for improper base-calling manually. All newly obtained sequences were deposited in GenBank (accession no.: *Pseudocalanus elongatus* Black Sea: PQ524996-PQ524998; *Pseudocalanus acuspes* South-Eastern Baltic: PQ525281-PQ525282).

The relationship of *Pseudocalanus* species was reconstructed based on COI sequences obtained from our samples and a set of sequences from the NCBI database. The data set encompassed full and partial mitochondrial DNA sequences (123 samples)

corresponding to specific haplotypes of *Pseudocalanus* species and an outgroup (see Table 2).

The sequences were first aligned with MAFFT v.7.48 (Kato, Toh, 2010) with option L-INS-I, and ambiguously aligned 5'- and 3'-terminal regions were trimmed. The length of the resulting matrix was 454 bp. Maximum-likelihood phylogenetic analysis was performed using IQ-TREE 1.6.12 [Trifinopoulos *et al.*, 2016] with a model TIM+I+G4 chosen by ModelFinder, an SH-like approximate likelihood ratio test with 10000 replicates, an ultrafast bootstrap with 10000 replicates, and approximate Bayes test for estimating branch support, unsuccessful iterations to stop tree searching set to 1000, and perturbation strength to 0.01. We calculated the Kimura two-parameter (K2P) distances using the MEGA 11 software [Kimura, 1980; Tamura *et al.*, 2021].

3. Results

3.1. Comparison of morphological characteristics

Under microscopic examination, all *Pseudocalanus* specimens from the Baltic Sea were identified as *P. acuspes*. We drew 39 female specimens of this species and 33 females of *P. elongatus* from the Black Sea and conducted measurements as described in the Materials and Methods section. The images of *Pseudocalanus* females from the Baltic Sea (Figs 2C, D; 3A–C) and the Black Sea (Figs 2A, 2; 3H–J) show a clear difference in the contours of the anterior cephalosome. The anterior cephalosomes of the Baltic specimens appear noticeably rounder in lateral view than those of the Black Sea specimens.

For the evaluation of external morphological differences between females of *P. acuspes* and *P. elongatus*, we focused on measuring total body length and lengths of specific body details that showed visible differences when comparing individuals of both species (Figs 2, 3). The measurements of the total body length (L), antennule length (A1L), prosome length (PrL), urosome length (UrL) and genital segment length (GL) (Fig. 3A, D), as well as their ratios, were performed, averaged and presented in Table 3. The full list of measurements can be found in Supplement 1.

The most pronounced difference was found in the length of the genital segment. In *P. acuspes*, it was (0.16 ± 0.01) mm, while in *P. elongatus* it was (0.13 ± 0.01) mm. Additionally, the length of antennule and urosome

Table 2. Sequences set from the NCBI database.
Таблица 2. Список нуклеотидных последовательностей из базы данных NCBI.

Species	NCBI ID
<i>Pseudocalanus acuspes</i> Arctic Ocean	MN831641.1, MN831487.1, MN831668.1
<i>Pseudocalanus acuspes</i> Baltic Sea	AF462317.1, HM770074.1
<i>Pseudocalanus acuspes</i> Barents Sea	KF991190.1, KF991189.1
<i>Pseudocalanus acuspes</i> Beaufort Sea	KU142110.1, KU142107.1, KU142061.1, KU142025.1, KU142013.1, KU142052.1, KU142003.1, KU142021.1, KU142008.1, KU142057.1, KU142113.1, KU142007.1
<i>Pseudocalanus acuspes</i> Bering Sea	KF836992.1, KF837014.1, KF836999.1, KF837000.1, KF837009.1, KF837012.1, KF837002.1, KF836996.1, KF836997.1, KF836984.1, KF836987.1, KF836986.1, KF837007.1, KF837003.1, KF837004.1, KF837001.1, KF837020.1, KF836991.1
<i>Pseudocalanus acuspes</i> Chukchi Sea	KU141966.1, KU141954.1, KU141997.1, KU141922.1, KU141895.1, KU141978.1, KU141931.1, KU141994.1, KU141848.1, KU141928.1, KU141832.1, KU141899.1, KU141937.1, KU141872.1, KU141827.1
<i>Pseudocalanus acuspes</i> Gulf of Alaska	KU141822.1, KU141823.1
<i>Pseudocalanus acuspes</i> Balsfjorden (Norwegian Sea)	KF991194.1, KF991193.1, KF991182.1, KF991181.1, KF991180.1, KF991179.1
<i>Pseudocalanus elongatus</i> Black Sea	KC305820.1 (AY604522.1), KC305830.1, KP136558.1
<i>Pseudocalanus elongatus</i> , Atlantic Ocean, Iceland	KF991177.1, KF991176.1, KF991175.1, KF991172.1
<i>Pseudocalanus elongatus</i> North Sea	JX995273.1 (KT208677.1), KT209067.1, JX995276.1,
<i>Pseudocalanus elongatus</i> Atlantic Ocean, East English Channel	KC305834.1, KC305833.1, KC305839.1, AY604523.1, KC305836.1, KC305841.1
<i>Pseudocalanus elongatus</i> Dutch Wadden Sea	AY144467.1
<i>Pseudocalanus elongatus</i> Arctic Ocean	MN831677.1, MN831675.1, MN831674.1, MN831672.1,
<i>Pseudocalanus elongatus</i> Norwegian_Sea	HM770078.1, HM770077.1
<i>Pseudocalanus major</i>	MN831643.1, MN831649.1, MN831652.1, MN831655.1, MN831659.1, MN831645.1
<i>Pseudocalanus mimus</i>	KU142146.1, KU142225.1, KU142242.1, KU142244.1, KU142245.1, AF513651.1
<i>Pseudocalanus minutus</i>	KU141577.1, KU141579.1, KU141611.1, KU141612.1, MN831518.1
<i>Pseudocalanus moultoni</i>	JX995277.1, JX995278.1, GU680119.1, MN831676.1
<i>Pseudocalanus newmani</i>	KU141641.1, KU141618.1, KU141621.1, KU141632.1, JX502997.1, KU141649.1, KU141679.1, KU141693.1, KU141706.1, KU141717.1, KU141721.1, KU141733.1, KU141751.1, KU141765.1, KU141781.1, KU141783.1, KU141792.1, KU141820.1, MN831491.1, MN831492.1

