

Research Article

To fit or to belong: characterization of the non-native invader *Eurytemora carolleeae* (Copepoda: Calanoida) in the Oder River system (Central Europe)Łukasz Sługocki^{1,2,*}, Anna Rymaszewska³ and Lucyna Kirczuk^{1,2}¹Department of Hydrobiology, Institute of Biology, University of Szczecin, Felczaka 3c, 71-712 Szczecin, Poland²Center of Molecular Biology and Biotechnology, University of Szczecin, Wąska 13, 71-715 Szczecin, Poland³Department of Genetics and Genomics, Institute of Biology, University of Szczecin, Felczaka 3c, 71-712 Szczecin, Poland

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OPEN ACCESS**Abstract**

The *Eurytemora affinis* species complex (Copepoda: Calanoida) is widespread in coastal and estuarine waters in the northern hemisphere. The species *Eurytemora carolleeae* belongs to this complex. It was recently described and recognized as an invasive copepod for European waters. Since then, it was found in a few European estuaries. Based on molecular and morphological studies, we observed that *E. carolleeae* has expanded into new freshwater habitats in Central Europe. We carried out detailed morphological analysis for this species which indicates the potential higher stability of the populations in freshwater habitats compared to brackish waters. Fluctuating asymmetry did not significantly differ between freshwater and brackish water habitats, but the share of deformed specimens was considerably larger in brackish waters. We also found that season and habitat influenced the morphology of *Eurytemora*. In the molecular analyses, we focused on mitochondrial (COI) as well as nuclear gene markers (18S rDNA, ITS1-2) in *E. carolleeae*. In addition, for the first time, we analyzed the cytochrome b gene of the latter species. A wide distribution of *E. carolleeae* in the Oder River system in Central Europe indicates that it is a suitable habitat for the present populations and suggests further expansion of the species into continental waters.

Key words: freshwater, brackish water, Crustacea, barcoding, morphological deformations, fluctuating asymmetry

Introduction

Invasive alien species pose a potential threat to native fauna and their biodiversity due to the remodeling of food webs and homogenization of ecosystems (Katsanevakis et al. 2014). Among the aquatic habitats, transitional waters are particularly prone to the expansion of non-indigenous species, caused by sea transport and ballast waters bringing foreign populations to new ecosystems (Cohen and Carlton 1998). In habitats such as estuaries and lagoons, one of the most successfully expanding organisms are those exhibiting high resistance to physiological stress related to changing environmental conditions, mainly salinity and temperature (Lee 1999). An example of such organisms is the *Eurytemora affinis* species complex (Copepoda: Calanoida).

The *E. affinis* species complex is widespread in coastal and estuarine waters in the northern hemisphere. In the last decade, following *E. affinis* (Poppe, 1880), two new species were isolated from the complex, namely *Eurytemora carolleeae* Alekseev and Souissi, 2011 and *Eurytemora caspica* Sukhikh and Alekseev, 2013. The former is considered as native to North American brackish waters (Alekseev and Souissi 2011) but becoming invasive to American freshwater basins (Lee et al. 2013). Furthermore, this species is treated as non-native and invasive to European estuaries and inland seas (Alekseev and Souissi 2011; Sukhikh et al. 2013; Labuce et al. 2018).

Studies conducted on *E. affinis* complex species demonstrate that they are characterized by great adaptability to waters with variable salinity conditions (Lee 1999, 2016; Souissi and Souissi 2020). Fast adaptation to changing conditions is a feature that favors the invasion of *Eurytemora* into freshwaters, which has been explored in North American river systems (Lee 1999; Winkler et al. 2008). Laboratory studies have shown that in freshwater habitats *E. affinis* experiences stronger osmoregulation stress (especially during low supply of food) than *E. carolleeae* (Lee et al. 2011, 2013; Cabrol et al. 2020); therefore, it is rarely found in freshwater habitats (Favier and Winkler 2014).

To date, the presence of *E. carolleeae* in Europe has been confirmed in the brackish waters of the Gulf of Finland, French estuaries (Gironde, Loire, Seine), Trave estuary (German), Amsterdam canals (the Netherlands) (Sukhikh et al. 2013, 2019, 2020), and the Gulf of Riga (Labuce et al. 2018, 2020). However, the range of the *E. affinis* complex in Central European river systems and lagoons is not clear yet. In the case of the Oder (Odra) River system (Chojnacki and Tyluś 2013) and the Vistula Lagoon (Paturej et al. 2017; Lajus et al. 2020), the presence of only *E. affinis* has been reported. Therefore, in this study, we investigated the occurrence of species from the *E. affinis* complex in one of the largest rivers of Central Europe, the Odra River, and its lagoon connecting the river mouth with the Baltic Sea. Although several non-indigenous species of invertebrates were found in this river (Gruszka 1999; Łabęcka et al. 2005; Grabowski et al. 2007; Czerniejewski et al. 2020), the presence of potentially invasive microinvertebrates is overlooked. We assume that the Odra system is especially prone to invasion by *E. carolleeae* because it has large trans-shipment ports (Szczecin-Świnoujście), is poorly saline, and eutrophic, which favors the growth of this species (Lee et al. 2013). The mouth section of the river, Szczecin Lagoon, is one of the largest in Europe and is characterized by low salinity. Therefore, it may be a reservoir and gateway to the invasion of *E. carolleeae* into the continental waters in Central Europe.

Despite the ability to survive in conditions outside the environmental optimum, aquatic organisms are affected by multiple stressors such as salinity, temperature, pollutants causing physiological stress (Lee 1999), or morphological changes occurring in the body (Lajus et al. 2003). Organisms

exposed to factors beyond the optimum environmental requirements undergo large asymmetry fluctuations (Palmer and Strobeck 1986). Therefore, fluctuating asymmetry (FA) has often been used as an indicator of phenotypic disruption caused by increased developmental stress (Palmer and Strobeck 1986). It is considered a good indicator of morphological alterations occurring in copepods and *Eurytemora* specifically, along with an increased environmental stress (Lajus et al. 2020). Thus, body measures can signal the state of certain populations. For newly arrived species, the stress might be so high that the morphological changes could be significant, as would be expected in the case of *E. carolleae* in newly inhabited waters.

Molecular analysis on the *E. affinis* complex mainly focuses on the analysis of a fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, which helps to identify closely related species through barcoding (Lee 1999; Sukhikh et al. 2013, 2019). Another mitochondrial gene, cytochrome *b* (cytb), is equally effective in species identification (Merritt et al. 1998; Parson et al. 2000), but was not commonly used in the studies on the genus (Sługocki et al. 2019). Several conservative nuclear genes, mainly 18S ribosomal RNA (rRNA), 28S rRNA, and internal transcribed spacer (ITS), were proposed as alternative markers to mitochondrial genes. These fragments of nuclear DNA (nDNA), inherited from both parents, have been shown to differ between species while allowing a wider range of amplification (Wu et al. 2015).

