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Taxonomic status of *Pisidium amnicum* (Müller, 1774) and *P. inflatum* Megerle von Mühlfeld in Porro, 1838 (Mollusca: Bivalvia: Sphaeriidae)

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ABSTRACT. In accordance with the taxonomic system adopted in Russia and used also in Estonia, the largest local forms of pea clams (Sphaeriidae) have been treated as two species, *P. amnicum* and *P. inflatum*. The aim of the study was to clarify if *P. amnicum* and *P. inflatum* are separate species. Populations of both forms from Estonia were analysed genetically and morphologically. Analysis of the COI and ITS-1 gene fragments revealed that these two forms were genetically indistinguishable. Although there were statistically significant differences in the relative proportions of the shell between their typical individuals, there occurred also intermediate values. Consequently, *P. inflatum* cannot be considered as a separate species and should be treated as an ecological variety of *P. amnicum* that inhabits mainly a characteristic sandy substrate.

Introduction

Pisidium amnicum (Müller, 1774), is the largest species among the pea clams (genus *Pisidium* Pfeiffer, 1821) with a length of up to 11 mm [Glöer, Meier-Brook, 2003]. It inhabits slow-flowing rivers, ditches and lakes [Kuiper, Wolf, 1970]. In the system adopted in Russia and used also in Estonia [e.g., Timm, 1975; Starobogatov, 1977], *P. amnicum s.l.* is split into two species, *P. amnicum s.str.* and *P. inflatum* Megerle von Mühlfeld in Porro, 1838. In Western Europe, *P. inflatum* is either treated as a more tumid form of *P. amnicum* i.e. *P. amnicum* var. *inflatum* [Clessin, 1887; Germain, 1930], considered a synonym of *P. amnicum* [Woodward, 1913], or ignored.

In the Russian system the species of the freshwater Bivalvia are primarily distinguished from each other, first of all, by comparing their external shell contour, i.e. by the „comparatorial method" [Logvinenko, Starobogatov, 1971; Shikov, Zatravkin, 1991]. The method was developed by B. M. Logvinenko and Ya. I. Starobogatov at the beginning of the 1970s [Logvinenko, Starobogatov, 1971]. It is based on the assumption that the shell growth curve corresponds to a logarithmic spiral [Thompson, 1945; Stasek, 1963; Raup, 1966]. This spiral is characterized by a constant angle between the radius and the tangent line drawn to any point of the spiral [Logvinenko, Starobogatov, 1971; Shikov, Zatravkin, 1991]. In order to use this method, the shell must be positioned on its anterior end in the plasticine or wax so that the edges of the anterior and posterior lateral teeth coincide with its optical axis [Logvinenko, Starobogatov, 1971; Timm, 1975; Stadnichenko, 1984; Korniushin, 2002]. In this case the outer contour (called the frontal section contour) will be drawn by means of the camera lucida and can be used as a species-specific model for comparing the contours of other specimens [Logvinenko, Starobogatov, 1971; Timm, 1975].

The adoption of this new method led to an increase in the number of species. According to V.I. Zhadin [1952], 16 species of pea clams were present in the European part of the ex-USSR, however, in the system based on the comparatorial method their number is 79–83 [Starobogatov *et al*., 2004; Kantor, Sysoev, 2005]. The method has been used not only for pea clams but also for other bivalves and even gastropods [Logvinenko, Starobogatov, 1971; Izzatullaev, Starobogatov, 1984].

The system based on the comparatorial method has met strong criticism as it is not in accordance with the Biological species concept [Graf, 2007]. The method has even been criticized by one of its developers, B. M. Logvinenko. His studies, based on protein electrophoresis, showed that several Unionidae species determined by the comparatorial method had no molecular biological differences [Kodolova, Logvinenko, 1973, 1974]. Voroshilova [2013] demonstrated that the contour of the frontal shell section does not always correspond to the logarithmic spiral and the values of the axis of the polar angle can overlap even in traditionally accepted species of the genera *Sphaerium* Scopoli, 1777, and *Margaritifera* Schumacher, 1816. It has been shown that there is no hiatus in the relative morphometric proportions between the species of the genus *Mar-*

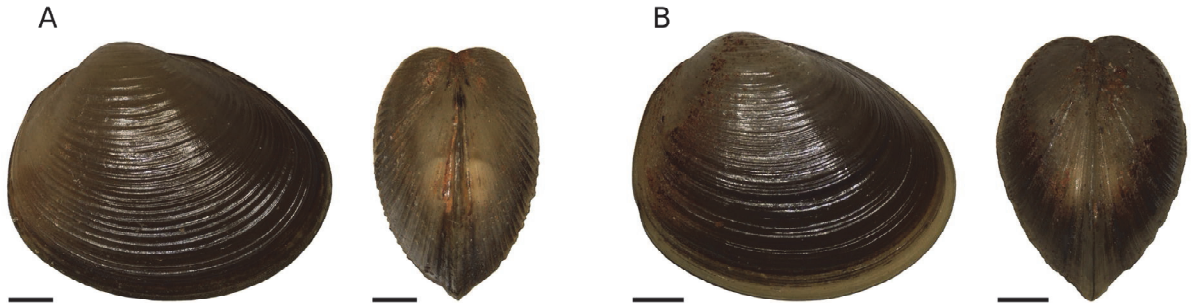

FIG. 1. *Pisidium amnicum* (**A**) and *P. inflatum* (**B**) in the lateral and posterior view. *Pisidium amnicum* was collected from Lake Peipsi, *P. inflatum* was collected from Lake Võrtsjärv. Scale – 1 mm.

