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Morphological Differentiation, Mitochondrial and Nuclear DNA Variability Between Geographically Distant Populations of *Daphnia galeata* and *Daphnia cucullata* (Anomopoda, Daphniidae)

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Although members of genus Daphnia (Anomopoda, Daphniidae) are the most common water invertebrates and are considered as model organisms for many taxonomic, ecological and evolutionary studies their systematics remains unresolved. Here, morphological differentiation and genetic polymorphism between the geographically distant populations of the sister species Daphnia galeata Sars, 1864 and Daphnia cucullata Sars, 1862 in the Curonian Lagoon, a large shallow freshwater lagoon of the Baltic Sea (Russia, Kaliningrad Oblast) and Novosibirsk Reservoir (Russia, Novosibirsk Oblast) are presented. The divergence between species and their populations was analyzed based on traditional morphological traits and a large set of morphometric traits describing the body shape. The traits describing the shape of head and helmet, and spine were the most variable morphological characters. Phylogenetic relationships between species and populations were constructed based on variation in mitochondrial 16S and 12S rRNA genes and nuclear ITS2 rDNA sequences. The mitochondrial DNA divergence between D. galeata and D. cucullata species was significant and reflected their monophyletic origin, whereas intraspecific genetic distances are estimated as insignificant.

Keywords: Daphnia galeata, Daphnia cucullata, morphological variation, mitochondrial DNA, nuclear DNA, genetic divergence

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Introduction

Cladoceran of genus *Daphnia* (Anomopoda, Daphniidae) are the most common invertebrates in water ecosystems. Many species of this genus are used as model organisms in the different field of biology including toxicology, biogeography, and evolutionary ecology. The most reliable taxonomic keys of some *Daphnia* species were developed by S.M. Glagolev (1986). However, the systematics of many *Daphnia* species complexes remains unresolved and morphological distinction between some species is often lacking. The main cause of taxonomic confusion consists in remarkable morphological plasticity in response to ecological and genetic factors. The body shape, helmet and tail spine sizes were shown to depend on water temperature, turbulence, quantity of available food, and presence of invertebrate and vertebrate predators (Hebert, Grewe, 1985; Mort, 1989; Sorensen, Sterner, 1992; Burns, 2000; Lass, Spaak, 2003; Laforsh, Tollrian, 2004). Both considerable morphological variability and similarity may be due to interspecific hybridization and introgression as it was shown for species of *Daphnia longispina* complex based on genetic studies (Taylor, Hebert, 1992; Colbourne, Hebert, 1996; Schwenk et al., 1998; Gieβler et al., 1999; Schwenk et al., 2000; Hobæk et al., 2004; Gießler, Englbrecht, 2009). At present time both mitochondrial and nuclear genetic markers have a wide use for delineation of *Daphnia* species and phylogenetic relations assignment between them (Taylor et al., 1996; Schwenk et al., 1998; Gieβler, 2001; Duffy et al., 2004; Petrusek et al., 2008). These studies deal with both geographically limited and distant *Daphnia* populations inhabiting different waterbodies of Western Europe and North America. Meanwhile, the study of genetic diversity of *Daphnia* populations from Russian water bodies is extremely shallow (Bychek, Müller, 2003; Kotov et al., 2006; Ishida, Taylor, 2007). Besides, often genetic studies of daphniids are not confirmed by analysis of the taxonomic traits, therethrough generate obvious mistakes in species identification. Different statistical methods on quantitative and qualitative morphological data sets were successfully applied to reveal traits useful for species delineation (Dodson, 1981; Schwartz et al., 1985; Benzie, 1988; Gieβler, 2001; Duffy et al., 2004).

The purpose of this study is to perform comparative morphological analysis of the body shape variability using multivariate statistical method and to evaluate the variability of the 16S and 12S mitochondrial DNA and the ITS2 nuclear DNA markers in geographically distant populations of sister species *D. galeata* Sars, 1864 and *D. cucullata* Sars, 1862 (*D. longispina* complex) from Novosibirsk Reservoir of West Siberia and the Curonian Lagoon of the Baltic Sea.

Materials and Methods

Study areas

Novosibirsk Reservoir (54°28′N, 82°23′E) is a large artificial water body in the Ob River's valley located in two regions: Novosibirsk Oblast and Altai Territory. Some reservoirs characteristics are given in Table 1. In winter this water body is covered by ice in the whole. According to literature data zooplankton community was originated from zooplankton of drowned flood-plane water bodies belonging to the river channel. The reservoir is used for recreation and fishing. In different periods of the reservoir's formation three species *D. longispina*, *D. cucullata*, and *D. hyalina* among genus *Daphnia* were identified (Solonevskaya, 1961; Bityukov, 1964; Pomerantseva, 1976; Kotikova, 1985). At present *D. cucullata* and *D. longispina* inhabit in the lacustrine part of the reservoir and *D. cucullata* has being dominated since 1995 (Ermolaeva, 2007).

Table 1. Some characteristics of the waterbodies investigated, specimens number in morphological and genetic data sets

Waterbody	Altitude (m a.s.l.)	Area (km^2)	Volume (km^3)	Mean depth (m)	Max depth (m)	*D. galeata*				*D. cucullata*			
						Morphology sample	16S	12S	ITS2	Morphology sample	16S	12S	ITS2
Novosibirsk Reservoir	113	1082	8.8	8.3	25.0	75	7	6	7	4	3	4	4
Curonian Lagoon	0	1584	6.2	3.8	5.8	71	17	19	10	31	3	3	3

The Curonian lagoon (55°18′N, 20°55′E) is a large shallow freshwater lagoon of the Baltic Sea is subjected to strong anthropogenic impact. Some characteristics of the lagoon are provided in Table 1. The continuing eutrophication of the lagoon is accompanied by water "hyperbloom" under the mass development of blue-green algae (Alexandrov, Dmitrieva, 2006). Their biomass significantly exceeds the level conditioning the secondary pollution of the water body in some year. According to hydrochemical data and the structural and functional characteristics of zooplankton, the Curonian Lagoon belongs to eutrophic water bodies with a transition to a hypereutrophic stage (Alexandrov et al., 2006; Semenova, Alexandrov, 2009). This water body is covered by ice for a short winter period. According to literature data several *Daphnia* species were registered in the Curonian Lagoon, namely *D. longispina*, *D. hyalina, D. cucullata, D. cristata*, and *D. pulex* (Szidat, 1926; Schmidt-Ries, 1940; Kiselite, 1957; Naumenko, 1994; Pliuraite, 2003). At present, *D. galeata* is dominant species and *D. cucullata* is subdominant one.