Our study aimed to determine the status of species within the *E. affinis* complex in the Odra River system, combining molecular and morphological approaches.

Materials and methods

Our research area covered the lower section of the Oder River, Dąbie Lake, and Szczecin Lagoon with straits (Poland). The Odra River mouth is a mixing zone for freshwaters flowing down the Oder and sea waters, which are connected through one of the three straits. Due to the excess load of nutrients, the Oder River and Szczecin Lagoon are strongly eutrophied. However, currently, the nutrient loads are reduced, comprising 30% lower nitrogen and 70% lower phosphorus than the proportions around 1990 (Friedland et al. 2019).

A total of 62 zooplankton samples were collected from ten sampling sites located in the Oder River system (Figure 1). Sample collection was carried out monthly in the warm season (June–August) during the years 2019–2020. Additionally, two samples were collected from a freshwater sampling station (Szczecin) during the cold season (December 2019) (Supplementary material Table S1).

Zooplankton samples were collected from the bottom of the water bodies to the surface using vertical hauls with a plankton net (mesh of 250 µm; d = 20 cm). Concentrated samples were added into a 110-ml tube and fixed in 96% ethanol. *Eurytemora* species were identified based on the morphological

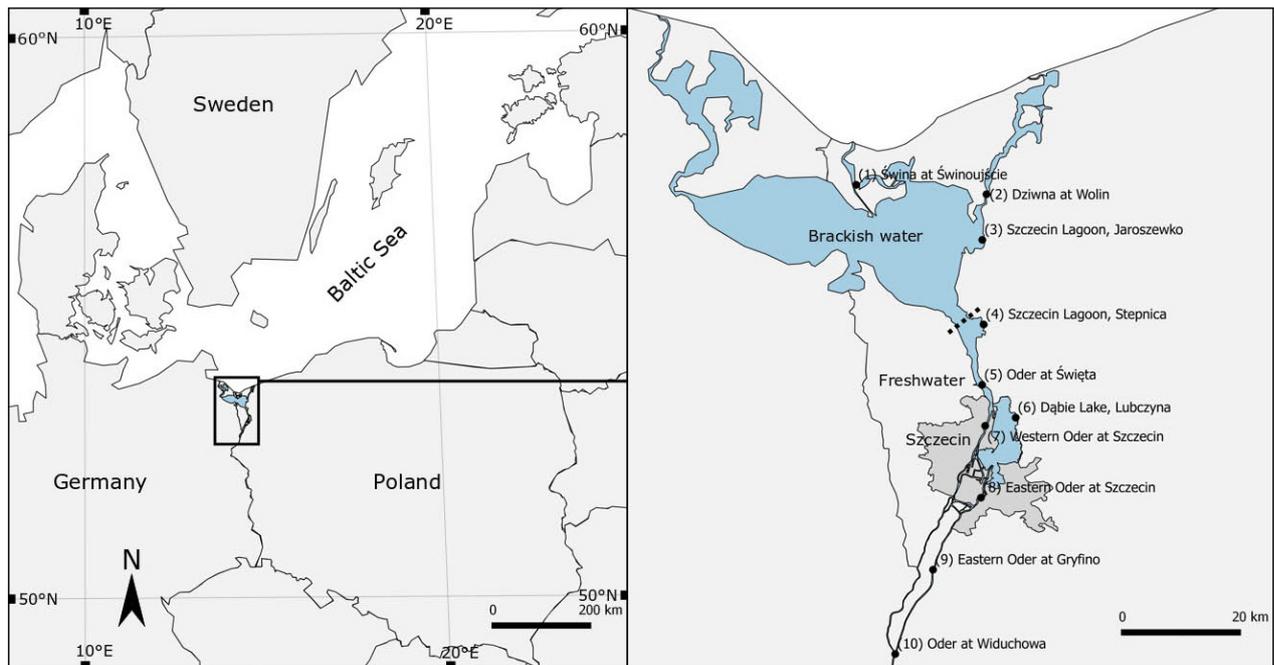


Figure 1. Map of sampling sites in the Oder River system (Central Europe). Dotted line shows the maximum range of brackish waters (Kowalewski and Kowalewska-Kalkowska 2011).

characteristics described by Alekseev and Souissi (2011). As the present work focused only on *E. affinis* complex, *Eurytemora velox* (Lilljeborg, 1853) found during the study was not taken into consideration.

The specimens were selected in a plankton chamber using a Zeiss Primo Vert reverse microscope (Germany), and measurements were performed only on females. Each specimen ($n = 91$) was dissected into parts, of which one was used for morphological studies (from the fourth somite to furca) and the rest for molecular analysis. Remains from the morphological analysis were transferred to glycerin and mounted in slides. Copepods were identified, and morphological measurements were made in multiple digital images obtained using a Nikon Eclipse 50i microscope (Japan) equipped with ToupView software (ToupTek Photonics, China). As the number of *E. affinis* s.str. specimens was insufficient (one adult female, and three adult males), we conducted our molecular and morphological analyses only on *E. carolleae*.

The fourth and fifth pair of legs and furca were inspected to determine the number of setae and spines. In the fifth legs, we measured the inner outgrowth (IO), distal segment (DS), long seta (LS), and spines (S1–S3) to characterize the morphological features (Figure 2). All traits were measured on both sides of the body (left and right). Specimens that were suspected to be mechanically damaged by sampling or manipulation in the laboratory were not included in the study.