РИС. 1. *Pisidium amnicum* (**A**) и *P. inflatum* (**B**) вид сбоку и сзади. *Pisidium amnicum* собран в Чудском озере, *P. inflatum* собран в оз. Выртсъярв. Масштаб 1 мм.

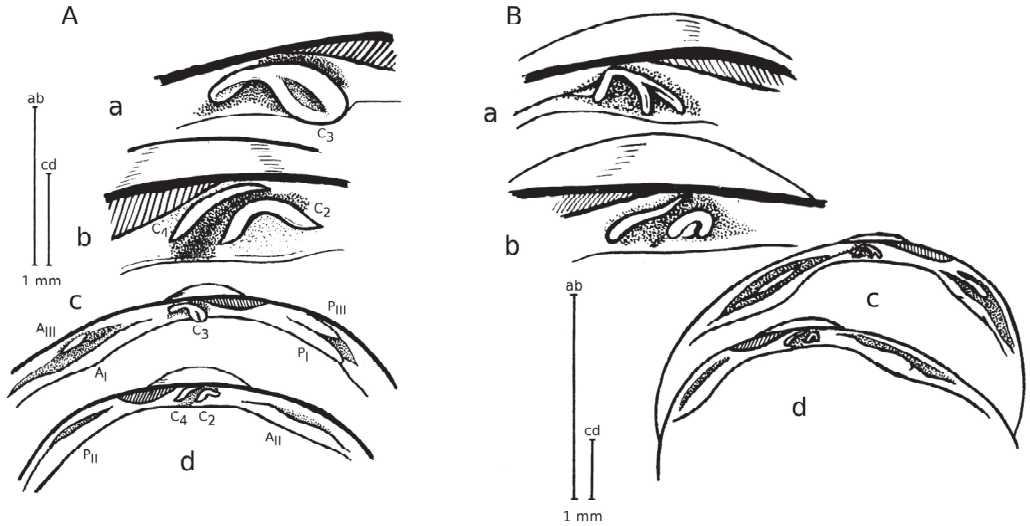

FIG. 2. Hinge teeth of *Pisidium amnicum* (**A**) and *P. inflatum* (**B**). C_3 – cardinal tooth of the right valve (**a**); C_2, C_4 – cardinal teeth of the left valve (**b**); A_I, A_{III} – anterior lateral teeth of the right valve (**c**); P_I, P_{III} – posterior lateral teeth of the right valve; A_{II} – anterior lateral tooth of the left valve (**d**); P_{II} – posterior lateral tooth of the left valve. Adapted from Timm [1975].

РИС 2. Замковые зубы *Pisidium amnicum* (**A**) и *P. inflatum* (**B**). C_3 – кардинальный зуб правой створки (**a**); C_2, C_4 – кардинальные зубы левой створки (**b**); A_I, A_{III} – передние латеральные зубы правой створки (**c**); P_I, P_{III} – задние латеральные зубы правой створки; A_{II} – передний латеральный зуб левой створки (**d**); P_{II} – задний латеральный зуб левой створки. По Timm [1975].

garitifera, determined by the comparatorial method [Sergeeva *et al*., 2008], and there occurs transition of the contour of the frontal shell section between them [Bolotov *et al.*, 2013].

Pisidium amnicum and *P. inflatum* are externally different. In the lateral view, *P. inflatum* has a higher and more triangular-shaped shell than *P. amnicum* [Pirogov, 1972; Timm, 1975, 1976; Starobogatov, 1977; Stadnichenko, 1984; Starobogatov *et al*., 2004] (Fig. 1). The umbo of *P. inflatum* is shifted more backwards than that of *P. amnicum*. Internally, *P. inflatum* is characterized by a thicker hinge plate and a more sharply bent cardinal tooth C_2 [Timm, 1975; Stadnichenko, 1984] (Fig. 2).

Pisidium amnicum and *P. inflatum* occur in different substrate types. *Pisidium amnicum* occurs more often in a silty substrate and is less frequent in a sandy substrate [Timm, 1975, 1976; Starobogatov, 1977]. *P. inflatum* is more often found in a sandy substrate and less in a silty substrate [Pirogov, 1972; Timm 1975, 1976; Starobogatov, 1977]. *Pisidium amnicum* has a Palaearctic distribution while *P. inflatum* occurs mainly in Europe and Western Siberia [Stadnichenko, 1984]. According to literature, when these species coexist there are no intermediate forms [Pirogov, 1972; Timm, 1976].

The aim of this study was to compare the morphometric and genetic characteristics of *P. amnicum* and *P. inflatum* and to clarify whether they are actually separate species.

Material and methods

The samples of pea clams were collected in July and August 2014 from lakes Peipsi and Võrtsjärv,

the Emajõgi River and the Amme Stream – all located in eastern Estonia. The samples were taken by a hand net with a mesh size of 1 mm from water to a depth of 1 m. The substrate type at the sampling spots of lakes Peipsi and Võrtsjärv was sand, in Amme Stream sand-gravel and in Emajõgi River mud. The collected specimens were fixed in 96% ethanol. In addition, 8 specimens, collected in 1964–1967 from Lake Peipsi, deposited at the Centre for Limnology in Estonia and determined by the late Viivi Timm, were used in morphometric analyses. *Pisidium amnicum* and *P. inflatum* were distinguished by the height to length ratio (H/L) and by the width to length ratio (W/L). According to Ya. I. Starobogatov [Starobogatov *et al*., 2004], H/L ratio for *P. amnicum s.str.* is not over 0.8 and W/L ratio is not over 0.63. These indices for *P. inflatum* are not less than 0.81 and 0.64, respectively. In present study, most of the individuals had H/L ratio over 0.81 but W/L ratio below 0.63, these specimens are placed into intermediate group *P.* „*amnicum-inflatum*“ (hereinafter for brevity's sake also *P*. „*amn.-infl.*“).