Sampling

For studies of morphological and genetic variability of *Daphnia* specimens in Novosibirsk Reservoir the zooplankton samples were taken in August, 2008 with the Apstein net (mesh size 250 μm). For studies of morphological variability of *Daphnia* in the Curonian Lagoon we used the samples collected from April to September, 2008. For study of their genetic polymorphism the samples were collected in May-June and September, 2009. In the Curonian Lagoon the samples were taken with a Van-Dorn bathometer.

The samples were preserved in 5 % (or 4 %) formalin solution with sucrose (Haney, Hall, 1973) for morphological and morphometric analyses. For genetic analysis of *Daphnia* species zooplankton samples were stored directly in ethanol (90-95 %) until DNA was extracted.

Morphological analysis

Daphnia species were identified according to the keys presented in the recent literature (Glagolev, 1986; Flößner, Kraus, 1989). Females of *D. galeata* и *D. cucullata* in the forth or fifth age-size groups were photographed for digital morphological analysis in lateral view under AxioScan microscope (Carl Zeiss, Germany) (×50 or ×100 magnitude) (for sample size see Table 1) To analyze a body shape 23 morphological measurements were made using the digital images with the AxioVision software. The morphometric characters were taken according to the set given in Zuykova, Bochkarev (2010). Three characters were additionally used, namely,

the distance from center of the eye to the point of tail spine attachment (*O.l.t.sp.*), the distance from the antennulae tip to the rostrum tip (*a.r.*) and the helmet angle (*helmet angle*).

A principal component analysis (PCA) was performed to estimate morphological variation just as it has been done for other *Daphnia* species (Schwartz et al., 1985; Benzie, 1988). This analysis calculates new variables (principal component) which are linear combinations of the original characters and allows distinguishing the most significant characters. Obtained variables were normalized and centered. The components were estimated as new traits, and then an average loading value, an error in mean, and a standard deviation were calculated for each sample. To estimate the significance of morphological divergence between all *Daphnia* samples based on the average loading values the Student *t*-test was applied (Efimov, Kovaleva, 2005). As the first principal component accounts for the most variation and explains the size variability, hence the body shape parameters between the *Daphnia* samples were analyzed in the space of the second and third PCA axes. The PCA variables were used as input in UPGMA analysis to estimate the divergence among the samples. All statistical analyses were performed using STATISTICA version 6.0 (StatSoft Inc., USA), SNEDECOR version 5.0 (ODS Soft, Novosibirsk, Russia), and PAST version 2.05 (http://palaeo-electronica.org) softwares.

DNA analysis

Ethanol-preserved animals were used for analysis of nucleotide polymorphism. Total DNA was extracted from a single individual (female or male) or an ephippium using a 5 % suspension of Chelex 100 resin (BioRad). Before use in PCR the extracted DNA was stored under -20˚C. The polymerase chain reaction was used to amplify the 16S and 12S mitochondrial genes and the ITS2 region of nuclear DNA including part of flanking 5.8S and 28S ribosomal RNA genes. The primers and conditions for PCRs in a 20 µl reaction volume were as following: 2-5 µl DNA homogenate, 0.2 µM dNTPs, 2 µl 10× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mMKCl), 2.5 mM $MgCl_2$, 0.5 µM of each primer and 1 unit of *Thermus aquaticus* DNA polymerase (*Taq*-pol).

The 16S gene was amplified using the originally designed primers:

16Sin-F 5'-TTTGTAAATGGCCGCAGTA-3' and

16Sin-R 5' -CGGTTTGAACTCAGATCAT-GTA-3'.

A thermocycler (BIS-N, Novosibirsk, Russia) was run for 2 min at 94 °C (1 cycle), followed by 30 s at 94 °C, 30 s at 56 °C, 1 min 45 s at 72 °C (35 cycles) and extension for 2 min at 72 °C.

The 12S gene was amplified using the primers:

12S-F 5'-ATGCACTTTCCAGTACATCTAC-3' and

12S-R 5'-AAATCGTGCCAGCCGTCGC-3' (Colbourne, Hebert, 1996). A thermocycler was run for 2 min at 94 °C (1 cycle), followed by 1 min 30 s at 94 °C, 45 s at 58 °C, 1 min 30 s at 72 °C (35 cycles) and extension for 6 min at 72 °C.

The ITS2 region was amplified using the specially designed forward primer 5.8Fr 5'-CCCTGAACGGTGGATCACTA-3' and a reverse primer according to Taylor et al. (2005) 28SD2BR 5'-TTAGAAGGAGTTTACCTCCCGCTTAGG-3'. A thermocycler was run at 2 min at 94 °C (1 cycle), followed by 1 min at 94 °C, 45 s at 53 °C, 1 min at 72 °C (35 cycles) and extension for 6 min at 72 °C.

The PCR products were separated on 1 % agarose 1× TAE gel (Low EEO Standart agarose, BIOZYM, Russia) in the presence of ethidium bromide and photographed under UV light. A 1-2 kb DNA ladder (MEDIGEN, Novosibirsk, Russia) was used for the estimation of the amplicon length.

The amplified products were purified using a kit from BIOSILICA (Novosibirsk, Russia) and both stands were sequenced on an automated sequencer ABI PrISM 3100 Avant Genetic Analyzer (Applied Biosystems, USA) using Big Dye terminator sequencing kit (Applied Biosystems, USA) at the *Center of DNA Sequencing* of Siberian Branch of the Russian Academy of Science (Novosibirsk, Russia, http://sequest.niboch.nsc.ru). The DNA sequences were first automatically aligned using the CLUSTALW algorithm and then manually edited. The nucleotide sequences of the newly analyzed specimens were deposited in GenBank (see Table 2 for accession numbers).

An estimation of the divergence between sequences and the construction of a neighbor-joining (NJ) phylogram based on Kimura 2-parameters (with pairwise deletion of the gaps and missing sites) was conducted in Molecular Evolutionary Genetics Analysis software version 4.0 (MEGA 4) (Saitou, Nei, 1987; Tamura et al., 2007). One thousand bootstrap replicates were run to assess the statistical support in the tree nodes. Additionally, we analyzed the phylogenetic relationships among individuals using minimum evolution (ME) and maximum parsimony (MP) methods. For comparative analysis the sequences of respective fragments for *Daphnia* species from GenBank database were included into analyses.

Results

Morphological variability

Morphological analysis of the *Daphnia* populations based on the main qualitative characters traditionally used in taxonomic keys (Glagolev, 1986) allowed identification of *D. galeata* and *D. cucullata* species in the Curonian Lagoon and Novosibirsk Reservoir. These characters included the shape of the antennulae mound, insertion and length of aesthetasks, presence of ocellus, the crest in frontal view, rostrum shape and length, head shape near the eye and the ventral margin of the head (Fig. 1, 2). In addition we use some traits of males (Fig. 1 K – P, U, V, Fig. 2 J). Subsequently, analysis of the body shape was carried out based on the morphometric traits describing body shape only.