To determine the morphological variation of the population, we analyzed the: (i) variability of the fifth leg size—by measuring all the traits of P5; (ii) FA of the fifth pair of legs—calculated as a percent of variance between the left and the right leg; and (iii) ratio between the parameters of the fifth

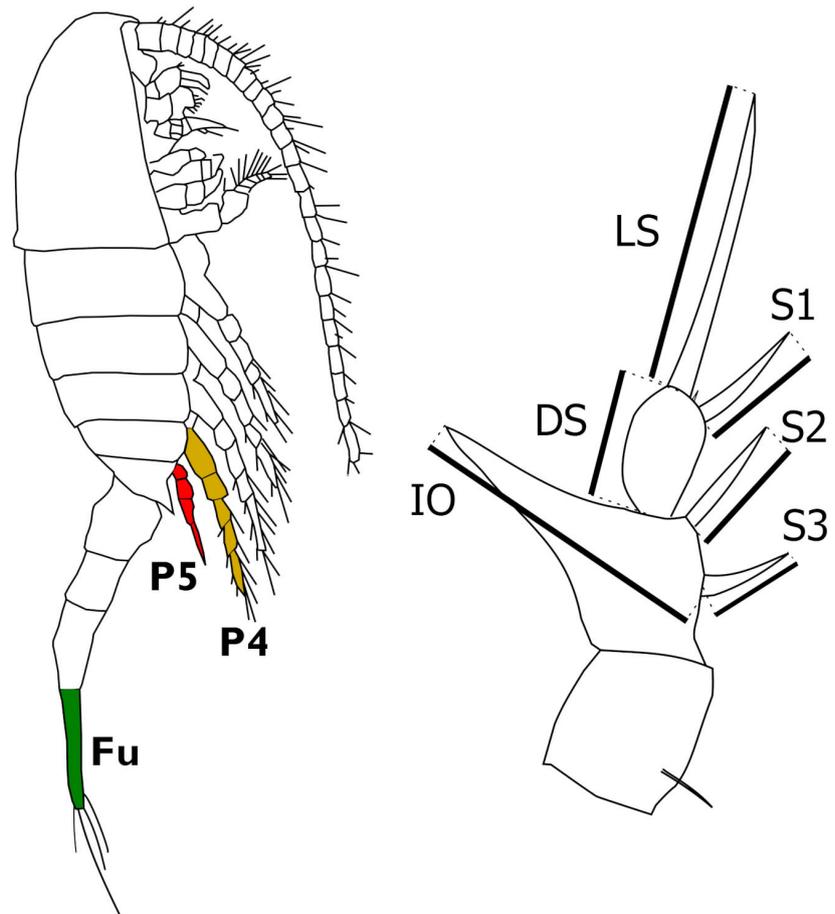


Figure 2. Morphological parts of the female body and features of the fifth legs used to analyze the variability of *Eurytemora*. P4 – fourth pair of legs, P5 – fifth pair of legs, Fu – furca, IO – inner outgrowth, DS – distal segment, LS – long seta, S1–S3 – spines.

legs—calculated as a proportion of certain traits of P5. In addition, during the morphological assessment, we observed that a large share of the population had various types of deformations; therefore, we took into account another factor—morphological deformations.

The *t*-test was used to find significant differences between morphological characteristics among seasons and type of water. To assess the heterogeneity of the of FA of P5, we used analysis of variance with Tukey’s honestly significant difference post hoc test (Statistica 12, StatSoft).

Total DNA was isolated from 45 single adult *E. carolleae* specimens collected from three localities (Dziwna, Święta, and Western Oder in Szczecin). Genetic material was obtained using the Tissue Genomic GPB Extraction Mini Kit (GenoPlast Biochemicals). mtDNA and nDNA sequences were used for molecular analyses. Polymerase chain reaction (PCR) was carried out using GoTaq® DNA Polymerase (Promega). The temperature–time profile of PCR was set as suggested by the manufacturer. The following primers were used for the amplification of DNA fragments: LCO1490/ HCO2198 (Folmer et al. 1994) for COI, CYTB151F/CYTB270R (Merritt et al. 1998) for *cytb*, ITS4/ITS5 (White et al. 1990) for ITS1-ITS2

and 18A1/1800 (Raupach et al. 2009) for 18S. The length of the amplified products is shown in Supplementary material Table S2. The PCR products were sequenced at Macrogen Europe (the Netherlands).

The nucleotide sequences of *E. carolleeae* were compared to each other and to the sequences of other species of the genus, namely *E. affinis*, *Eurytemora lacustris*, and *Eurytemora foveola*, as well as to that of the species *Temora longicornis* and *Heterocope appendiculata*, which constituted an outgroup (depending on the availability of appropriate sequences in the database). All the sequences used for molecular analysis are deposited in GenBank. The accession numbers of all sequences used for molecular analyses are listed in Table S2. The method of Maximum Likelihood, implemented in MEGA 7, was used to analyze the relationship (Kumar et al. 2016). Best-fit substitution models were calculated using the algorithm implemented in MEGA 7 for every set of sequences independently: Hasegawa-Kishino-Yano model with discrete Gamma distribution [HKY+G] (Hasegawa et al. 1985) for COI; Tamura 3-parameter model [T92] (Tamura 1992) for *cytb* and 18S rRNA. Due to indels ITS1–ITS2 sequences were validated with Gblocks (parameters used were Minimum Number of Sequences for a Conserved Position, Minimum Number of Sequences for a Flank Position, Maximum Number of Contiguous Nonconserved Positions, Minimum Length of a Block, Allowed Gap Positions) (Castresana 2000). The analysis gave 86% of the aligned nucleotide positions in 12 blocks. The sequences prepared in this way were used to generate phylograms (Kimura 2-Parameter model [K2P] (Kimura 1980). Bootstrap values for the trees were obtained from 1500 randomly generated trees. The genetic distance between polymorphic sequences within a species as well as between species was calculated in MEGA 7 using K2P.

Environmental factors were measured on the same day the invertebrate samples were collected (Table S1). Temperature and the levels of salinity, nitrate, ammonium, and chlorophyll *a* were measured in situ using a Hydrolab DS5 multiparameter probe (USA). Based on the salinity values, we categorized the samples into freshwater and brackish water (if the value of the practical salinity units (PSU) on the station was > 1, we classified the station as brackish). Another factor considered in the analysis was the season – winter (cold season; December–March) and summer (warm season; June–August).

Results

Spatial distribution

The species *E. carolleeae* was observed in high frequency (the percentage of occurrence on site) from Szczecin to straits connecting the waters of the Szczecin Lagoon with the Baltic Sea (stations 1–8) (Table S1). The highest occurrence of *E. carolleeae* was observed in Dziwna in Wolin (100%) followed

Table 1. Percentage of deformed appendages in the female individuals of *Eurytemora carolleae* Alekseev and Souissi, 2011 depending on the water characteristics of the Oder River system.