Morphometric analyses

Nine morphometric variables of the shell were measured: length (L), height (H), width (W), commissural height (HC), length of shell's posterior part from the umbo (LP), length of the hinge (LH), height of the hinge plate (HH), length of the ligament pit (LL), height of the ligament pit (HL). The following indices were calculated: H/L, W/L, W/H, HC/H, LP/L, LH/L, HH/H and HL/LL. Measurements were made using the program NIS-Elements Documentation 3.1 (Nikon Corporation, Tokyo, Japan). For each specimen, the contour of the frontal shell section was drawn and compared with the reference curves. The reference curves were drawn after two specimens collected from Lake Peipsi in 1965–1966. These specimens were chosen because they were identified by Viivi Timm, who was one of the students of Ya. I. Starobogatov. A total of 102 specimens were analysed morphometrically.

Anatomical analyses

The number of inner demibranch filaments in front of the outer demibranch anterior edge was counted. According to Korniushin [1991], this characteristic is stable during ontogeny and is also species-specific. In order to count filaments, the demibranches were stained with Grenacher's borax carmine. A total of 48 specimens were analysed anatomically.

Statistical analyses

Statistical differences between the forms were tested using the non-parametric Kruskal-Wallis test [Kruskal, Wallis, 1952]. Principal component and canonical discriminant analyses were carried out using log-transformed morphometric variables. Statistical analyses were performed using the program MYSTAT 12 (Systat Software Inc., San Jose, USA). The results were considered statistically significant at p-value <0.05.

Genetic analyses

The DNA extraction and PCR of a fragment of the mitochondrial gene cytochrome c oxidase subunit I (COI) were performed at the University of Tartu. The COI fragment was sequenced by the company Macrogen (Amsterdam, Holland). The PCR of nuclear gene internal transcribed spacer 1 (ITS-1) was carried out at the Estonian University of Life Sciences and it was sequenced at the Estonian Biocentre, core laboratory (Tartu, Estonia).

A total of 10 specimens were examined genetically. GenBank accession numbers and voucher numbers are reported in Table 1. For DNA extraction, the whole soft body of small specimens and approximately 1 mm^3 piece of larger specimens were used.

The COI fragment was amplified using universal primers LCO1490: 5' GGTCAACAAATCATAAAGATATTGG-3' and HC02198: 5' TAAACTTCAGGGTGACCAAAAAATCA- 3' [Folmer *et al*., 1994]. The PCR was carried out in a final volume of 25 µl containing 5 µl 5x HOT FIREPol Blend Master Mix 10 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 0.5 µl (20µM) forward and reverse primers,18 µl distilled water and 1 µl DNA template. Initial incubation for 15 min at 95°C was followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min. Final elongation was performed at 72°C for 10 min. The PCR products were visualized by electrophoresis through 1% agarose gel. The PCR products were purified with SAP/EXOI treatment using 1 µl enzyme FastAP TM Thermosensitive Alkaline Phosphatase 1 U/µl (Thermo Scientific, Waltham, USA) and Exonuclease I 20 U/µl (Thermo Scientific, Waltham, USA).

The ITS-1 gene amplification was carried out using universal primers 18S: 5'-TAACAAGGTTTCCGTAGGTG-3' and 5.8S: 5'-AGCTRGCTGCGTTCTTCATCGA-3' [White *et al*., 1996]. The PCR was performed in a total volume of 10 µl containing of 2 µl 5x HOT FIREPol Blend Master Mix 10 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 0.5 µl forward and reverse primers, 5 µl distilled water and 2 µl DNA template. The PCR programme included initial incubation at 95°C for 15 min, followed by 34 cycles consisting of denaturation at 95°C for 15 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min. Final elongation was performed at 72°C for 2 min. The PCR products were purified using 1 µl of enzyme FastAP TM Thermosensitive Alkaline Phosphatase 1 U/µl (Thermo Scientific,

Table 1. List of studied *P. amnicum s.l.* taxa. Voucher specimens are stored in the collection of Centre for Limnology, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences.

Spec. Code	Voucher No.	COI GenBank Acc. No.	ITS-1 GenBank Acc. No.	Locality
V1	TAAM<EST> 15-1059-a-01	KX351303	KX351313	Estonia, Lake Võrtsjärv
V2	TAAM<EST> 15-1059-a-02	KX351304	KX351314	Estonia, Lake Võrtsjärv
V3	TAAM<EST> 15-1059-a-03	KX351305	KX351315	Estonia, Lake Võrtsjärv
V4	TAAM<EST> 15-1059-a-04	KX351306		Estonia, Lake Võrtsjärv
V5	TAAM<EST> 15-1059-a-05	KX351307		Estonia, Lake Võrtsjärv
V6	TAAM<EST> 15-1059-a-06	KX351308		Estonia, Lake Võrtsjärv
P1	TAAM<EST> 15-1059-a-07	KX351309	KX351316	Estonia, Lake Peipsi
P2	TAAM<EST> 15-1059-a-08	KX351310	KX351317	Estonia, Lake Peipsi
A1	TAAM<EST> 15-1059-a-09	KX351311	KX351318	Estonia, Amme Stream
E1	TAAM<EST> 15-1059-a-10	KX351312		Estonia, Emajõgi River

Table 2. List of COI and ITS-1 gene fragment sequences obtained from GenBank.