The body shape of *D. galeata* from the Curonian lagoon was found to be remarkably changeable. At first, this can be explained by seasonal variability, because the morphological analysis was carried out with the samples taken during the whole growing season. The most significant morphological differences concerned helmet size and form. So, *D. galeata* specimens collected in April were characterized by a rounded head or had a very small helmet (Fig. 1 A, F). The individuals collected in May had both a large and medium-scale helmet; in September the individuals with a large helmet were registered only. Thus, the *D. galeata* specimens from the Curonian lagoon were divided into three groups with respect to their helmet size and shape. *D. cucullata* specimens presented the separate forth group (Fig. 1 N – P, V). The sample of *D. galeata* in Novosibirsk Reservoir was more homogeneous. The only significant difference among individuals was related to the helmet size (Fig. 2 A – F). The second group in Novosibirsk Reservoir was presented by *D. cucullata* specimens (Fig. 2 G, H).

Figure 3a displays the morphological divergence between all groups and samples generated by principal component analysis at the space of the first two axes. The first PCA axis was formed by approximately equal positive loadings of all characters and this axis reflects a dimensional variability (69.01 %) in the common *Daphnia* samples (Table 3). The most remarkable differences were registered between all samples of *D. galeata* and *D. cucullata* and between the populations of these species (Table 4). The most significant divergence among all *D. galeata* samples was found between the rounded head

Table 2. List of specimens' designations and corresponding GenBank accession numbers of Daphnia species genetically examined in this study

Species	Label	Country	Location	Developmental stage	Sex	GenBank accession numbers		
						16S	12S	ITS2
1	2	3	4	5	6	7	8	9
D. cucullata	NRCu1	Russia	Novosibirsk Reservoir	adult	female	HM067407	HM100080	HM161684
D. cucullata	NRCu2	Russia	Novosibirsk Reservoir	adult	female	HM067408	HM100081	HM161685
D. cucullata	NRCu3	Russia	Novosibirsk Reservoir	adult	female	HM067409	HM100082	HM161686
D. cucullata	NRCu4	Russia	Novosibirsk Reservoir	adult	female	-	HM100083	HM161687
D. galeata	NRG1	Russia	Novosibirsk Reservoir	adult	female	HM067430	HM100087	HM161698
D. galeata	NRG2	Russia	Novosibirsk Reservoir	adult	female	HM067431	HM100088	HM161699
D. galeata	NRG3	Russia	Novosibirsk Reservoir	adult	female	HM067432	HM100089	HM161700
D. galeata	NRG4	Russia	Novosibirsk Reservoir	adult	female	HM067433	HM100090	HM161701
D. galeata	NRG5	Russia	Novosibirsk Reservoir	adult	female	HM067434	HM100091	HM161702
D. galeata	NRG6	Russia	Novosibirsk Reservoir	ephippium		HM067435	HM100092	HM161703
D. galeata	NRG7	Russia	Novosibirsk Reservoir	ephippium		HM067436	-	HM161704
D. cucullata	CoLCu15	Russia	Curonian Lagoon	adult	female	HM067410	HM100084	HM161681
D. cucullata	CoLCu16	Russia	Curonian Lagoon	adult	female	HM067411	HM100085	HM161682
D. cucullata	CoLCu17	Russia	Curonian Lagoon	adult	female	HM067412	HM100086	HM161683
D. galeata	CoLG1	Russia	Curonian Lagoon	juv	female	HM067413	HM161705	HM161688
D. galeata	CoLG2	Russia	Curonian Lagoon	juv	female	HM067414	HM161706	HM161689
D. galeata	CoLG3	Russia	Curonian Lagoon	adult	female	HM067415	HM161707	HM161690
D. galeata	CoLG4	Russia	Curonian Lagoon	adult	female	HM067416	HM161708	HM161691
D. galeata	CoLG5	Russia	Curonian Lagoon	adult	female	HM067417	HM161709	HM161692
D. galeata	CoLG7	Russia	Curonian Lagoon	adult	female	HM067418	HM100093	HM161693
D. galeata	CoLG8	Russia	Curonian Lagoon	juv	female	HM067419	HM100102	-
D. *galeata*	CoLG9	Russia	Curonian Lagoon	adult	female	-	HM100094	-
D. *galeata*	CoLG10	Russia	Curonian Lagoon	juv	female	HM067420	HM100095	-
D. *galeata*	CoLG11	Russia	Curonian Lagoon	adult	female	HM067421	HM100096	-
D. *galeata*	CoLG14	Russia	Curonian Lagoon	juv	male	HM067422	HM100103	-
D. *galeata*	CoLG18	Russia	Curonian Lagoon	adult	female	HM067423	HM100097	-
D. *galeata*	CoLG19	Russia	Curonian Lagoon	juv	male	HM067424	HM100104	-
D. *galeata*	CoLG20	Russia	Curonian Lagoon	adult	female	HM067425	HM100098	HM161694
D. *galeata*	CoLG21	Russia	Curonian Lagoon	adult	female	HM067426	HM100099	-
D. *galeata*	CoLG22	Russia	Curonian Lagoon	juv	male	HM067427	HM100105	HM161695
D. *galeata*	CoLG23	Russia	Curonian Lagoon	adult	female	HM067428	HM100100	HM161696
D. *galeata*	CoLG24	Russia	Curonian Lagoon	juv	male	HM067429	HM100101	HM161697

Continued table 2

1	2	3	4	5	6	7	8	9
D. *galeata*	Canada		Guelph Lake			AF064187*		
D. *galeata*	Russia		Volga River			AY115492*		
D. *cucullata*	Germany		Thalersee				FJ943783*	
D. *cucullata*	Germany		Klostersee				FJ943782*	
D. *cucullata*	Netherlands		Tjeukemeer				FJ943784*	
D. *cucullata*	Germany		Hattstein Weiher				FJ178307*	
D. *cucullata*	Czech Repub.		Medlov Pond				AF277270*	
D. *cucullata*	Slovenia		Lake Bled				AF277269*	
D. *cucullata*	Russia		Lake Glubokoe				AF277272*	
D. *galeata*	Canada							AY730382*
D. *galeata*	USA							AY730396*
D. *galeata*	USA							AY730397*
D. *galeata*	Japan							AY730398*
D. *galeata*	England							AY730401*
D. *cucullata*	Germany							AY730402*