also differ significantly between the two species. The length of antennule was (1.06 ± 0.06) mm in *P. acuspes* and (1.16 ± 0.04) mm in *P. elongatus*, and the length of urosome was (0.42 ± 0.02) mm and (0.37 ± 0.02) mm, respectively (Table 3). Shorter antennules in *P. acuspes* extend to the distal edge of the second somite of the urosome, while in *P. elongatus*, they reach the distal edge or extend beyond the third somite. We found one female *P. elongatus*, with antennules even longer than its body. The total body length of *P. acuspes* was only slightly greater than that of *P. elongatus* (Table 3).

The ratios between different body parts also show significant variations between species, as shown in Table 3. Specifically, the ratio of the prosome to the urosome length was averaged 2.0 ± 0.08 for *P. acuspes* and 2.3 ± 0.05 for *P. elongatus*. The statistical analysis confirmed significant differences between *P. acuspes* and *P. elongatus* in terms of A1L/L, PrL/UrL, and GL/PrL ratios ($p < 0.001$). To visually evaluate the discrepancies in body proportions between species, we compared the measured ratios pairwise (Fig. 4). We have identified the following ranges of ratios between A1L,

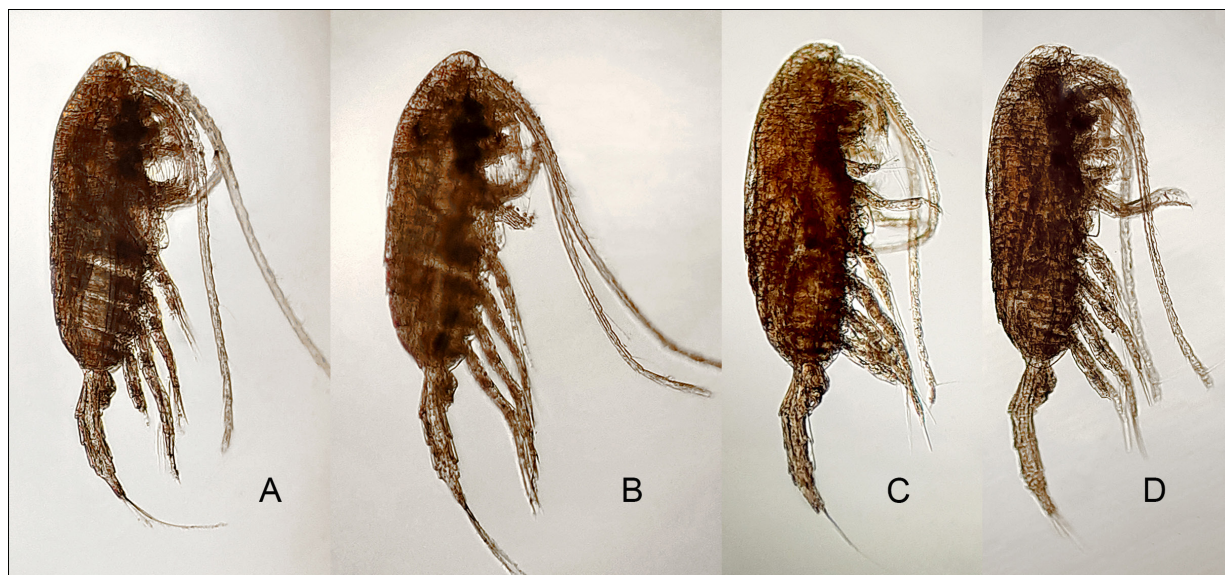


Fig. 2. Adult females of *Pseudocalanus elongatus*, Black Sea (A, B) and *Pseudocalanus acuspes*, Baltic Sea (C, D). A magnification of 40x.
Рис. 2. Взрослые самки *Pseudocalanus elongatus*, Черное море (A, B) и *Pseudocalanus acuspes*, Балтийское море (C, D). Увеличение 40х.

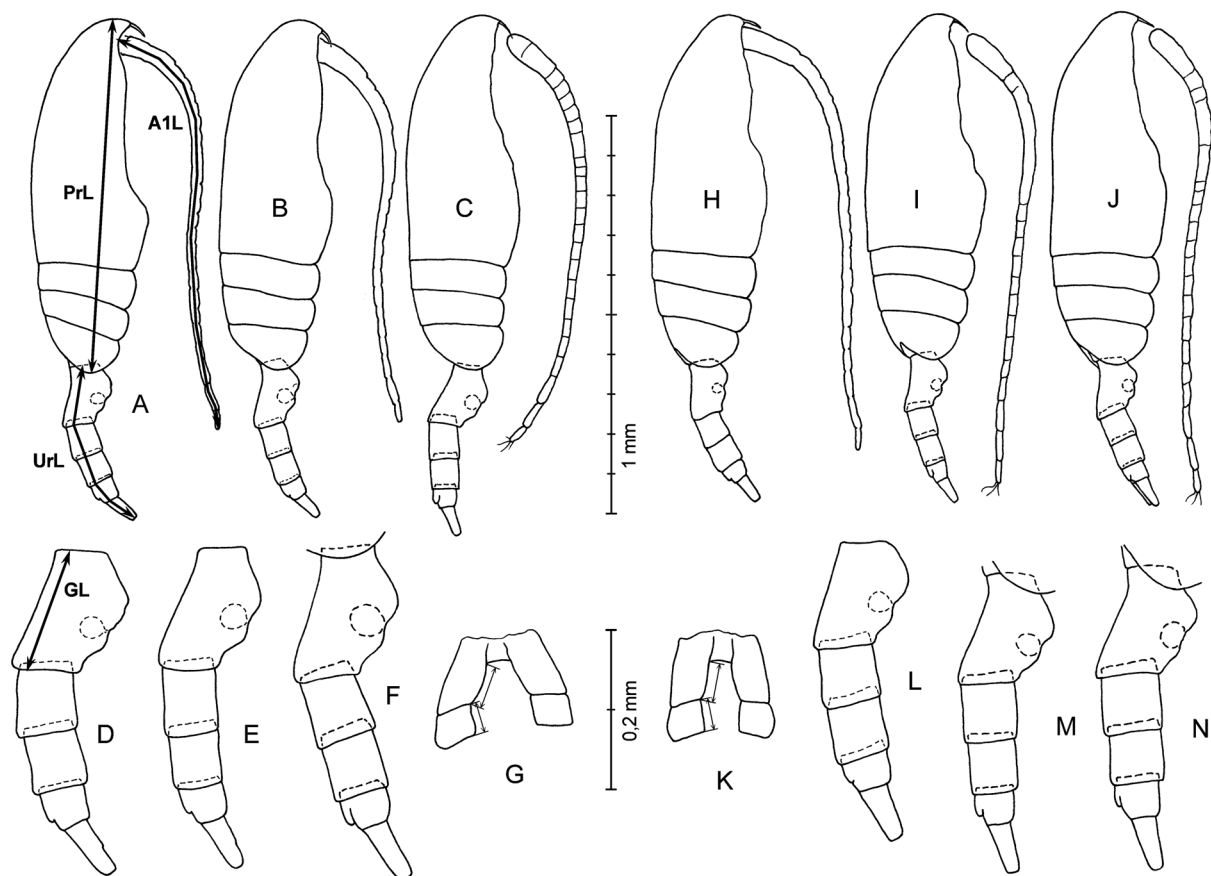


Fig. 3. Adult females of *Pseudocalanus acuspes*, Baltic Sea (A–G), and *Pseudocalanus elongatus*, Black Sea (H–N). A–C, H–J — lateral view; D–F, L–N — urosome, lateral view; G, K — coxal and basal segments of the fourth swimming legs. Arrows show the lengths of their inner edges that have been used for measurements. Scale 1 mm — for A–C, H–J; 0.2 mm — for the remaining images. A1L — antennule length; PrL — prosoma length; UrL — urosome length.