Species	COI GenBank Acc. No.	ITS-1 GenBank Acc. No.	Locality	References
Eupera cubensis (Prime)		AY093501	Cuba	Lee, Ó Foighil, 2003
E. ferruginea (Kraus)	KF483420		Uganda	Clewing *et al.,* 2013
E. ferruginea	KF483421		Uganda	Clewing *et al.,* 2013
E. platensis Doello-Jurado		AY093502	Argentina	Lee, Ó Foighil, 2003
Pisidium (*Cyclocalyx*) *casertanum* (Poli)		AY093515	Germany	Lee, Ó Foighil, 2003
P. (*C.*) *casertanum*	KF483386		Germany	Clewing *et al.,* 2013
P. (*C.*) *globulare* Westerlund	KF483423		Germany	Clewing *et al.,* 2013
P. (*C.*) *henslowanum* (Sheppard)	KF483398		Macedonia	Clewing *et al.,* 2013
P. (*C.*) *hibernicum* Westerlund		AY093522	Germany	Lee, Ó Foighil, 2003
P. (*C.*) *lilljeborgii* Esmark & Hoyer		AY093521	Russia	Lee, Ó Foighil, 2003
P. (*C.*) *lilljeborgii*	KF483406		Norway	Clewing *et al.,* 2013
P. (*C.*) *milium* Held		AY093523	Germany	Lee, Ó Foighil, 2003
P. (*C.*) *nitidum* Jenyns		AY093526	Germany	Lee, Ó Foighil, 2003
P. (*C.*) *nitidum*	KF483385		Germany	Clewing *et al.,* 2013
P. (*C.*) *obtusale* (Lamarck)	KF483387		Germany	Clewing *et al.,* 2013
P. (*C.*) *personatum* Malm		AY093527	Germany	Lee, Ó Foighil, 2003
P. (*C.*) *personatum*	KF483389		Georgia	Clewing *et al.,* 2013
P. (*C.*) *subtruncatum* Malm		AY093528	Germany	Lee, Ó Foighil, 2003
P. (*C.*) *supinum* Schmidt		AY093529	Germany	Lee, Ó Foighil, 2003
P. (*C.*) *supinum*	KF483388		Germany	Clewing *et al.,* 2013
P. (*Pisidium*) *amnicum* (Müller)		DQ062573	N/A	Steiner, unpublished
P. (*P.*) *amnicum*		DQ062574	N/A	Steiner, unpublished
P. (*P.*) *dubium* (Say)		AY093533	USA	Lee, Ó Foighil, 2003

Table 3. Summary of the morphometric shell characteristics and the results of the Kruskal-Wallis test.

Index	*P. amnicum*		*P. "amnicum-inflatum"*		*P. inflatum*			
	n=19		n=71		n=12			
	Mean±SD	Min–Max	Mean±SD	Min–Max	Mean±SD	Min–Max	H	p-value
H/L	0.79±0.013	0.75–0.80	0.83±0.016	0.81–0.87	0.87±0.015	0.83–0.88	60.353	<0.0001
W/L	0.54±0.022	0.49–0.57	0.57±0.024	0.50–0.62	0.66±0.020	0.64–0.69	46.58	<0.0001
W/H	0.68±0.029	0.63–0.73	0.69±0.027	0.62–0.74	0.76±0.022	0.73–0.80	30.98	<0.0001
HC/H	0.96±0.010	0.93–0.98	0.97±0.009	0.95–0.99	0.96±0.007	0.95–0.97	10.449	0.005
LP/L	0.34±0.014	0.32–0.37	0.34±0.017	0.29–0.37	0.34±0.017	0.30–0.37	2.02	0.364
LH/L	0.53±0.019	0.50–0.56	0.54±0.020	0.49–0.58	0.54±0.014	0.52–0.57	2.275	0.321
HH/H	0.072±0.010	0.048–0.087	0.088±0.008	0.069–0.11	0.09±0.007	0.078–0.10	31.712	<0.0001
HL/LL	0.28±0.042	0.2–0.36	0.31±0.034	0.23–0.44	0.29±0.021	0.26–0.33	10.176	0.006

Table 4. Number of inner demibranch filaments in the three forms of *P. amnicum s.l.* and the results of the Kruskal-Wallis test.

	P. amnicum		*P. "amnicum-inflatum"*		*P. inflatum*			
	n=8		n=36		n=4			
Character	Mean±SD	Min–Max	Mean±SD	Min–Max	Mean±SD	Min–Max	H	p-value
Number of filaments	14.38±2.07	11–18	14.17±2.51	9–18	13.25±1.50	11–14	0.72	0.70

Waltham, USA) and 1 µl Exonuclease I 20 U/µl (Thermo Scientific, Waltham, USA). The PCR products were visualized by electrophoresis through 1% agarose gel. The DNA sequences were aligned by the ClustalW method using the program Mega 6.0 [Tamura *et al*., 2013].