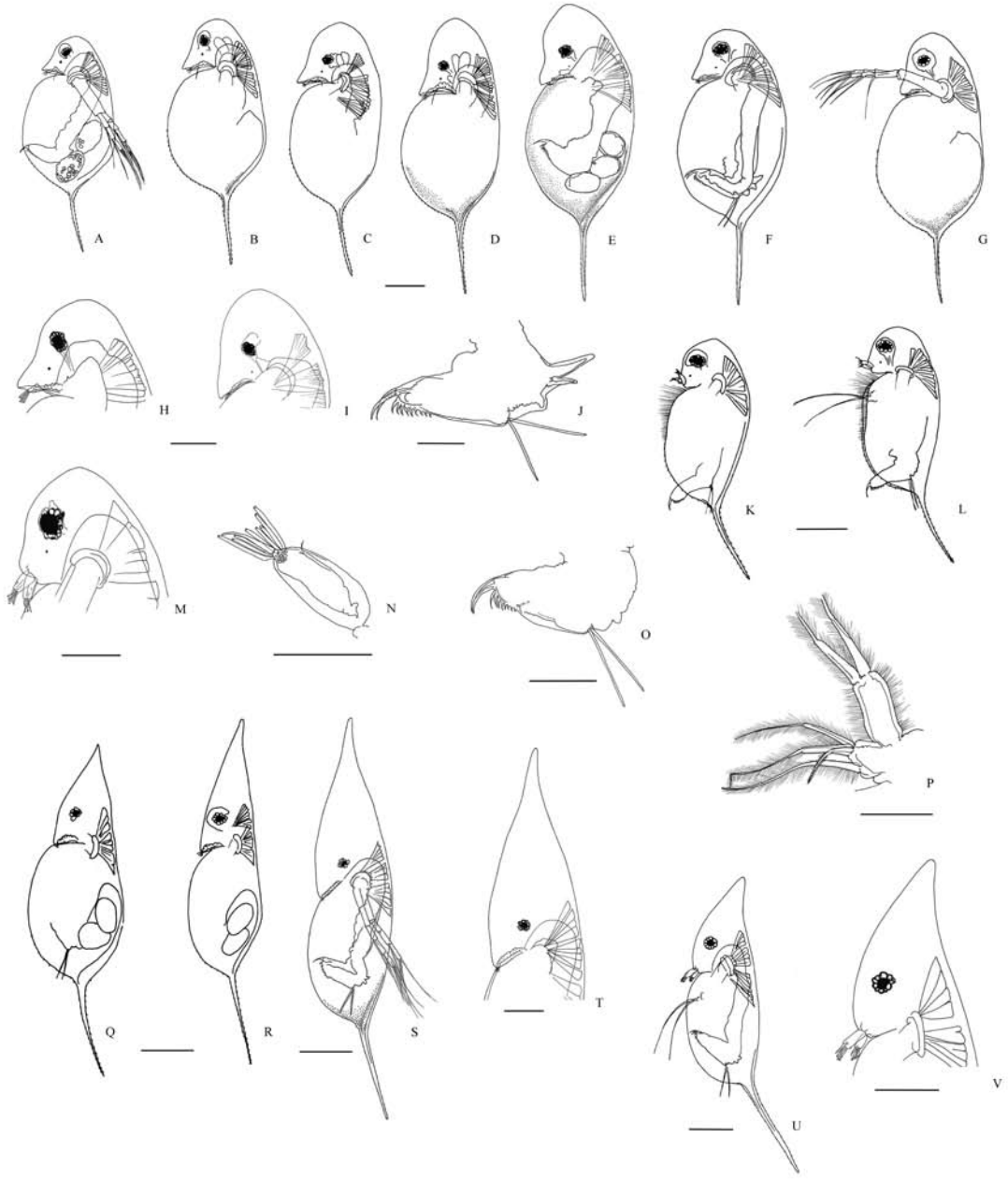

Fig. 1. *Daphnia* morphology from the Curonian Lagoon. *D. galeata* A-P: A-G. female, lateral view; H, I. Head, female, lateral view; J. Postabdomen, female, lateral view; K, L. male, lateral view; M. Head, male, lateral view; N. Antenna I, male; O. Postabdomen, male; P. Limb I, male; *D. cucullata* Q-V: Q-S. female, lateral view; T. Head, female, lateral view; U. male, lateral view; V. Head, male, lateral view. Scale bars 200 μm for A-I, K, L, R, S; 100 μm for J, M-P, T-V

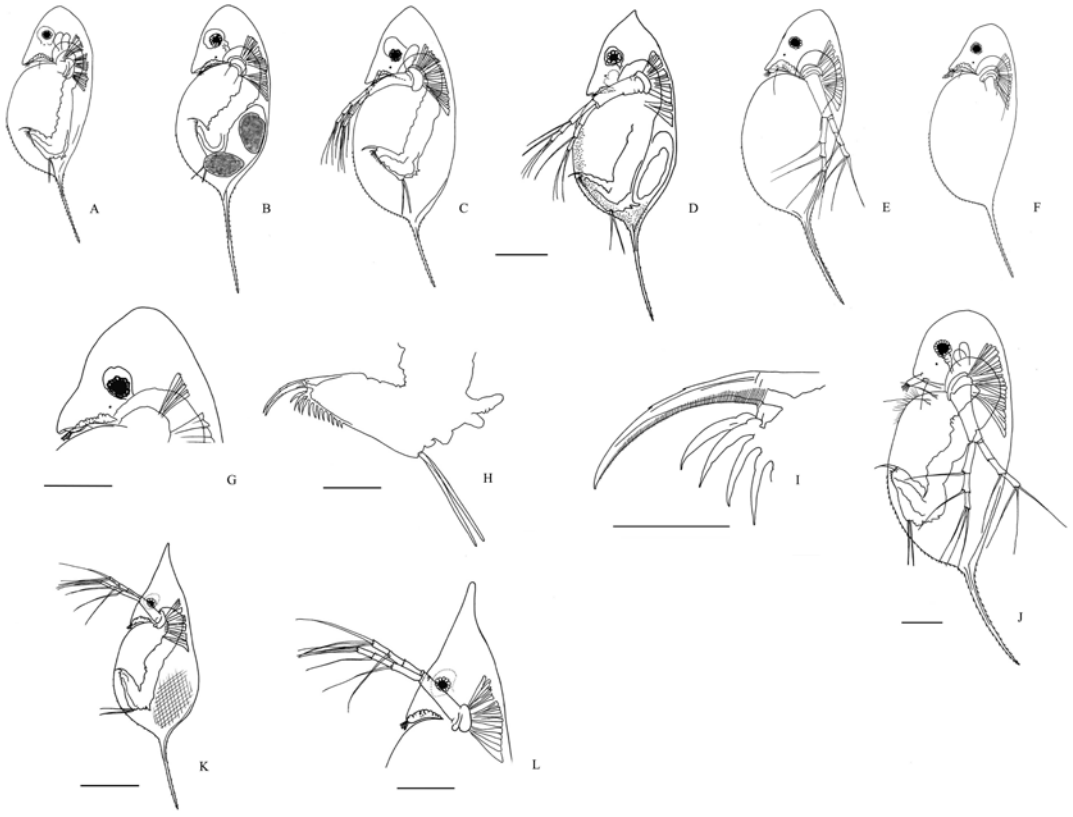

Fig. 2. *Daphnia* morphology from Novosibirsk Reservoir. *D. galeata* A-J: A-F. female, lateral view; G. Head, female, lateral view; H. Postabdomen, female, lateral view; I. Postabdominal claw, female; J. male, lateral view; *D. cucullata* K,L: K. female, lateral view; L. Head, female, lateral view. Scale bars 200 μm for A-G, K; 100 μm for H, J, L; 50 μm for I

form and an intermediate one inhabiting the Curonian Lagoon.