Рис. 3. Взрослые самки *Pseudocalanus acuspes*, Балтийское море (A–G), и *Pseudocalanus elongatus*, Черное море (H–N). A–C, H–J — вид сбоку; D–F, L–N — уросома, вид сбоку; G, K — коксальный и базальный сегменты четвертых плавательных ног (стрелками показана длина их внутренних краев). Масштаб 1 мм — для A–C, H–J; 0,2 мм — для остальных рисунков. A1L — длина антеннулы; PrL — длина просомы; UrL — длина уросомы.

Table 3. The length of the body and body details (mm), and their ratios in females of *P. acuspes* and *P. elongatus*.
Таблица 3. Длина тела, его отдельных частей (мм) и их соотношения у самок *P. acuspes* и *P. elongatus*.

Character	<i>P. acuspes</i> , April 2017			<i>P. acuspes</i> , November 2021			<i>P. elongatus</i> , February 2024		
	n	range	mean \pm SD	n	range	mean \pm SD	n	range	mean \pm SD
L	23	1.17–1.44	1.30 \pm 0.07	16	1.20–1.42	1.28 \pm 0.06	33	1.10–1.35	1.22 \pm 0.06
A1L	21	0.97–1.21	1.07 \pm 0.06	15	0.98–1.17	1.06 \pm 0.06	24	1.07–1.25	1.16 \pm 0.04
PrL	23	0.80–0.97	0.88 \pm 0.05	16	0.80–0.97	0.87 \pm 0.04	33	0.78–0.96	0.86 \pm 0.04
UrL	23	0.38–0.52	0.44 \pm 0.03	16	0.39–0.48	0.42 \pm 0.02	33	0.33–0.42	0.37 \pm 0.02
GL	23	0.14–0.19	0.16 \pm 0.01	16	0.14–0.18	0.16 \pm 0.01	33	0.12–0.15	0.13 \pm 0.01
PrL/UrL	23	1.83–2.11	2.00 \pm 0.08	16	1.96–2.19	2.06 \pm 0.07	33	2.20–2.39	2.29 \pm 0.05
A1L/L (%)	21	77.1–86.5	81.4 \pm 2.6	15	78.1–85.6	83.0 \pm 2.1	24	92.8–100.7	96.5 \pm 1.8
GL/UrL (%)	23	35.0–37.6	36.4 \pm 0.7	16	35.8–37.9	36.9 \pm 0.7	33	34.6–37.5	36.1 \pm 0.7
GL/PrL (%)	23	16.9–20.1	18.2 \pm 0.8	16	16.8–19.1	18.0 \pm 0.7	33	15.2–16.4	15.7 \pm 0.4

n — numbers of specimens; L — total body length; A1L — antennule 1 length; PrL — prosome length;
UrL — urosome length; GL — genital segment length; PrL/UrL — ratio of prosome length to urosome length; A1L/L (%) — ratio of antennule 1 length to total body length; GL/UrL (%) — ratio of genital segment length to urosome length; GL/PrL (%) — ratio of genital segment length to prosome length.

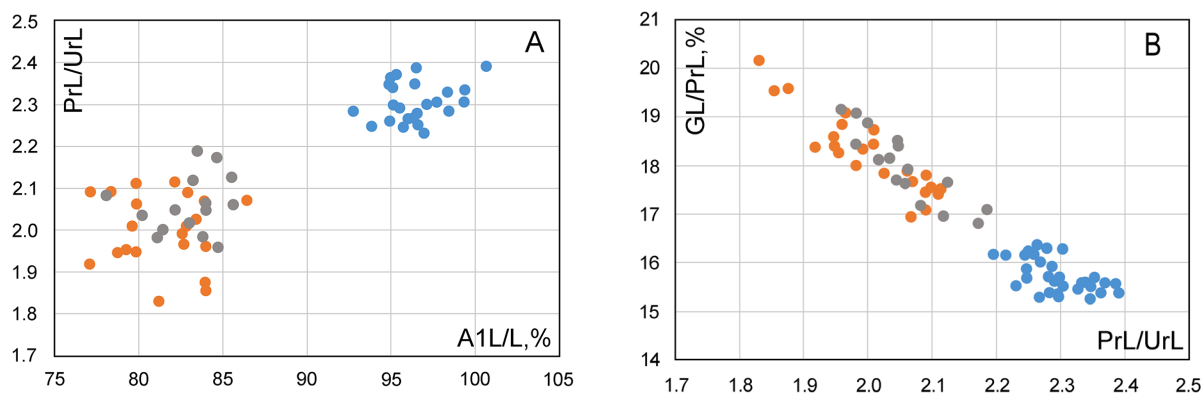


Fig. 4. Pairwise comparison of morphometric ratios with statistically significant differences between females of *P. acuspes* (orange, April 2017, and grey, November 2021, circles) and *P. elongatus* (blue circles). Relationship between the ratios PrL/UrL and A1L/L (A), GL/PrL and PrL/UrL (B).