Phylogenetic relationships were examined with maximum parsimony (MP) method for the COI and ITS-1 gene fragments. Additional sequences of other *Pisidium* species, *P.* (*P.*) *dubium* from the subgenus *Pisidium* and several species from the subgenus *Cyclocalyx*, as well as three species from genus *Eupera* as an outgroup were obtained from GenBank. Accession numbers of DNA sequences obtained from GenBank are reported in Table 2. Phylogenetic tree was constructed using program Mega 6.0 [Tamura *et al*., 2013]. Heuristic searches were conducted using 100 random stepwise addition and tree bisection-reconnection (TBR) branch-swapping. Sequence gaps were considered as missing data. Bootstrap values were obtained from 1000 replicates. Branches with bootstrap values lower than 50% were condensed.

Results

Morphological characteristics

In total, 19 specimens belonged to the typical form *P. amnicum,* 71 specimens belonged to the form *P.* „*amnicum-inflatum*“ and 12 specimens belonged to the typical form *P. inflatum*. The Kruskal-Wallis test revealed that the following indices differed between the forms: height to length ratio (H/L), width to length ratio (W/L), width to height ratio (W/H), commissural height to shell height ratio (HC/H), height of the hinge plate to shell height ratio (HH/H), height of ligament pit to length of ligament pit ratio (HL/LL) (Table 3). The ratio of the length of the posterior part (from the umbo) to total shell length (LP/L), and the ratio of the length of the hinge to total shell length (LH/L) were not significally different between the forms.

Comparison of the contour of the frontal shell section with reference contours revealed intermediate forms. Each sample contained specimens whose curvature coincided with the reference curve, as well as transition forms. Hence, the comparatorial method is inapplicable for distinguishing these two forms.

The difference in the number of inner demibranch filaments in front of the outer demibranch anterior edge was not statistically significant between the forms of *P. amnicum s.l.* (Table 4).

Principal component analysis

The first principal component explained 71.89% and the second principal component explained 15.69% of the total variation. The eigenvalues for

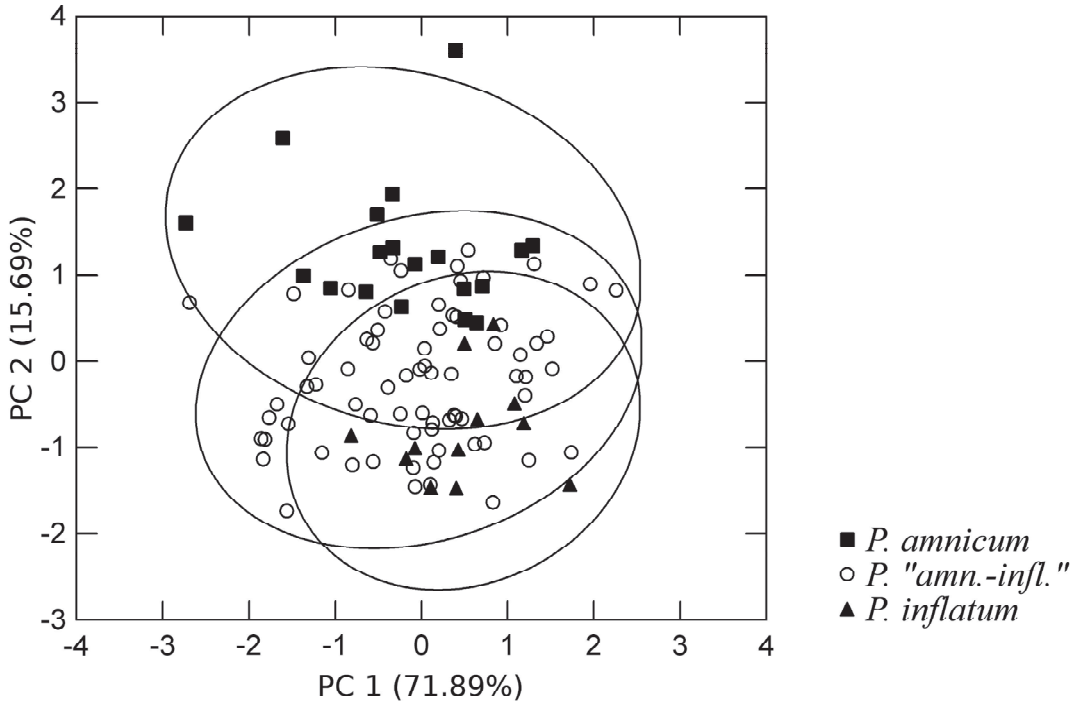

FIG. 3. Scatterplot of the first two principal component scores.

РИС. 3. Диаграмма рассеяния значений первых двух главных компонент.

Table 5. Logarithm transformed loadings of the first two principal components.

Character	PC 1	PC 2
Log length (L)	0.928	0.324
Log height (H)	0.983	0.106
Log width (W)	0.923	-0.04
Log commissural height (HC)	0.983	0.087
Log posterior part length (LP)	0.798	0.481
Log hinge length (LH)	0.901	0.286
Log hinge plate height (HH)	0.538	-0.711
Log ligament pit length (LL)	0.745	-0.508
Log ligament pit height (HL)	0.729	-0.459
% of the total variance	71.89	15.69

the first two principal components were 6.47 and 1.41. The first principal component was strongly correlated with shell height (H), commissural height (HC), length (L), width (W) and hinge length (LH) (Table 5). The three forms were more distinctly separated by the second principal component, which was strongly positively correlated with length of the posterior part of the shell from the umbo (LP) and negatively correlated with the height of the hinge plate (HH). There was an overlap of 95% confidence ellipses of the three forms (Fig. 3).