With respect to the second and third PCA axes the morphological divergence between all *D. galeata* samples was smaller, except the rounded head form from the Curonian Lagoon (Fig. 3 b). The second PCA axis (12.44 %) loaded primarily on the head characters (*l.cap.*, *m.v.cap.*), the eye position (*O.m.v.*), helmet size and form (*l.helm.*, *m.v.helm.*, *helmet angle*), and length tail spine (*l.t.sp.*). The third PCA axis (4.30 %) was formed by the loadings of the characters of the eye (*O*, *O.m.v.cap*), helmet form (*m.v.helm.*, *helmet angle*), rostrum form and length (*r.m.v.*, *a.r.*) and the carapace characters (*w.br.*, *r.W.v.*, *w.cap.d.*) (Table 3). Almost all samples and forms significantly differed with respect to the loadings into the third PCA axis, except the *D. cucullata* samples (Table 4).

A dendrogram constructed using average values of the first three principal components suggested that there are three main distinct clusters (Fig. 4). The first cluster consisted of the *D. galeata* specimens from both water bodies. The *D. cucullata* populations comprised the second cluster. Finally, the rounded head form of *D. galeata* from the Curonian Lagoon was separated into a distinct group, mainly due to head shape near the eye and the ventral margin of the head.

Mitochondrial DNA variability

16S mtDNA. For thirty *Daphnia* individuals 481 bp of the 16S gene were sequenced. Additional

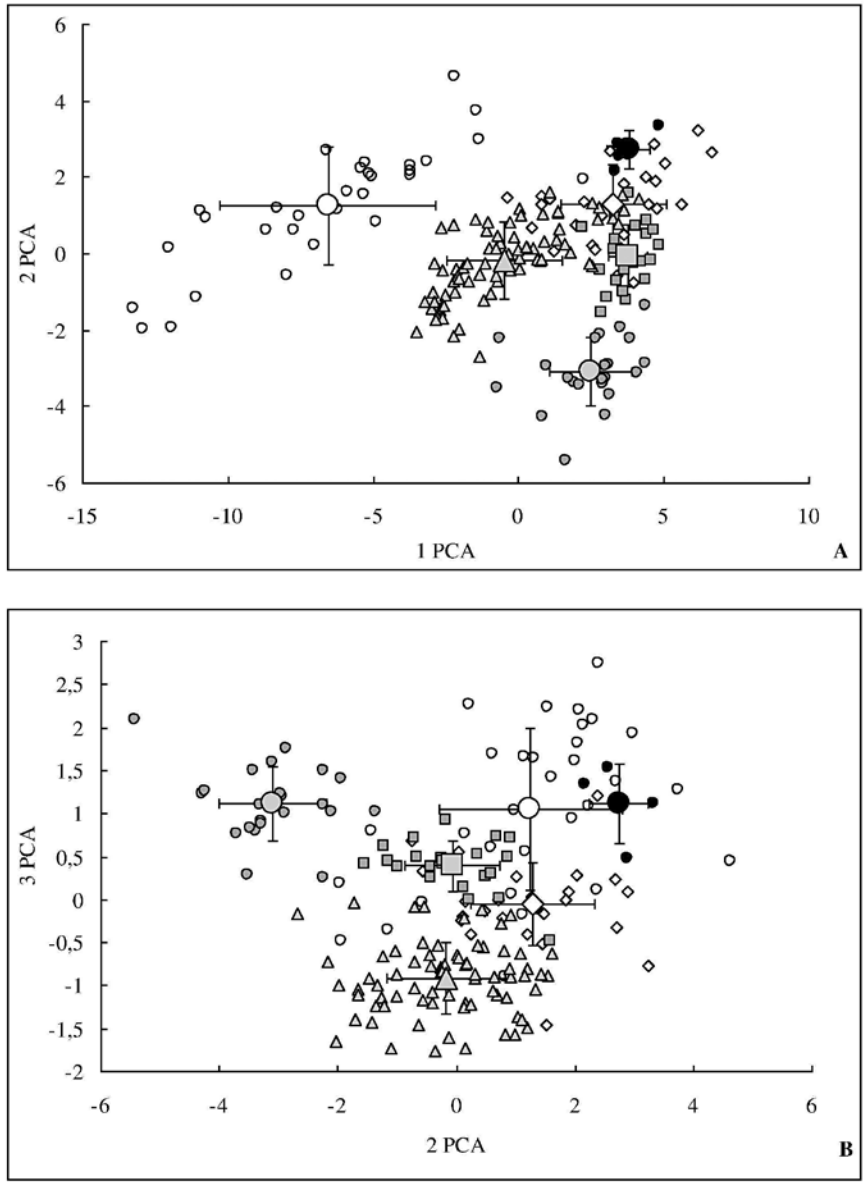

Fig. 3. Plot of clouds distributions and centroids of the common samples of *D. galeata* and *D. cucullata* from the Curonian Lagoon (CoL) and Novosibirsk Reservoir (NR) according to the morphological variables in the space of the first and second (A) and second and third (B) PCA axes; ± standard deviation. Open cirles – *D. cucullata* (CoL), black circles – *D. cucullata* (NR); grey circles – rounded form of *D. galeata* (CoL), open diamonds – helmeted form of *D. galeata* (CoL), grey squares – intermediate form of *D. galeata* (CoL), grey triangles – *D. galeata* (NR)

2 sequences for *D. galeata* were obtained from GenBank database (Table 2). The pairwise distances for the 16S fragment within *D. galeata* and *D. cucullata* species were 0.002 and 0.004, respectively. The divergence between these species was 0.022. There were 7 conservative sites through multiple alignment 522 nucleotides of length. The overall transition/transversion bias was $R = 3.862$.

A neighbour-joining analysis (the 16S sequence for *Eubosmina coregoni* was used as outgroup, GenBank #EU650747) produced a tree with the high bootstrap support for two branches corresponding to *D. galeata* and *D. cucullata* species, 89 and 88 %, respectively (Fig. 5). The topology indicated monophyletic origin of these groups. However, two *D. cucullata* specimens (NRCu2 and NRCu3) from Novosibirsk Reservoir formed a separate group with high bootstrap support, 85 %. Minimum evolution and maximum parsimony analyses (trees not presented) resulted in identical topologies with slightly less bootstrap support for the branches.

