

Evaluation of the Applicability of the Comparatory Method for Species Diagnosis of Unionidae (Bivalvia) by Genetic Analysis

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Abstract—Five genetically distant groups of mussels possessing high intragroup homogeneity were identified among 65 specimens of 14 East European Unionidae “comparatory species” by genetic analysis of nuclear and mitochondrial markers. By shell morphology other than the shape of the convex contour of the shell, the identified groups correspond to five “taxonomic species” according to Zhadin’s classification. The use of the comparatory method for Unionidae species identification is unjustified.

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The comparatory method instead of general morphological approach was used for identification of freshwater Unionidae in the late 1960s to early 1970s in

the Soviet Union. The comparatory method uses a microscope and drawing apparatus to compare the convex contours of the cross-section of the shell. Fol-

Table 1. Taxonomy of Unionidae from the river Ivitsa according to the classifications of Zhadin [4] and Starobogatov–Bogatov [3] and the list of specimens collected for genetic analysis in this study

| Species according to [4] | “Comparatory” taxa according to [3] | Specimens |
|--|--|----------------------------------|
| <i>Colletopterum anatinum</i> (L. 1758) | <i>C. anatinum</i> | C-41 |
| | <i>C. nilssoni</i> (Kuster, 1838) | C-7, C-18, C-26, C-29 |
| | <i>C. ponderosum</i> (C. Pfeiffer, 1825) | C-3, C-9, C-12, C-20, C-33 |
| | <i>C. piscinale</i> (Nilsson, 1822) | C-2, C-6, C-8, C-16, C-22 |
| <i>Pseudanodonta complanata</i> (Rossm., 1835) | <i>P. complanata</i> | P-2, P-7, P-12, P-13, P-16 |
| | <i>P. elongata</i> (Hollandre, 1836) | P-1, P-4, P-8, P-11, P-18 |
| | An intermediate form between <i>P. complanata</i> and <i>P. elongata</i> | P-14 |
| <i>Unio pictorum</i> (L., 1758) | <i>U. (Unio) pictorum</i> | U-7, U-17, U-18, U-25, U-30 |
| | <i>U. (U.) protractus</i> (Lindholm, 1932) | U-5, U-8, U-24, U-40, U-41 |
| <i>Unio tumidus</i> (Phil. in Retz, 1788) | <i>U. (Tumidiana) tumidus</i> | U-78, U-42 |
| | <i>U. (T.) longirostris</i> (Rossm., 1836) | U-11, U-37, U-62, U-64, U-72 |
| | <i>U. (T.) conus</i> (Spengler, 1793) | U-10, U-12, U-44, U-67, U-83 |
| <i>Unio crassus</i> (Phil. in Retz., 1788) | <i>Crassiana crassa</i> | Cr-1, Cr-7, Cr-17, Cr-48 |
| | <i>Cr. musiva</i> (Spengler, 1793) | Cr-8, Cr-32, Cr-34, Cr-41, Cr-49 |
| | <i>Cr. nana</i> (Lamarck, 1819) | Cr-3, Cr-24, Cr-27, Cr-33, Cr-51 |
| | An intermediate form between <i>Cr. musiva</i> and <i>Cr. crassa</i> | Cr-11, Cr-52, Cr-53 |

Hereinafter, the names of genera of the subfamily Anodontinae (*Colletopterum* and *Pseudanodonta*) are given according to [3].

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Fig. 1. Phylogenetic tree of East European Unionidae constructed from ITS1 and 16S rDNA fragments. Here and in Fig. 2, italics designate species names for genetic groups of mussels according to Zhadin [4].

lowing Thompson's theoretical ideas [1], the shape of the convex contours of shell valves in *Bivalvia* were postulated to be species specific [2].

Using the comparative method resulted in a noticeably increased number of recognized genera and species of freshwater Unionidae. For example, in Northeastern Europe, the last revision using the comparative method revealed 19 species that belong to five genera [3]; conversely, eight Unionidae species that

belong to two genera were registered in this region before using this method [4].

In recent years, inapplicability of the comparative method for taxonomy has been considered by some malacologists [5, 6]. Since comparative species were identified using the diagnostically unreliable ratio of the shell height to width, attempts to prove inappropriateness of the comparative method by genetic methods [7–9] failed [10].