	Sample size	Fourth pair of legs	Fifth pair of legs	Furca	All considered appendages
Brackish water (warm season)	28	10.7	7.1	10.7	28.6
Freshwater	63	4.8	6.3	6.3	15.9
– warm season	29	5.9	2.9	5.9	14.7
– cold season	34	3.4	10.3	6.9	17.2
All samples	91	6.6	6.6	7.7	19.8

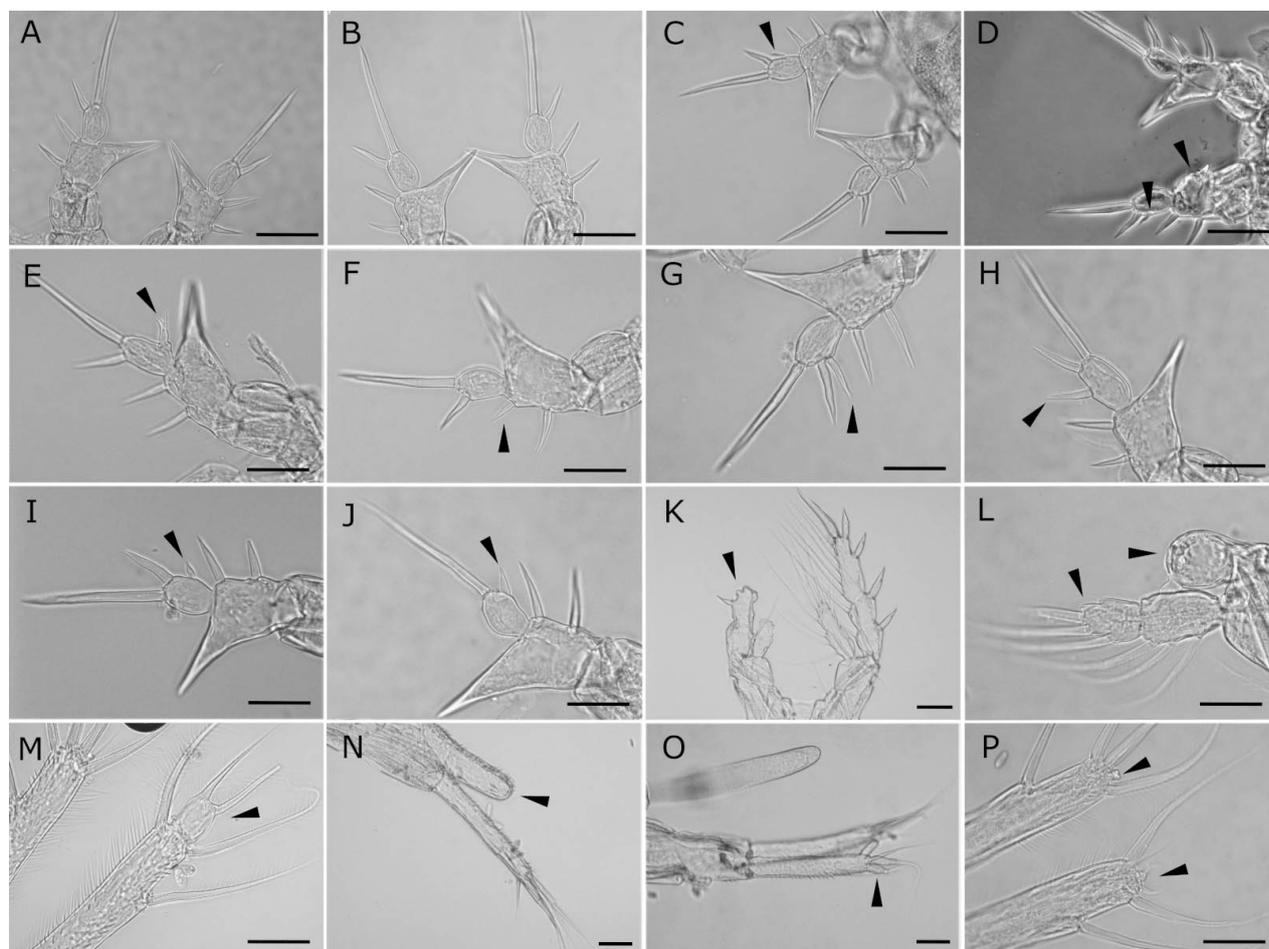


Figure 3. Morphological deformations of *Eurytemora carolleae* in the Odra River system: A, B – regular fifth pair of legs; C–J – fifth legs; K, L – fourth legs; M–S – furca. Arrows indicate anomalies and deformations. Scale bar: 50 μ m.

by Western Odra in Szczecin (88%), whereas the lowest frequency was found in Eastern Oder in Szczecin (17%) followed by Dąbie Lake (67%). The species *E. affinis* s.str. was found at only one station (Świna), which is the most saline, with a very low frequency (17%).

Morphological parameters

Morphological deformations

Among the 91 females of *E. carolleae* subjected to morphological analysis, almost 20% showed at least one morphological deformation out of the three parameters (Table 1 and Figure 3). The largest share of deformed specimens was recorded in brackish waters (warm season) (28.6%), which