Рис. 4. Попарное сравнение морфометрических соотношений со статистически значимыми различиями между самками *P. acuspes* (оранжевые, апрель 2017 г., и серые, ноябрь 2021 г., кружки) и *P. elongatus* (синие кружки). Взаимосвязь между соотношениями PrL/UrL и A1L/L (A), GL/PrL и PrL/UrL (B).

PrL, UrL, and the GL, which can be used to distinguish between two species:

P. acuspes — PrL/UrL < 2.2; A1L/L(%) < 87; GL/PrL > 16.8;

P. elongatus — PrL/UrL > 2.2; A1L/L(%) > 92; GL/PrL < 16.5.

There are no statistically significant differences between the ratios of body detail lengths in *P. acuspes* females from Baltic Sea sampled in spring and autumn ($p > 0.1$).

3.2. COI sequence analysis and phylogenetic reconstruction

The partial COI mtDNA gene was utilized to identify *Pseudocalanus* specimens. They were compared with homologous sequences found in the GenBank for this genus.

The lengths of gene fragments from Baltic Sea *Pseudocalanus* were 454 bp and 481 bp. The length of the

resulting alignment matrix was 454 bp. By Blast search the best species match scores yielded *P. acuspes* with 96.38–99.78% identity. Phylogenetic reconstruction for partial mtCOI proved that *Pseudocalanus* sequences from the Baltic Sea clustered with *P. acuspes* in the ML tree with support values up to 99% (Fig. 5). The Baltic Sea and Balsfjorden (Norwegian Sea) samples formed a separate subgroup (evolutionary distance 0.014) with 99% support. The samples from the Barents Sea, Bering Sea, Chukchi Sea, Gulf of Alaska and the Arctic Ocean form a common subgroup with 99% support and an evolutionary distance of 0.008. This subgroup is represented on a pie chart in the tree (Fig. 5). It is impossible to identify separate subgroups for geographical regions within this clade (see the detailed tree in Supplement 2).

We calculated the Kimura two-parameter (K2P) distances for the mtCOI fragment to estimate the range of genetic differences within *P. acuspes* and between