Discriminant analysis

The three forms were distinguished statistically significantly (Wilks' lambda: 0.174; approx. F-ratio: 14.145; $p<0.0001$). The eigenvalues for the first two discriminant functions were 2.8 and 0.52. Specimens pertaining to the typical forms *P. amnicum* and *P. inflatum* were 100% correctly classified and specimens of the form *P.* „*amnicum-inflatum*" were 89% correctly classified. The first discriminant function explained 84.4% and the second function explained 15.6% of the total variation. The first function was strongly positively correlated with shell length (L) and negatively correlated with commissural height (HC). The second function was strongly positively correlated with shell width (W) and negatively correlated with commissural height (HC) (Table 6). There was an overlap of 95% confidence ellipses of the three forms (Fig. 4).

Genetic analyses

The length of the sequenced mitochondrial COI fragment used in the analyses was 608 nucleotides. Alignment of the sequences revealed 5 variable sites of which only one site was informative for parsimony analysis (Table 7). Three haplotypes were found, which differed by two (0.33% divergence) or four (0.66% divergence) sites. Haplotype I was the most abundant and included specimens from all three *P. amnicum s.l.* forms.

The length of the sequenced nuclear gene ITS-1 used in the analyses was 424 nucleotides. The ITS-

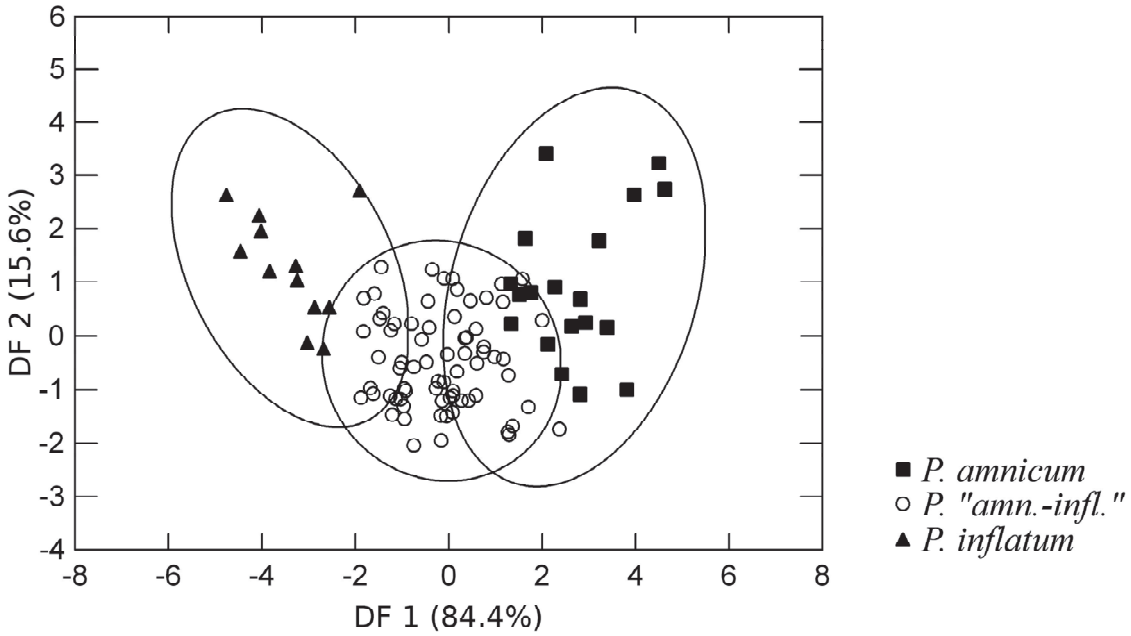

FIG. 4. Scatterplot of the first two discriminant function scores.

РИС. 4. Диаграмма рассеяния значений первых двух дискриминантных функций.

1 gene was succesfully sequenced for 6 specimens (2 specimens from each form of *P. amnicum s.l.*). All sequences were genetically identical.

The results of phylogenetic analyses showed that the subgenus *Pisidium s.str.* formed a well supported clade in both, COI and ITS-1 dataset, which is congruent with previous molecular studies (Lee, Ó Foighil, 2003; Schultheiß *et al*., 2008; Clewing *et al*., 2013). In COI dataset, *P. amnicum s.l.* clade was sister to a *polytomous* clade consisting of six branches which supported taxa of the *Pisidium* subgenus *Cyclocalyx* (Fig. 5). *Pisidium „amn.-infl.*“ from Emajõgi River and *P. amnicum* from Lake Peipsi occupied a well-supported basal position within *P. amnicum s.l.* clade.

Pisidium dubium, a North-American representative of the subgenus *Pisidium s.str.*, was basal to the *P. amnicum s.l.* clade in ITS-1 dataset (Fig. 6). Two *P. amnicum* sequences obtained from GenBank formed a moderately supported clade, sister to other *P. amnicum s.l.* taxa.

Discussion

Morphometric analyses showed that the *P. inflatum* form differs from *P. amnicum s.str.* in a higher hinge plate, which is in accordance with relevant literature data [Pirogov, 1972; Timm, 1975; Stadnichenko, 1984]. Differences in the position of the umbo, as suggested by earlier authors [Timm, 1975; Stadnichenko, 1984], were not confirmed. Some morphometric indices of shell characteristics showed statistically significant differences between

Table 6. Logarithm transformed scores of the first two discriminant functions.