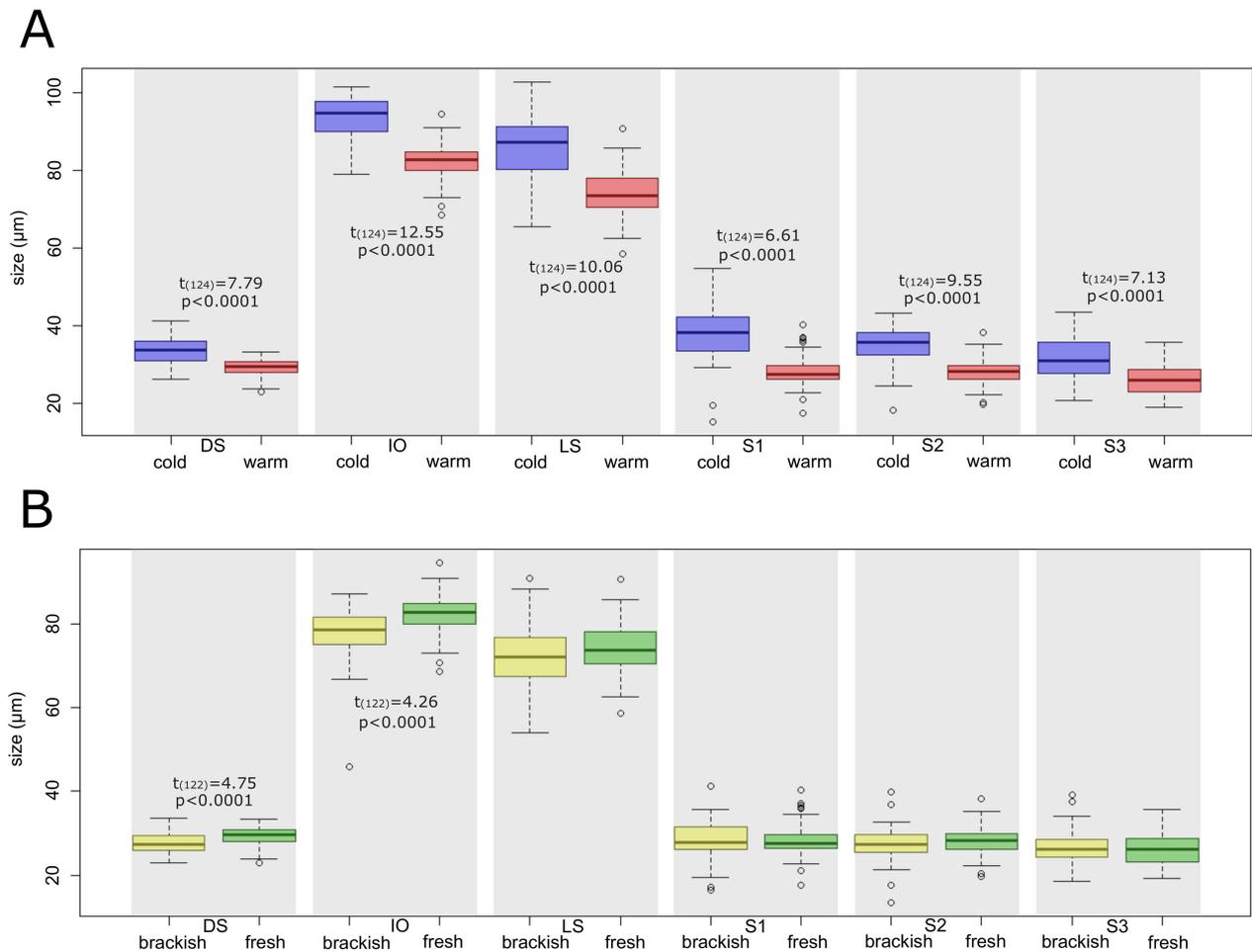


Figure 4. Boxplot distribution of the size of fifth leg parameters. A – cold versus warm season; B – freshwater versus brackish water. DS – distal segment, IO – inner outgrowth, LS – long seta, S1–S3 – spines. A paired *t*-test was used to compare the differences between parameter size in different types of water (only significant values are shown; $p < 0.05$).

was considerably higher compared to freshwater (mean value for the cold and warm season) (15.9%). Compared to the same research period (warm season), the proportion of deformed individuals in freshwaters was even lower and amounted to 14.7%, while in the winter period it was 17.2%. The share of deformed appendages in *Eurytemora* was similar for all the examined body parts (ca. 7%).

Variability of the fifth leg size

The parameter fifth leg size was mainly associated with the season rather than with the salinity of water bodies (Figure 4). In cool waters, each of the tested parameters was characterized by statistically significantly larger values than in warm waters ($p < 0.001$). In freshwater, the sizes of DS and IO were significantly greater than in brackish waters ($p < 0.001$), while the other parameters were similar.

Fluctuating asymmetry of the fifth pair of legs

The variability of parameters between the left and the right leg was not significant in any of the studied groups – with respect to either seasons or

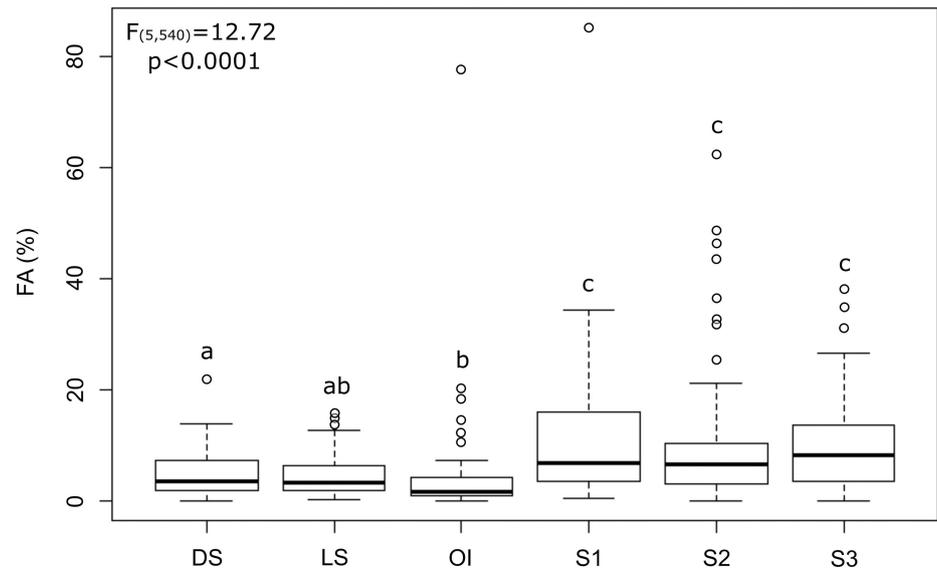


Figure 5. Boxplot distribution of fluctuating asymmetry of fifth leg parameters (left–right ratio). DS – distal segment, IO – inner outgrowth, LS – long seta, S1–S3 – spines. Different letters indicate significant ($p < 0.05$) differences between the values of asymmetry (Tukey’s honestly significant difference test).

water types ($p > 0.05$, data not shown); however, for all three spines (S1–S3), FA was significantly higher compared to the other parameters ($p < 0.05$) (Figure 5). The lowest FA values were determined for IO and the highest for S3.

Ratio between the parameters of the fifth legs

The largest differences between the parameter ratios were found in the comparison based on the season. The differences in IO/S1, IO/S2, IO/S3, LS/S1, LS/S2, S1/DS, S1/S3, S2/DS, and S3/DS ratios were high between seasons ($p < 0.05$) (Figure 6). On the other hand, much smaller differences were found between freshwaters and brackish waters in the case of IO/S3, LS/DS, LS/S3, S2/S3, and S3/DS ratios ($p < 0.05$).