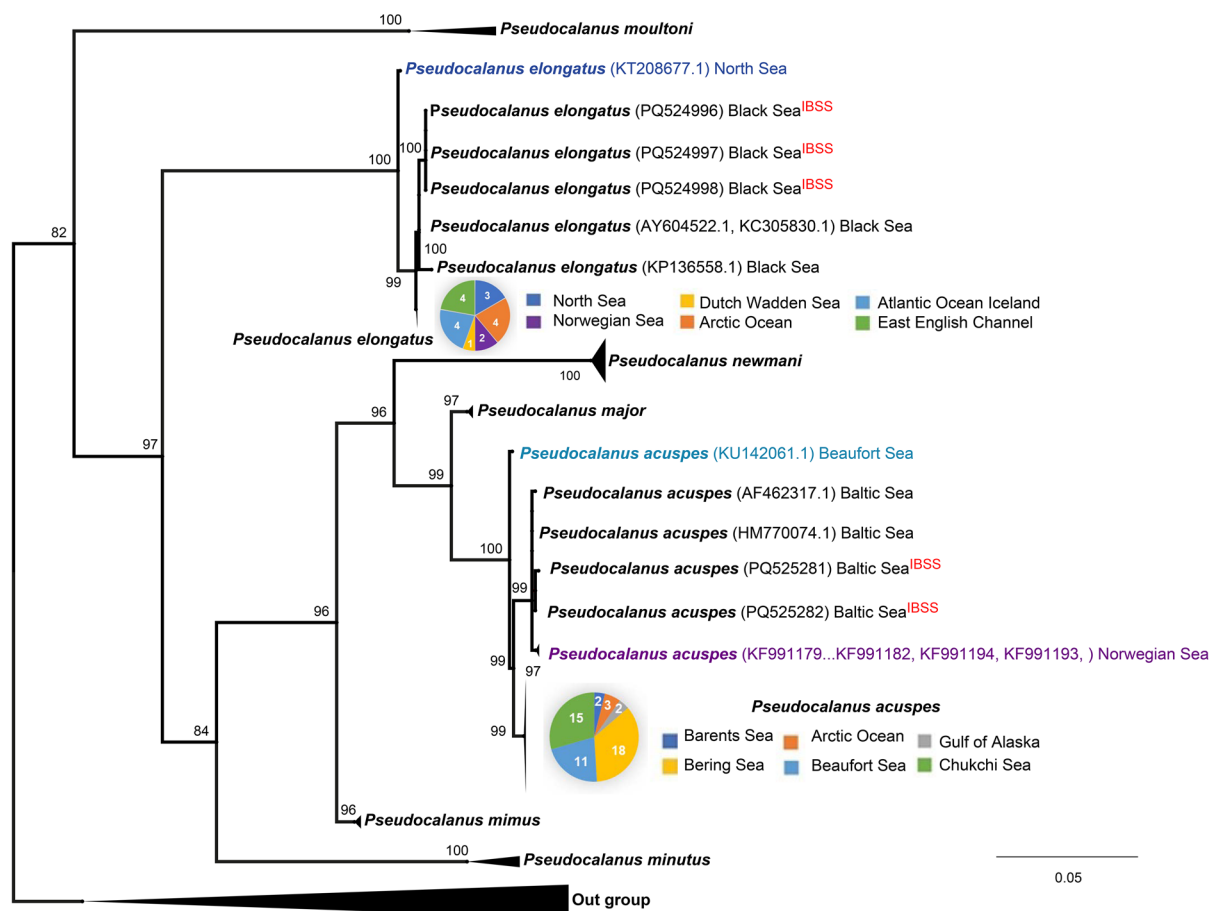


Fig. 5. Phylogenetic reconstruction of the *Pseudocalanus* tree based on partial COI mtDNA gene sequences from samples collected from the Black Sea and Baltic Sea, as well as sequences available in the NCBI database for the genus. Ultrafast bootstrap support (Maximum likelihood) is provided at nodes, with only values above 85% shown. Sequences obtained in this study are marked with IBSS. Non-differentiated subgroups of *P. elongatus* and *P. acuspes* are presented in the form of pie charts. The pie charts show the proportion of haplotypes in different geographical regions. Geographical regions are represented by color in the legend, and the number of haplotypes for each region is shown on the pie chart. A detailed tree can be found in the Supplement 2

Рис. 5. Филогенетическая реконструкция дерева *Pseudocalanus* с использованием фрагмента последовательностей митохондриального гена COI из образцов, собранных в Черном и Балтийском морях, а также последовательностей, доступных в базе данных NCBI для этого рода. Поддержка сверхбыстрой начальной загрузки (максимальное правдоподобие) представлена в узлах, при этом показаны только значения выше 85%. Последовательности, полученные в настоящем исследовании, отмечены как IBSS. Недифференцированные подгруппы *P. elongatus* и *P. acuspes* представлены в виде круговых диаграмм. Круговые диаграммы показывают соотношение гаплотипов в разных географических регионах. Географические регионы обозначены цветом в условных обозначениях, а количество гаплотипов для каждого региона показано на круговой диаграмме. Подробное дерево можно найти в дополнительных материалах (Приложение 2).

species. The results, grouped by region, are presented in the Supplement 3 (Table 1). In general, the intraspecific variability among *P. acuspes* individuals in the COI region ranges from 0 to 3.16% for the entire available dataset. The overall array of K2P values falls into two distinct ranges corresponding to the subgroups noted above. Within these ranges, the K2P value does not exceed 1% with few exceptions. The genetic differences in the COI fragment between *P. acuspes* from the Baltic Sea and Balsfjorden (Norwegian Sea), compared with samples from other parts of the Arctic Ocean and the Northwest Pacific Ocean, vary by 2–3%. K2P distances between *P. acuspes* and other *Pseudocalanus* species most often ranged from 10 to 20%. The smallest genetic differences in the COI fragment were found between *P. acuspes* and *P. major*. The K2P values for them range from 3 to 5%.

The mtCOI fragments obtained from Black Sea *Pseudocalanus* were 500 bp, resulting in an alignment matrix of 448 bp. The highest Blast match scores were obtained with *P. elongatus*, which showed a sequence identity range of 99.20 to 99.80%. The Black Sea *Pseudocalanus* COI sequences group with *Pseudocalanus elongatus* in the phylogenetic tree with strong support (Fig. 5). The Black Sea samples have formed a distinct subgroup with an evolutionary distance of 0.014 and 92% support. Three haplotypes were revealed within that subgroup. Our samples belong to one haplotype, which is noticeably distinct from early-obtained sequences. The samples from the North East Atlantic Ocean, the North Sea, the Norwegian Sea, and some other Arctic regions formed a common subgroup with strong support. Determining distinct subgroups based on geographical regions within this clade is not feasible.