12S mtDNA. For the 12S gene 7 sequences of 610 bp for *D. cucullata* and 24 sequences of 608 bp for *D. galeata* were obtained. Additional 17 sequences for both species from GenBank database were included into analysis. The sequence for *E. coregoni* was chosen as outgroup (GenBank #AF494467). The within-specific pairwise distances for the 12S fragment were 0.002 for *D. galeata* and 0.003 for *D. cucullata*. The divergence between species was 0.083. If the sequences obtained from GenBank database were eliminated from the analysis the genetic distances within and between species were 0.001 and 0.075, respectively. There were 8 conservative sites through 12S multiple alignment 743 nucleotides of length. The overall transition/transversion bias was R = 2.356.

NJ-tree agreed in topology with NJ-tree based on 12S sequences (Fig. 6). There were two

Table 3. Component loadings of the morphological characters of the common Daphnia samples into the first three PCA axes. Major loadings are asterisked.

Character	Loadings		
	1 PCA	2 PCA	3 PCA
L	0.24*	0.00	0.10
o.t.sp	0.23*	-0.08	0.05
W	0.23*	-0.08	0.10
w.br.	0.21*	-0.07	0.35*
w.cap.	0.24*	0.02	-0.09
l.cap.	0.21*	0.24*	0.01
l.helm.	-0.15	0.38*	-0.14
O	0.19	-0.12	0.35*
lr	0.21*	0.14	-0.15
O.m.v.	0.16	0.31*	0.15
r.m.v.	0.22*	0.04	-0.22*
m.v.cap.	0.19	0.30*	-0.03
m.v.helm.	-0.05	0.53*	0.22*
Or	0.24*	0.06	-0.10
O.w.cap.	0.22*	0.15	-0.22*
cap.d.	0.21*	0.19	-0.01
r.W.v.	0.21*	-0.16	0.29*
w.cap.d.	0.22*	-0.14	0.30*
l.t.sp.	0.14	0.30*	-0.06
d.l.t.sp.	0.23*	-0.06	0.05
v.l.t.sp.	0.23*	-0.05	-0.07
helmet angle	0.19*	-0.22*	-0.36*
a.r.	0.19*	-0.17	-0.42*
l.cl.	0.18*	-0.06	-0.14
Cumulative %	69.01	12.44	4.30

Table 4. *P*-values between average values of the first three eigenvectors for pairwise comparison of the common samples of *Daphnia* (*t*-test)

Eigenvectors	*P*-value														
	1-2	1-3	1-4	1-5	1-6	2-3	2-4	2-5	2-6	3-4	3-5	3-6	4-5	4-6	5-6
1	-	-	**0.001**	0.001	-	0.001	**0.001**	**0.001**	-	**0.001**	**0.001**	-	0.001	**0.001**	**0.001**
2	**0.001**	0.001	-	0.001	-	**0.001**	**0.001**	**0.001**	**0.001**	0.001	-	**0.001**	0.001	-	**0.001**
3	**0.001**	0.001	0.001	0.001	**0.001**	0.001	-	**0.001**	-	0.01	**0.001**	0.001	**0.001**	-	**0.001**

Note: number samples see Fig. 4; differences assuming *CD*-test are marked by bold type.

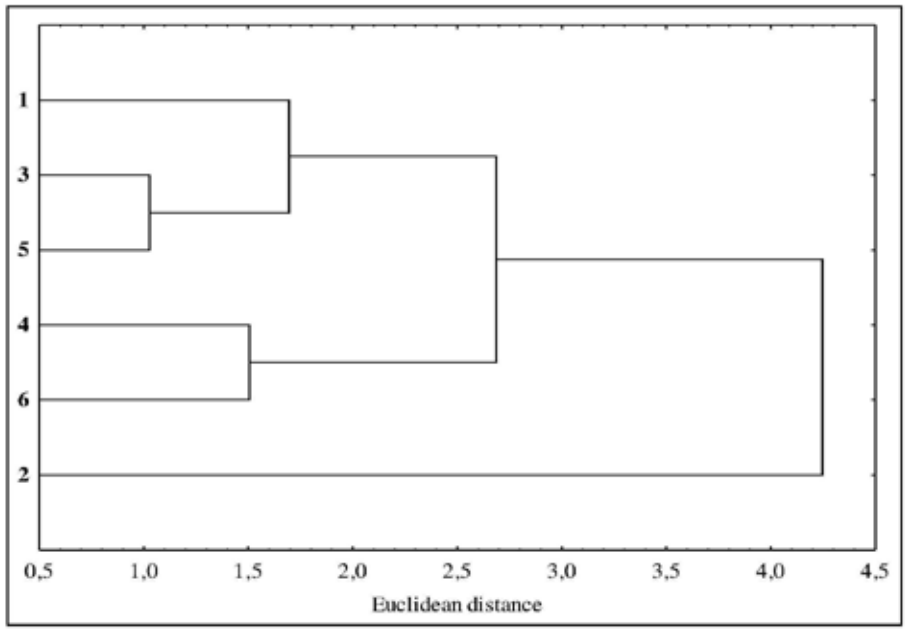

Fig. 4. UPGMA-dendrogram based on morphometric data for six samples of *D. galeata* and *D. cucullata* from the Curonian Lagoon (CoL) and Novosibirsk Reservoir (NR) (Euclidean distance between the average loading values into the second and third PCA axes). 1 – helmeted form of *D. galeata* (CoL), 2 – rounded form of *D. galeata* (CoL), 3 – intermediate form of *D. galeata* (CoL), 4 – *D. cucullata* (CoL), 5 – *D. galeata* (NR), 6 – *D. cucullata* (NR)

clusters with bootstrap support of the branches for *D. galeata* 99 % and *D. cucullata* 100 %. The topology of the 12S NJ-tree also indicated the monophyletic origin of these species.

ITS2 nuclear DNA. Between 1075 and 1087 bp of the ITS2 region were sequenced for 7 specimens of *D. cucullata* and for 17 specimens of *D. galeata*. Additional 6 ITS2 sequences were obtained from GenBank database and *D. longispina* ITS2 sequence (Poland, GenBank #AY730404) was used as the outgroup. Pairwise distances within *D. galeata* and *D. cucullata* species ranged from 0.002 to 0.05, respectively, with divergence between these species 0.013. There were 11 conservative sites in the ITS2 region through multiple alignment 1131 nucleotides of length. The overall transition/transversion bias was $R = 1.652$.

The phylogenetic relationships between *D. galeata* and *D. cucullata* species identified using the ITS2 region (tree is not presented) were generally consistent with the branching topology of trees based on mitochondrial DNA. The ITS2 sequences were also subjected to NJ and ME analyses. All methods produced a nearly identical topology with respect to species. But the support for a branch that resolves the position both species was lost. One *D. cucullata* specimen (NRCu3) from Novosibirsk Reservoir clustered together with *D. galeata*.