Molecular characteristics

COI/mtDNA

The COI gene was analyzed at 624 bp, and six original sequences (haplotypes) were determined (Table S3). The obtained nucleotide sequences were compared to the corresponding *E. carolleae* sequences from different regions, and their variability was determined. The average genetic distance for *E. carolleae* from Poland was 0.6% (S.E. 0.2). Within the species, the distance was within 0.5% (S.E. 0.2) between the Polish and Russian sequences, and 3.3% (S.E. 0.7) between the Polish and US sequences. Interspecies differences were found as well. The genetic distance between *E. carolleae* and *E. affinis*, the closest related species, was on average 15.1% (S.E. 1.8), while between *E. carolleae* and *E. foveola* the distance was 23.2% (S.E. 2.4). On the phylogenetic tree, these species form the clades of common origin,

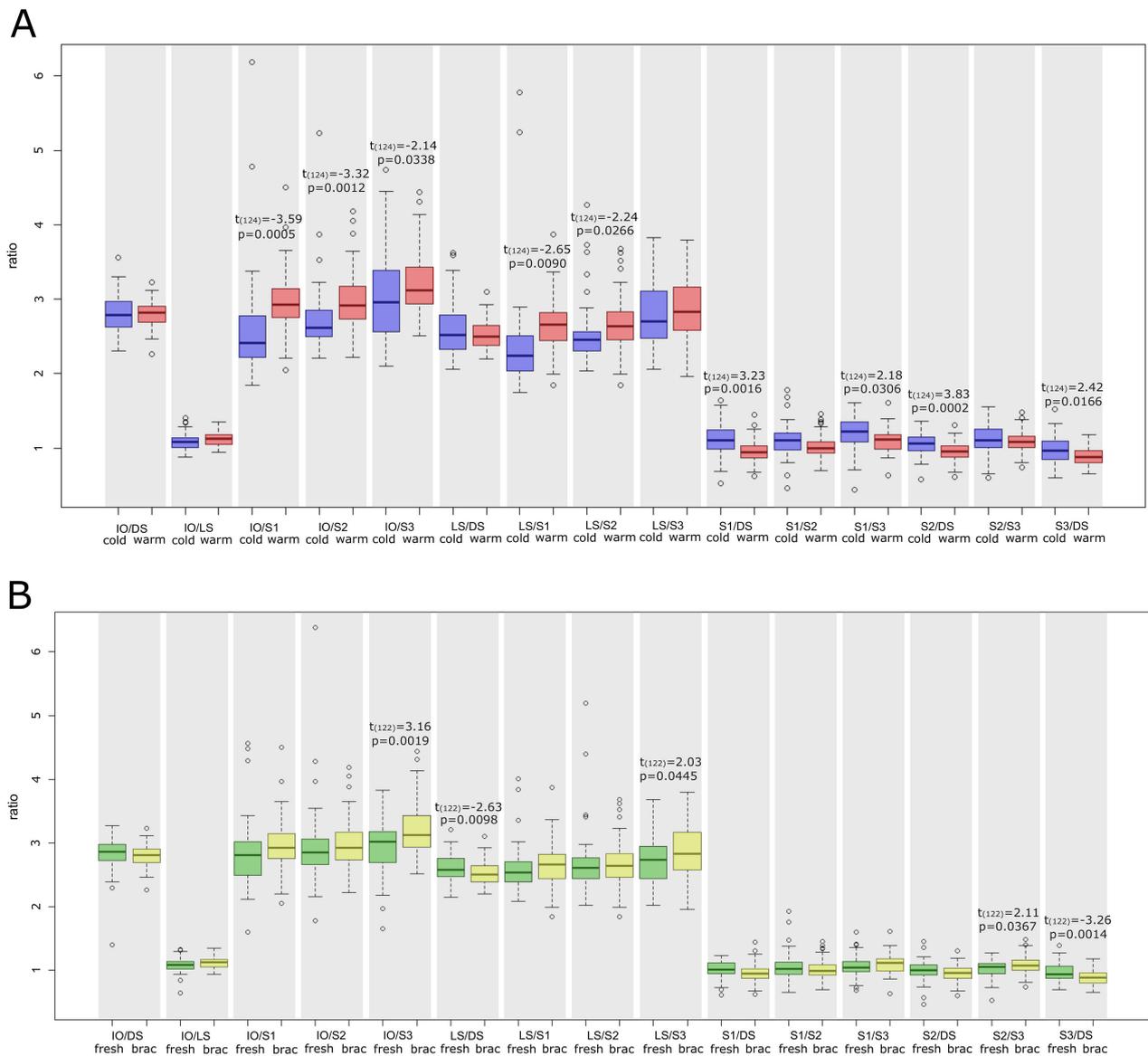


Figure 6. Boxplot distribution of the ratios of fifth leg parameters. A – cold versus warm season; B – freshwater versus brackish water. DS – distal segment, IO – inner outgrowth, LS – long seta, S1–S3 – spines. A paired *t*-test was used to compare the differences between parameter ratios in different types of water (only significant values are shown; $p < 0.05$).

while the other species of the genus *Eurytemora* (*E. lacustris*, *E. foveola*) are grouped separately, forming a monophyletic group (Figure 7).

cytb/mtDNA

Due to the lack of data in GenBank for *Eurytemora* species, cytb analysis was carried out only for the sequences of *E. carolleeae* and the previously described *E. lacustris* and *E. velox* (Slugocki et al. 2021 *unpublished*). The genetic distance within the species was in the range of 0.3% (S.E. 0.6)–1.5% (S.E. 0.7), while the distance between *E. carolleeae* and *E. velox* was 26.4% on average (S.E. 3.3) and that between *E. carolleeae* and *E. lacustris* was 23.3% on average (S.E. 3.0). The sequence system on the phylogenetic tree was identical to that of the tree constructed based on COI (data not shown).

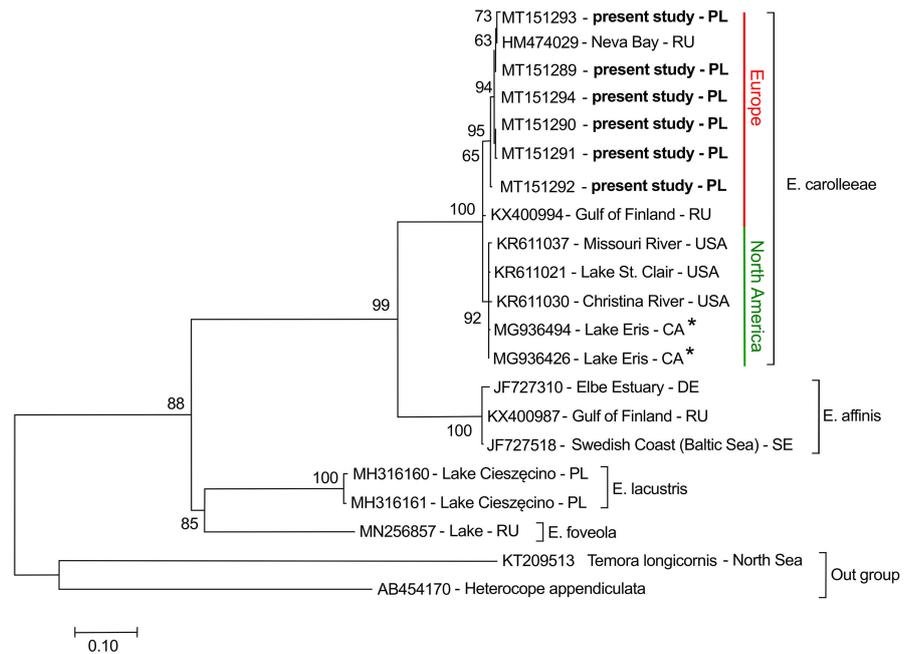


Figure 7. Evolutionary relationship of taxa based on COI sequences. The analyzes were performed on the basis of own sequences and those deposited in GenBank. Country codes for individual tanks according to international lists. Asterisks indicate records from GenBank that were described as a *Eurytemora affinis*.