The K2P distances for the mtCOI fragment among *P. elongatus* ranges from 0 to 2.5%, but most values do not exceed 0.9% (see Supplement 3, Table 2). Only one from the North Sea (KT208677.1) and one from the Black Sea (KP136558.1) demonstrated higher K2P distances (1.3–2.5%) from other *P. elongatus*. The COI sequences we obtained from *P. elongatus* in the Black Sea differ from those previously reported for this region, with K2P distance values ranging from 0.22 to 1.13%. Interspecific distances of *P. elongatus* from other species of the genus range from 15 to 23%.

4. Discussion

Analyzing mitochondrial COI gene fragments and morphological characteristics of *Pseudocalanus* specimens from geographically isolated areas of the Atlantic Ocean basin confirmed their species identity — *P. elongatus* in the Black Sea and *P. acuspes* in the Baltic Sea. Based on original drawings and photographs, external morphological differences have been identified among these two species. Females of *P. acuspes* (Baltic Sea) exhibit the following distinct features compared to *P. elongatus* (Black Sea): 1) more rounded anterior cephalosome in lateral view; 2) shorter antennules that reach the distal edge of the second urosomal segment (averaging 82% of total body length for *P. acuspes* versus 96% for *P. elongatus*); 3) shorter prosomes (averaging 2.0 times longer than urosome for *P. acuspes* versus 2.3 for *P. elongatus*); and 4) larger genital segments.

The study on the genetic diversity of *Pseudocalanus* was conducted in the Black Sea in 2006 by Unal et al. They analyzed mtCOI sequences from 15 female individuals collected from a single location in the southwest deep-sea region. The authors noted low intraspecific variation of the Black Sea *P. elongatus* compared to populations from the English Channel. Only two haplotypes were found in the Black Sea, with most of the mtCOI sequences being identical, but they did not match the sequences of this gene in the English Channel samples. The phylogenetic tree of *Pseudocalanus* we have reconstructed also allows us to distinguish a separate group of Black Sea *P. elongatus*. The genetic divergence between the COI sequences of this species from the Black Sea and those from other parts of the ocean is, on average, one-third greater than the divergence between COI sequences from all other locations.

A study of mtCOI in five species of *Pseudocalanus* from the North Atlantic Ocean demonstrated the greatest genetic diversity in the sample of *P. acuspes*, with a high degree of haplotype divergence within a single population, compared to other *Pseudocalanus* species [Aarbakke et al., 2014]. The number of *P. acuspes* haplotypes within the distinct geographic region is significantly higher compared to *P. elongatus*. The intraspecific variability in *P. acuspes* shows a wider range of K2P distances compared to *P. elongatus*. However, in the Baltic Sea, the genetic diversity within the *P. acuspes* population is exceptionally low [Holmborn et al., 2011]. Reduced genetic diversity is a common feature of many inhabitants of the Baltic Sea,

as it is a marginal ecosystem for them due to its extremely low water salinity [Johannesson, Andre, 2006; Holmborn et al., 2011]. Based on mtCOI variation, we found that populations of *P. acuspes* from the Baltic Sea and Balsfjorden (Norwegian Sea) form a distinct well-supported subgroup within the species cluster on the phylogenetic tree. These two geographically isolated populations seem to have a common origin, which supports the idea that *P. acuspes* is part of the glacial relict fauna of the Baltic Sea [Peters et al., 2006; Grabbert et al., 2010]. The isolation of these populations may have occurred less than nine thousand years ago, after the connection between the Baltic Sea and the North Atlantic/Arctic waters was closed.

Thus, the studied populations of both *Pseudocalanus* species are genetically distinct from those living in other parts of their geographical range. Therefore, further validation of the conclusions about morphological differences between the two species is necessary. In particular, it is essential to assess the extent of variation in phenotypic traits across the entire range of each species. No comprehensive modern morphological descriptions and illustrations of *P. elongatus*, originally described off the southeastern coast of Norway in the Oslo Fjord [Boeck, 1865] and also from the Black Sea, are available in the literature. In the Black Sea *P. elongatus* was first registered by Karavaev, who mentioned longer antennules than those of *Pseudocalanus* described by Giesbrecht from Kiel Bight (Baltic Sea) [Karavaev, 1895: 137]. Thus some morphological differences between *P. acuspes* and *P. elongatus* were noted more than 100 years ago. Our study confirmed the difference in antennule length between two species.