Character	DF 1	DF 2
Log length (L)	4.907	0.528
Log height (H)	0.457	1.144
Log width (W)	-1.84	1.976
Log commissural height (HC)	-3.604	-3.13
Log posterior part length (LP)	-0.035	0.612
Log hinge length (LH)	-0.078	-0.617
Log hinge plate height (HH)	-0.112	-0.576
Log ligament pit length (LL)	0.079	0.217
Log ligament pit height (HL)	0.1	-0.151
% of the total variance	84.4	15.6

Table 7. Nucleotide differences for studied COI fragment.

Specimen	Haplotype	Variable site position				
P. inflatum (V1)	I	135	179	318	390	564
P. inflatum (V2)	I	T	T	A	A	G
P. „amn.-infl.“ (V3)	I	•	•	•	•	•
P. „amn.-infl.“ (V4)	I	•	•	•	•	•
P. „amn.-infl.“ (V5)	I	•	•	•	•	•
P. „amn.-infl.“ (V6)	I	•	•	•	•	•
P. „amn.-infl.“ (P1)	I	•	•	•	•	•
P. amnicum (A1)	I	•	•	•	•	•
P. amnicum (P2)	II	•	C	•	•	A
P. „amn.-infl.“ (E1)	III	C	•	T	G	A

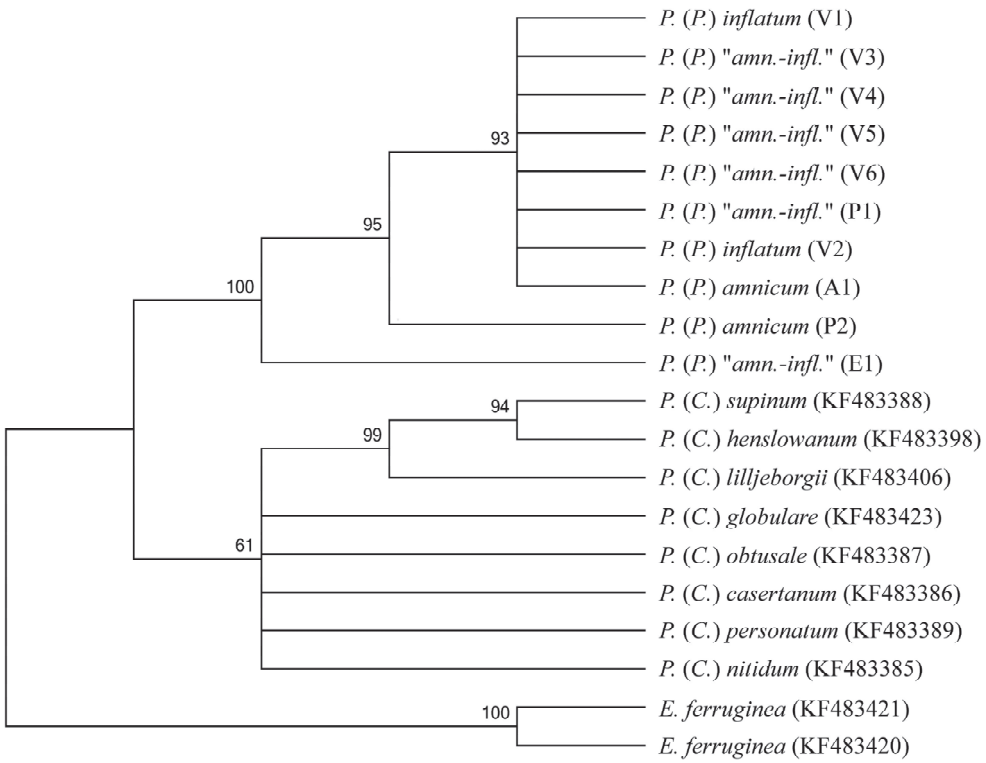

FIG. 5. Maximum parsimony strict consensus tree with bootstrap values of the COI dataset. Sequences obtained from GenBank are indicated with accession numbers.

РИС. 5. Строго консенсусное наиболее экономное дерево с бутстрап поддержками, основанное на данных COI. Для последовательностей из GenBank приведены инвентарные номера.

the three forms of *P. amnicum s.l.*, however, there occurred intermediate values. Discriminant analyses indicated that typical individuals of *P. amnicum s.str.* and *P. inflatum* were morphologically distinct and can be identified with multivariate methods.

One of the characteristics of *P. inflatum* is the shape of cardinal tooth C_2 on the left valve: it is more sharply bent than that of *P. amnicum,* according to Timm [1975] and Stadnichenko [1984]. In this study no differences were found in the hinge teeth between these forms: these teeth were evenly rounded or sharply bent in both forms.

The family Sphaeriidae is characterized by the plasticity of morphological and anatomical traits [Holopainen, Kuiper, 1982; Korniushin, 1996]. Variability in morphological characteristics may be related to the bottom substrate. It has been shown that the specimens of *P. subtruncatum* Malm, 1855 living in a silty substrate have a lower shell than those living in a sandy substrate [Funk, Reckendorfer, 2008]. Shell morphology is also affected by water movement: specimens living in open shore exposed to waves have a thicker and more triangularily shaped shell than those inhabiting low exposure areas [Korniushin, 1996]. It has been shown that the unionids living in areas exposed to waves have a higher and heavier shell than the specimens living in less exposed areas [Hinch, Bailey, 1988]. Shell morphology can be also affected by parasite infection. Specimens of the family Unionidae affected by trematodes have a longer and wider shell than uninfected specimens [Zieritz, Aldridge, 2011].