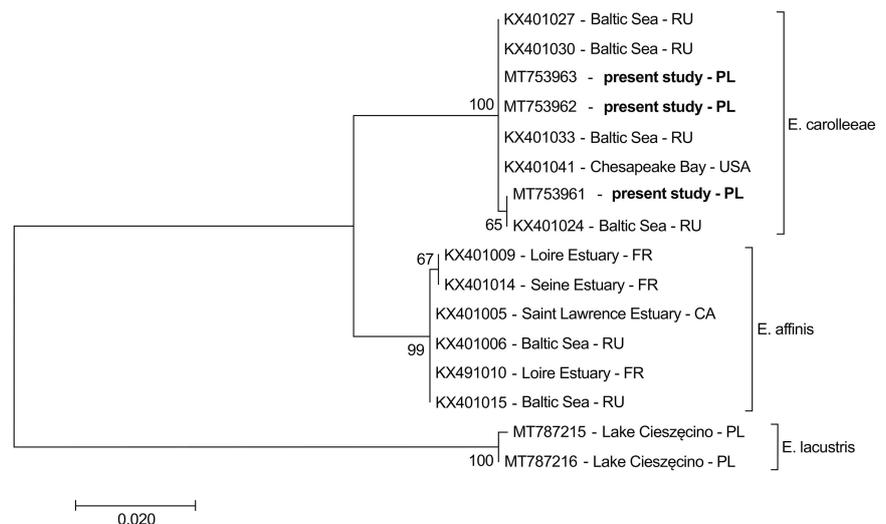


Figure 8. A phylogram constructed on the basis of diagnostic nDNA fragments – ITS1–5,8S rRNA-ITS2. The analyses were performed on three species for the genus *Eurytemora*, the only ones available from GenBank.

ITS/nDNA

The ITS1–ITS2 sequences were obtained for 12 *E. carolleae* specimens. Differences in the fragment length resulting from indel-type mutations, as well as those concerning individual nucleotides (SNP), were observed between *E. carolleae*, *E. lacustris*, and *E. affinis* species. Within the amplified ITS sequence of *E. carolleae*, three genetic variants (alleles) were distinguished.

Original sequences from a given geographical region were selected for constructing the phylogenetic tree and estimating the genetic distance (Table S2, Figure 8). Sequences of *E. carolleae* from the USA, Russia, and

Poland are monophyletic. Pairwise distance between Polish and Russian or American sequences within the species was 0.1%, while the distance between *E. carolleae* and *E. affinis* was 3.7% (S.E. 0.7) and that between *E. carolleae* and *E. lacustris* was 17.3% on average (S.E. 1.6).

18S rRNA/nDNA

Amplification of the gene encoding the small nuclear subunit of the 18S rRNA with primers resulted in a fragment 1,691-bp long, which corresponds to almost the entire length of the gene. The resulting nucleotide sequence was compared to the corresponding ones deposited in GenBank for the species *E. affinis*, *E. carolleae*, and *E. lacustris*. Between the analyzed sequences, 23 SNP-type mutations were observed. The genetic distance between *E. carolleae* and *E. lacustris* calculated from the 18S rRNA gene sequence was 1.3% (S.E. 0.3). No nucleotide differences were observed between *E. carolleae* and *E. affinis* in the analyzed 1,691-bp segment. The phylogenetic tree (data not shown) constructed for the genus *Eurytemora* indicated the high affinity of *E. carolleae* and *E. affinis*, which confirms our previous analyses, both based on nDNA (ITS sequences) and mtDNA (COI or cyt b).

Discussion

The species *E. carolleae* was dominant in the *E. affinis* complex in the Odra River system. *Eurytemora affinis* has only been recorded in the most saline part of the river system. This study reports for the first time the expansion of invasive *E. carolleae* into the freshwater basins in Central Europe. Due to the lack of extended research on the calanoids of the Odra River system in recent years, it is impossible to determine when *E. carolleae* inhabited and spread into the waters of the lower section of the Odra River, Szczecin Lagoon, and Dąbie Lake.

In North America, *E. affinis* is rarely found in freshwaters, while *E. carolleae* inhabits both brackish waters and freshwaters (Winkler et al. 2008; Lee et al. 2011; Engström-Öst et al. 2017), which indicates the spatial segregation of this species. In European waters, the spatial segregation of *E. affinis* populations was observed in Scheldt estuary (Belgium/the Netherlands) (Gasmi et al. 2014). It was suspected that the tributaries of this estuary could be inhabited by *E. carolleae*, but this was not confirmed morphologically or genetically. In the present study, we demonstrated the spatial segregation between *E. affinis* and *E. carolleae* in the Oder River system. However, due to the low salinity of this river system, the border between *E. affinis* and *E. carolleae* lies at the most saline junction of sea and lagoon waters.

Lee et al. (2013) found that high concentrations of specific food could enable the invaders of *Eurytemora* from brackish water to greatly extend into freshwater habitats. Eutrophic waters of the Odra River system seem to be a suitable habitat for the expansion of *E. carolleae*. Based on the

history of its expansion after connecting certain water bodies in America (Mills et al. 1993), further expansion of *E. carolleae* upstream of the Odra River can be expected. We speculate that this expansion process may be strengthened by the construction of dams in the lower section of the river system, which often leads to the homogenization of zooplankton communities in the river networks (Sługocki et al. 2021).

Szczecin Lagoon is subject to fluctuations by environmental factors, including salinity, which is largely dependent on meteorological conditions (Osadczyk et al. 2007). The level of salinity is an important factor determining the structure of invertebrate communities (Devreker et al. 2004). Due to climate changes, increasing water level, more frequent northern winds (BACC II Author Team 2015; Männikus et al. 2020), and the deepening of the fairway (Kotowska et al. 2019), which will facilitate water injection into the Szczecin Bay, changes may occur in the spatial distribution of some estuarine species, including those from the *E. affinis* complex. Such changes, particularly toward species with optimum salinity higher than that of the Szczecin Lagoon, will probably support *E. affinis* s. str. populations.