Grabbert and co-authors [Grabbert et al., 2010] failed to successfully distinguish *P. elongatus* from the North Sea and *P. acuspes* from the Baltic Sea based on morphometric ratios. In the North Sea, they observed strong seasonal variations in the length of the prosome in *P. elongatus*, possibly related to temperature fluctuations throughout the year, which was reflected by a broad variability of obtained PrL/UrL ratios. For *P. acuspes* from the Baltic Sea, this relationship remained relatively constant for most of the year, except for April, when the total prosome length for females (1.5 mm) was higher than the rest of the year, resulting in lower PrL/UrL [Grabbert et al., 2010]. In our study in the Baltic Sea, we found no statistically significant differences between the morphometry of *P. acuspes* females sampled in spring and autumn.

Frost [1989] identified only one primary taxonomic feature to distinguish *P. acuspes* females from *P. elongatus*: the more rounded anterior cephalosome in lateral view in *P. acuspes*. He mentioned the Black Sea population of *P. elongatus*, noting that he did not have specimens from the Black Sea at his disposal; however, his specimens from the Alboran Sea and northern Adriatic Sea (Mediterranean Sea) agree with his other materials [Frost, 1989: 536]. The length of the antennules and the prosome-to-urosome length ratio in existing images of this species from northern regions [Sars, 1903; Frost, 1989; Markhaseva et al., 2012] are comparable to those of *P. acuspes* (Table 4). The exception is a specimen from the Beaufort Sea [Frost, 1989: fig. 13A], which

Table 4. Ratios A1L/L and PrL/UrL for females of *P. acuspes* and *P. elongatus* calculated based on the drawings from Giesbrecht [1882]; Sars [1903]; Frost [1989]; Markhaseva *et al.* [2012].Таблица 4. Соотношения A1L/L и PrL/UrL у самок *P. acuspes* и *P. elongatus*, рассчитанные на основе рисунков из работ: Giesbrecht [1882]; Sars [1903]; Frost [1989]; Markhaseva *et al.* [2012].

Species	A1L/L (%)	PrL/UrL	Region	References
<i>P. acuspes</i>	86.8	1.97	Baltic Sea, Kiel Bay	Giesbrecht, 1882: pl. II, fig. 17
<i>P. acuspes</i>	87.6	2.16	Baltic Sea, Kiel Bay	Frost, 1989: fig. 13A
<i>P. acuspes</i>	87.3	2.33	Beaufort Sea	Frost, 1989: fig. 13B
<i>P. acuspes</i>	reaching to	2.07	White Sea	Markhaseva <i>et al.</i> , 2012: fig. 1, L=1.20 mm
<i>P. acuspes</i>	about middle	1.99	White Sea	Markhaseva <i>et al.</i> , 2012: fig. 1, L=1.55 mm
<i>P. acuspes</i>	of Ur2–Ur3	1.97	White Sea	Markhaseva <i>et al.</i> , 2012: fig. 1, L=1.65 mm
<i>P. elongatus</i>	82.6	1.94	Oslofjord, Norway	Sars, 1903: pl. X
<i>P. elongatus</i>	87.7	2.08	Oslofjord, Norway	Frost, 1989: fig. 10A

has a more elongated prosome than the other females listed in the table. Some of these values may have been overestimated due to the authors not accounting for the curvature of the urosome. It can be assumed that some differences between Black Sea *P. elongatus* and specimens from the northern parts of its range (possibly including the Mediterranean Sea) demonstrate the potential variability of this species under the unique conditions of the Black Sea. This is further influenced by the extremely restricted exchange of Black Sea waters with the Atlantic Ocean and, consequently, the significant isolation of the Black Sea population of *P. elongatus*. Further research is necessary to explore the morphological variability of *P. elongatus* from different areas of the Atlantic Ocean (Black Sea, North Sea, Skagerrak, Kattegat).

Thus, we found several differences in the morphometric ratios of body measurements between *P. acuspes* from the Baltic Sea and *P. elongatus* from the Black Sea. Specifically, for *P. acuspes*, the ratios are PrL/UrL < 2.2, A1L/L (%) < 87; GL/PrL > 16.8. For *P. elongatus*, these ratios are PrL/UrL > 2.2; A1L/L (%) > 92; GL/PrL < 16.5. However, these differences need to be confirmed by additional morphological investigations of these species from different parts of their range.

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Supplementary data. The following materials are available online.

Supplement 1. The full list of morphometric measurements of *Pseudocalanus* body parts and ratios.

Supplement 2. Phylogenetic reconstruction of the *Pseudocalanus* tree (expanded) based on mitochondrial COI gene sequences from samples collected from the Black Sea and the Baltic Sea, as well as sequences available in the NCBI database

for this genus. Ultrafast bootstrap support (Maximum likelihood) is provided at nodes, with only values above 85% shown.

Supplement 3. The Kimura two-parameter (K2P) distances (in %) of partial COI mtDNA gene between *P. acuspes* (Table 1) and *P. elongatus* (Table 2), grouped by region, and on *Pseudocalanus* spp.

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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