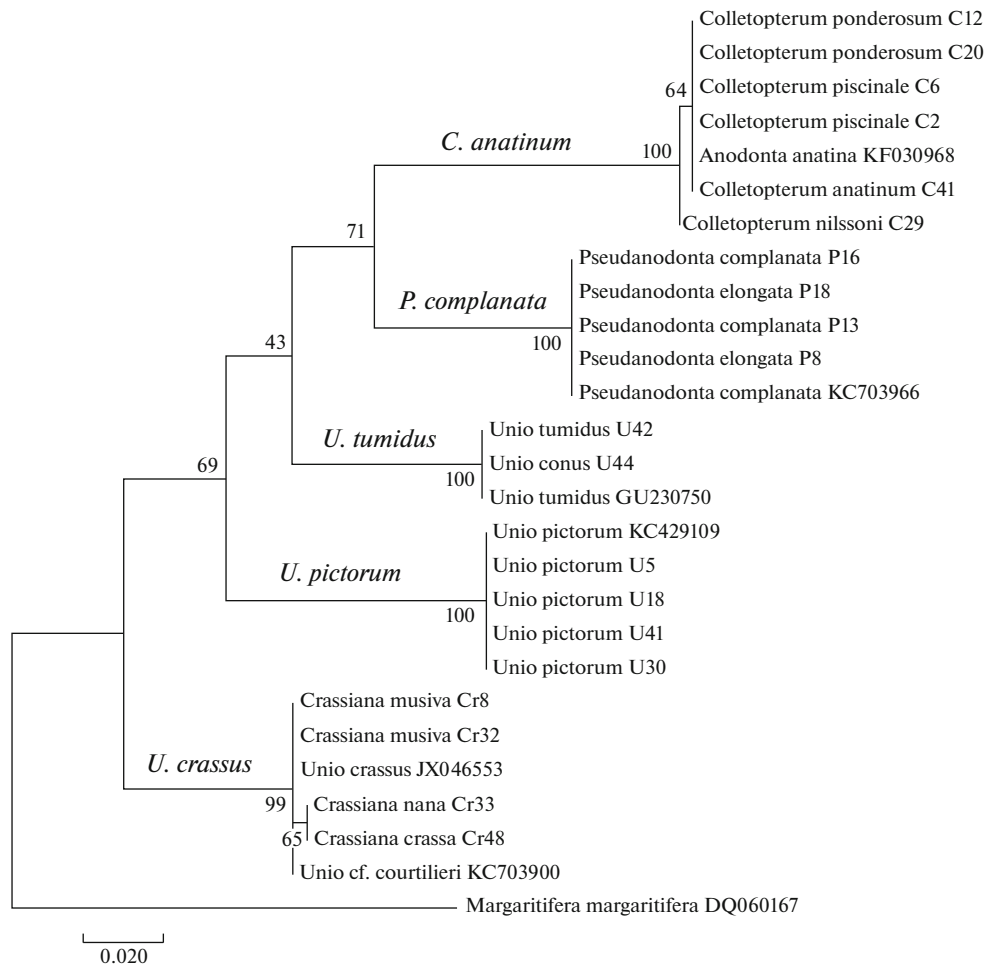


Fig. 2. Phylogenetic tree of East European Unionidae constructed from the COI gene fragment.

The aim of this study was to perform a genetic analysis using nuclear and mitochondrial markers in order to evaluate the applicability of the comparative method for species identification of bivalves as exemplified by Eastern European Unionidae.

Mussels were collected in May 21–22, 2016 from the typically lowland Ivitsa River (Volga basin, Rameshkovskii raion, Tver oblast). Genetic analysis was performed for 65 specimens. We identified five species among these 65 specimens according to Zhadin's classification from shell morphology other than the convex contour of the shell [4]. At the same time, the modified comparative method [10] recognizes 14 Unionidae species (Table 1) that belong to five comparative groups from four genera (*Unio*, *Crassiana*, *Pseudanodonta* and *Colletopterum*) and two subgenera of the *Unio* genus (*Unio* s. str. and *Tumidiana*) according to the classification of Starobogatov–Bogatov [3]. The results were verified by genetic studies conducted at the Moscow State University.

Genomic DNA was extracted from the leg tissue using the Diatom DNA Prep 100 kit (Laboratory

Isogen, Russia) according to the manufacturer's recommendations. The ITS1, COI, and 16S fragments were generated by PCR amplification. Direct and reverse sequencing was performed using an ABI PRISM 3730 automatic sequencer (Applied Biosystems, United States). Nuclear and mitochondrial markers were used to detect presence or lack of hybridization between the studied species and presence of mitochondrial introgression.

All sequences were aligned using the ClustalW algorithm implemented in MEGA7. Two multiple alignments were constructed: 602 bp in length for ITS1 and 16S rDNA fragments using 60 specimens (Fig. 1) and 295 bp in length for the COI fragment using 20 specimens (Fig. 2). Phylogenetic trees were constructed using the maximum likelihood algorithm implemented in MEGA7 with 500 bootstrap replications. We used the sequences of Unionidae representatives with known phylogenetic taxonomy derived from GenBank, including sequences of *Margaritifera margaritifera* (L.) as an outgroup.

Molecular genetic analysis using nuclear and mitochondrial markers has demonstrated that the five studied comparative groups of mussels are genetically distant from each other but possess high intragroup homogeneity (Figs. 1, 2). Hybrids among the examined samples were not revealed. Importantly, the genetic groups of mussels that we identified according to major morphological features other than the curvature of the outer contours of the shells corresponded to five “taxonomic” species according to Zhadin [4]. This fact confirms the validity of the traditional morphological approach in Unionidae species identification. Therefore, based on rules of the Zoological nomenclature, the Unionidae malacofauna in the Ivitsa River is represented by five species that belong to three genera (Table 1). The names of comparative species (given in brackets) should be regarded as invalid: *C. anatinum* (= *C. ponderosum*, *C. piscinale*, *C. nilssoni*) of the *Colletopterum* genus; *Ps. complanata* (= *Ps. elongata*) of the *Pseudanodonta* genus; *U. pictorum* (= *U. protractus*), *U. tumidus* (= *U. longirostris*, *U. conus*) and *U. crassus* (= *Crassiana musiva*, *Cr. nana*) of the *Unio* genus.

Hence, the comparative method cannot be used in Unionidae species identification. At the same time, studying the convex contours of the cross-section of shell valves can be useful in investigation of growth patterns, shape formation and intraspecific variation of bivalves.

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