Discussion

The use of traditionally taxonomic keys has allowed identification of *D. galeata* and *D. cucullata* species in the Curonian Lagoon and Novosibirsk Reservoir. We suppose that enormous morphological variability, nomenclatural problems and the use of the inappropriate key for the identification of species within *Daphnia longispina* complex by previous studies could result in delineation of *D. longispina* and *D. hyalina* species in the investigated water bodies. The remarkable fact was that *D. galeata* was not recognized in the species composition of

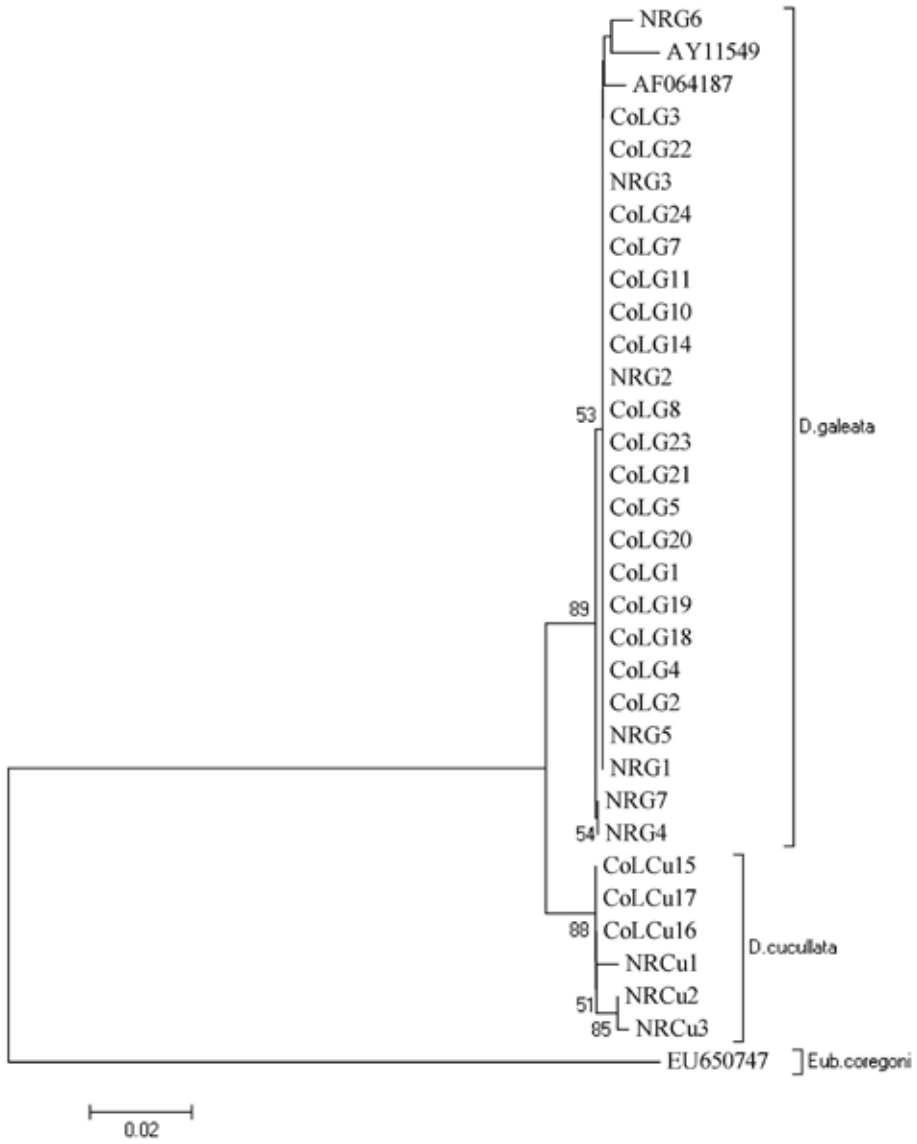

Fig. 5. A phylogenetic tree constructed using the neighbor-joining method (NJ) based on mitochondrial 16S rDNA sequences for *D. galeata* and *D. cucullata*. The NJ was rooted with *Eubosmina coregoni*. The number above the branches represents the bootstrap confidence limit (1000 replicates)

zooplankton community in Novosibirsk Reservoir until recently (Ermolaeva, 2007). Based on the morphometric analysis we have shown that the geographically distant populations of *D. galeata* differed between each other based on head length, shape of the ventral margin of the head, helmet length, slope and shape, the position and diameter of the eye, rostrum shape, some characters of the carapace and length tail spine. *D. cucullata* was characterized by less interpopulation morphological variability compared with *D. galeata*. However, despite the marked differences the geographically distant populations of these *Daphnia* species clustered together confirming their species identity.

Interpopulation variability of the 16S and 12S mitochondrial genes for the studied species is negligible and the consistency in the topology of the