The size of the *Eurytemora* body is strongly related to that of the appendages, as demonstrated in the example of *E. lacustris* (Poppe, 1887) (Sługocki 2020). Our results showed that season had a greater impact on the morphological features of *E. carolleae* than the habitat type. In the cold season, the size of all the studied morphological traits was the highest which was also related to the changes in proportions (values of ratio) among the traits. The variability of the size of the body and its appendages may be caused by factors such as temperature or oxygen concentration. These factors could affect the body size of the organisms which has already been demonstrated in both terrestrial and aquatic organisms (Kozłowski 1992; Karpowicz et al. 2020). Pierson et al. (2016) investigated *E. carolleae* populations in Chesapeake Bay (USA) in winter concluded that colder conditions resulted in larger individuals of the species. However, in present study both season and water type had no impact on FA in P5 of female *E. carolleae*. Nevertheless, the highest differentiation was noted in the spines (S1–S3). According to Lajus et al. (2020), the FA of the spines is mainly forced by stochastic components. Moreover, Lajus et al. (2020) revealed that the populations of *E. carolleae* in the Baltic Sea (Gulf of Finland) had lower FA values than in the native habitats (Chesapeake Bay, USA). The authors suspected global warming as a factor which elevated the temperatures in Chesapeake Bay beyond the optimal conditions of *E. carolleae*. Our results indicate the limited impact of temperature on FA, which could suggest that other factors may play a role in shaping the FA of *Eurytemora* in the Oder River system. Further investigations are necessary to evaluate the main factors that influence the FA of these species.

We found a high share of morphologically deformed specimens of *E. carolleae* in the samples. The deformations were two times more frequent

in brackish water than in freshwater. However, molecular analyses showed no differences between deformed and normal individuals. Behrends et al. (1997) studied *Acartia* population in the Baltic Sea and showed a high proportion of deformations in the fifth pair of female legs. For various species of the genus *Acartia*, the percentage of abnormalities of the fifth pair of legs ranged from 0% to 16%. The authors concluded that deformations were not caused by contamination, and a high proportion of deformations is natural for this species. Souissi and Souissi (2020) found that under laboratory conditions, *E. affinis* do not demonstrate morphological deformations, even when factors as temperature and salinity were stressful. The inlet section of the Odra system is characterized by large salinity fluctuations, which is mainly the result of storm inflow (Kowalewski and Kowalewska-Kalkowska 2011). For some zones of the Szczecin Lagoon, the amplitude of salinity changes may amount to 2 PSU for just a few hours. Hence, it is presumed that a high share of deformed individuals in the brackish waters may be related to large fluctuations of salinity or other water components accompanying this fluctuation, causing morphological alterations. Stability of populations at a given site is often determined by their abundance (Czerniawski et al. 2016; Karpowicz et al. 2020). However, the abundance of copepods at a given sampling site largely varies depending on the season, time of day, or sampling method (Czerniawski et al. 2016; Labuce et al. 2020). Therefore, it seems that morphological features indicating whether the conditions are suitable for a given organism provide necessary information for assessing population stability, especially if there are no historical data. In our study, we found that *E. carolleae* exhibited greater morphological stability in freshwater than in brackish water. It could mean that the freshwater section of the Oder River provides the optimal environment for the invasive *Eurytemora*, which might lead to further expansion of this species.

Recent separation of *E. carolleae* from *E. affinis* species complex could cause problems with morphological identification of the species, especially in the non-native regions. Therefore, molecular tools can be useful in the proper identification of species. Due to the fact that *E. carolleae* is relatively a newly described species, there are artifacts in the databases and literature that should be systematically verified. For example, we suspect that *E. affinis* found in freshwater habitats of the Oder River system in 2003–2005 (Chojnacki and Tyluś 2013) was in fact an *E. carolleae*. A similar problem was pointed out by Vasquez et al. (2016) when analyzing the *Eurytemora* collection obtained from Laurentian Great Lakes in the 1950s. Moreover, the authors showed that COI analysis indicated sequential differences between *E. affinis* and *E. carolleae* at a level of 14%, and in our study, similar to the work of Sukhikh et al. (2019), the genetic distance was slightly higher (15%). Phylogenetic trees based on COI distinguished two subclades of *E. carolleae*, separating native and non-native regions. Although

previous studies demonstrated rapid evolutionary processes in this clade (Lee 1999, 2016) it is still not precisely known when the *E. carolleae* species settled in new European habitats.

The databases for the COI of *Eurytemora* are relatively rich (Winkler et al. 2011; Sukhikh et al. 2013, 2019, 2020; Vasquez et al. 2016; Sługocki et al. 2019). Unfortunately, for other genes or noncoding, diagnostic DNA fragments, there are few or no data. The *cytb* gene sequence of *E. affinis* species complex has not been reported so far, whereas a few sequences have been reported for ITS and 18S rRNA (Sukhikh et al. 2019, 2020). Therefore, new records for *cytb* expand the range of available molecular markers, which allows for even more in-depth research on this taxon.

Molecular data analyses of the ITS1–5.8S rRNA–ITS2 region of several species belonging to the genus *Eurytemora* (*E. affinis*, *E. carolleae*, and *E. lacustris*) enabled species-level differentiation, as previously demonstrated by Sukhikh et al. (2020). Due to the variability of these nuclear sequences, which come from both parents, as opposed to mitochondrial markers, it was possible to perform rapid molecular identification within the genus. For the conservative 18S rRNA gene, identification is possible for higher taxonomic units. Although we performed differentiation within the genus *Eurytemora*, the sequences of 18S rRNA gene did not differ between *E. affinis* and *E. carolleae* species which was also previously reported by Sukhikh et al. (2020).

Regardless of the marker used, we observed a high affinity for *E. carolleae* and *E. affinis*. At the same time, our molecular analyses, as well as those of other researchers reported in recent years (e.g. Vasquez et al. 2016; Sukhikh et al. 2020), clearly indicated the need for both classical and molecular studies. This seems to be important for studying the invasions and biodiversity of aquatic ecosystems, and especially microinvertebrates that are often overlooked. Due to the new invasion of *E. carolleae* in the freshwater habitats of Central Europe, it is necessary to monitor the invasion and assess the impact of this taxon on the native fauna.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Locations sampled within the Oder River system for populations of *Eurytemora affinis* complex in years 2019–2020.

Table S2. List of accession numbers for *Eurytemora* spp. sequences from West Pomerania (PL) and sequences from other regions (GenBank) used in molecular analyses.

Table S3. Sequence polymorphism for the COI fragment of *E. carolleae*.

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