I observed transition forms, regarding the frontal shell section contour, between the two studied typical forms, *P. amnicum* and *P. inflatum*. Hence the comparatorial method cannot be used for distinguishing between *P. amnicum* and *P. inflatum*. Lack of the hiatus between species determined by the comparatorial method has also been reported for the families Margaritiferidae [Bolotov *et al*., 2013] and Unionidae [Bogatov, 2014].

According to COI fragment sequences, genetic variation between the forms *P. amnicum s.str.* and *P. inflatum* was ten times as small as the 3% interspecific threshold proposed by Hebert *et al*. [2003]. As is known, there is no fixed threshold for discrimination of species. A small, less than 1% genetic divergence between species has been described for lepidopterans [Hebert *et al*., 2003] and birds [Johnson, Cicero, 2002]. Although, one specimen of *P. amnicum* and one *P. „amnicum-inflatum*“ specimen formed separate branches in the maximum parsimony tree of the COI dataset, their difference from other *P. amnicum s.l.* taxa was only

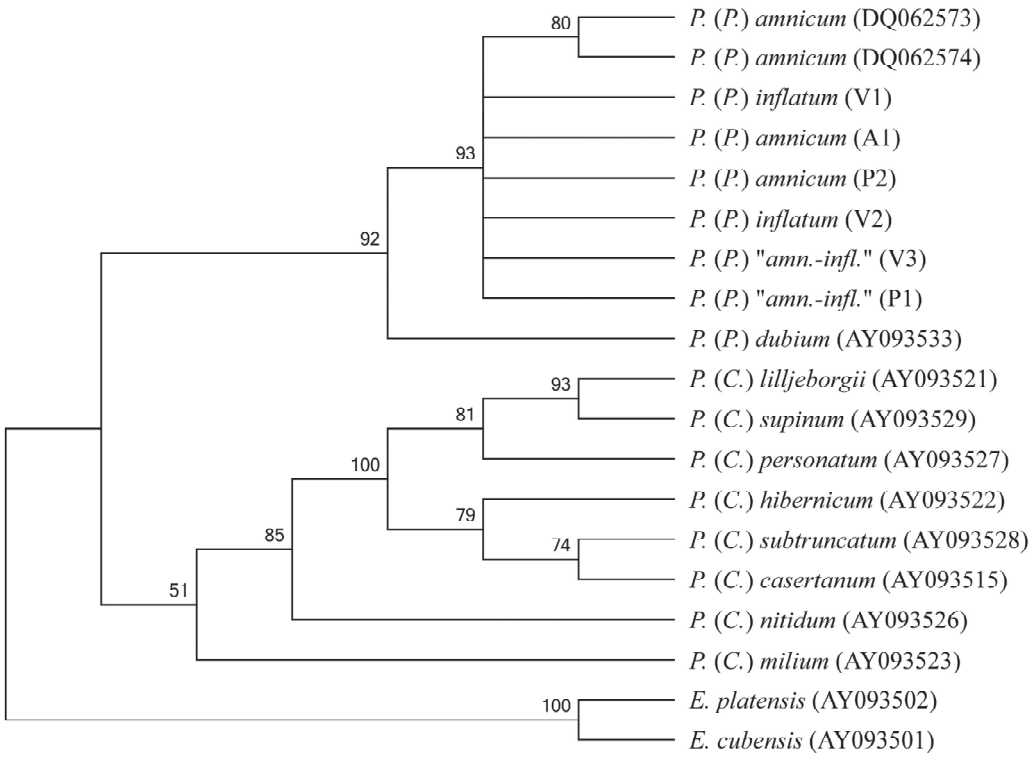

FIG. 6. Maximum parsimony strict consensus tree with bootstrap values of the ITS-1 dataset. Sequences obtained from GenBank are indicated with accession numbers.

РИС. 6. Строго консенсусное наиболее парсимонийное дерево с бутстрап поддержками, основанное на данных ITS-1. Для последовательностей из GenBank приведены инвентарные номера.

0.33–0.66% and overlapped intraspecific variability. Consequently, these forms cannot be considered as separate species.

Conclusion

This study showed that according to morphometrical, anatomical and genetic data, *P. amnicum* and *P. inflatum* cannot be considered as separate species. *Pisidium inflatum* should be treated as an ecological form living mainly in a sandy substrate, as well as a synonym of *P. amnicum.* The taxonomic status of other pea clam species distinguished by the comparatorial method should be determined in further studies.

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Таксономический статус *Pisidium amnicum* (Müller, 1774) и *P. inflatum* Megerle von Mühlfeld in Porro, 1838 (Mollusca: Bivalvia: Sphaeriidae)

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РЕЗЮМЕ. В соответствии с таксономической системой, принятой в России и Эстонии, крупные локальные формы шаровок (Sphaeriidae) рассматривались как два вида, *P. amnicum* и *P. inflatum*. Целью работы было выяснение, являются ли *P. amnicum* и *P. inflatum* самостоятельными видами. Популяции обеих форм из Эстонии были проанализированы с помощью морфологических и молекулярных методов. Анализ фрагментов генов COI и ITS-1 показал, что обе формы генетически не различаются. Хотя были найдены статистически достоверные различия в пропорциях раковин типичных особей, существовали также и особи с промежуточными значениями. Соответственно, *P. inflatum* нельзя считать отдельным видом и он должен рассматриваться как экологический вариетет *P. amnicum*, населяющий, в основном, характерные песчаные субстраты.