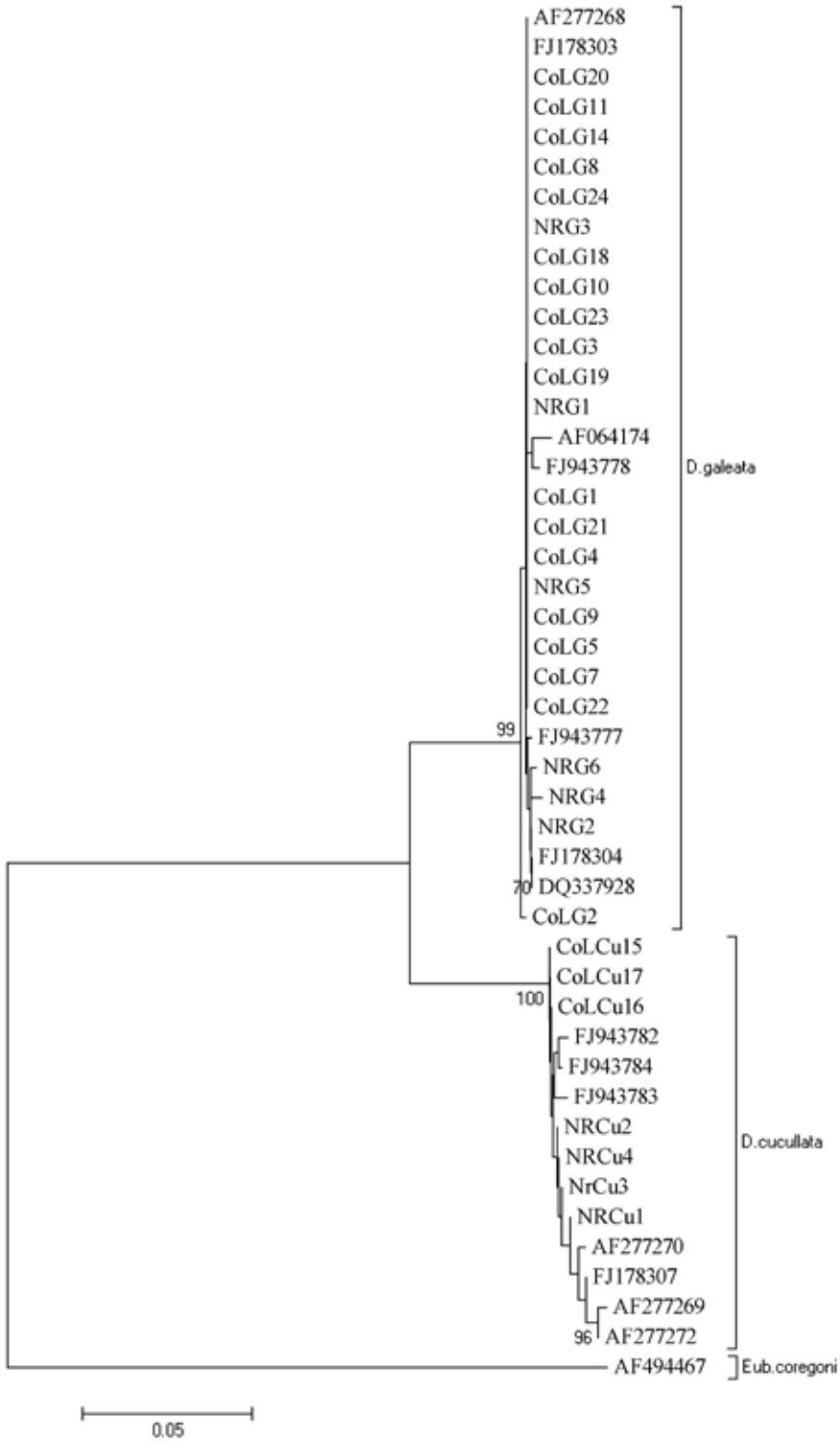

Fig. 6. A phylogenetic tree constructed using the neighbor-joining method (NJ) based on mitochondrial 12S rDNA sequences for *D. galeata* and *D. cucullata*. The NJ was rooted with *Eubosmina coregoni*. The number of the branches represents the bootstrap confidence limit (1000 replicates)

NJ-trees for both markers was found. Additional analyses of the phylogenetic relationships between closely related species *D. galeata* and *D. cucullata* using the minimum evolution and maximum parsimony methods also showed a concordant topology. Meanwhile, the intraspecific genetic distances for the 16S gene were higher than those for the 12S gene but the interspecific genetic distances were lower. An additional point is that the genetic divergence within *D. cucullata* was more significant as compared with *D. galeata*, whereas the morphology of the first species was less variable. The deletion from the analysis of the sequences obtained from GenBank database for the 12S gene resulted in reduction of the differences between the *D. cucullata* specimens inhabiting Novosibirsk Reservoir and the Curonian Lagoon. Our data are consistent with data on the phylogenetic relationships of *D. cucullata* and *D. galeata* populations in the water bodies of Western Europe, which also marked monophyletic and sister relationships (Schwenk et al., 2000; Petrusek et al., 2008). We did not find divergence between European and Siberian *D. galeata* populations using both mitochondrial markers, as it was shown earlier for European and North American populations (Taylor et al., 1996). However, we found that 16S gene was scarce conservative sites in comparison with the 12S gene, whereas for the North American *D. laevis* complex has been shown opposite (Taylor et al., 1998).

A clear resolution between the ITS2 sequences of nuclear DNA for *D. galeata* and *D. cucullata* species from both Novosibirsk Reservoir and the Curonian Lagoon was not found. The genetic divergence was lower than it was calculated for mitochondrial DNA. The possibility of interspecific hybridization is suggested by the lack of divergence among the ITS2 sequences of specimens from the studied populations. This finding, in turn, indicates also that both species are insufficiently isolated from each other and demonstrate sister relationship. The existence of hybridization between different populations of these species has been previously shown using other DNA markers (Schwenk et al., 1998; Gieβler et al., 1999; Schwenk et al., 2001; Taylor et al., 2005; Ishida, Taylor, 2007; Petrusek et al., 2008; Gieβler, Englbrecht, 2009).

Conclusion

The most important finding of our study is the absence of any significant morphological and genetic divergence between the geographically distant *D. cucullata* and *D. galeata* populations. The existence of separate phylogenetic lineage of *D. cucullata* in Novosibirsk Reservoir may be a result from flooding from various water bodies during the process of its formation. Mitochondrial and nuclear DNA significant variation among different morphotypes of *D. galeata* from the Curonian Lagoon was absent too. Such low level of the divergence within these morphs may be due to either their conspecific or hybrid origin of the intermediate morphs with inheritance of maternal mitogenome of *D. galeata*. On the other hand, the rounded head morph of *D. galeata* from the Curonian Lagoon observed at the beginning of spring enormously distinguished it from both other morphs and *D. cucullata* based on morphometric analysis. However, its specific delineation remains in abeyance.

In general, we have demonstrated significant morphological and genetic similarity among the geographically distant *D. galeata* and *D. cucullata* populations from two large water bodies in Russia.

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Морфологическая изменчивость и генетический полиморфизм географически удаленных популяций *Daphnia Galeata* и *Daphnia Cucullata* (Anomopoda, Daphniidae)

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Несмотря на то, что представители р. Daphnia (Anomopoda, Daphniidae) являются одними из наиболее распространенных водных беспозвоночных и используются в качестве модельных организмов в таксономических, экологических и эволюционных исследованиях, их систематика остается весьма запутанной. Настоящее исследование посвящено изучению морфологической дифференциации и генетической изменчивости географически удаленных популяций сестринских видов Daphnia galeata Sars, 1864 и Daphnia cucullata Sars, 1862 (Anomopoda, Daphniidae) из пресноводной части Балтийского моря – Куршского залива (Россия, Калининградская область) и Новосибирского водохранилища (Россия, Новосибирская область). Морфологическая дивергенция между видами и их популяциями оценивалась по диагностическим признакам и на основании анализа изменчивости формы тела по набору морфометрических признаков. Самыми изменчивыми были признаки, характеризующие форму головы, шлема и хвостовой иглы. Реконструкция филогенетических отношений между видами выполнена на основе изменчивости 16S и 12S генов митохондриальной ДНК и фрагмента ITS2 ядерной ДНК. Дивергенция между видами D. galeata и D. cucullata на основе генов митохондриальной ДНК была значительной и свидетельствует об их монофилетическом происхождении, тогда как внутривидовые генетические дистанции оцениваются как незначительные.

Ключевые слова: Daphnia galeata, Daphnia cucullata, морфологическая изменчивость, митохондриальная ДНК, ядерная ДНК, генетическая дивергенция.