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Research Article

Shell morphological versus genetic identification of quagga mussel (*Dreissena bugensis*) and zebra mussel (*Dreissena polymorpha*)

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Abstract

Herein we provide first genetic evidence that *Dreissena bugensis* Andrusov, 1897 has reached the German Danube system. The occurrence of this species at several sampling sites along a 152 km river stretch of the upper Danube River thereby proved to be wrongly estimated by using solely shell morphology features for species identification. 17.5% of the dreissenid specimens (*Dreissena bugensis*, N=26, *Dreissena polymorpha*, N=34), identified according to exterior shell characteristics, were assigned to the wrong species. In contrast to RFLP identification, comparisons of shell dimension measurements by discriminant function analysis were not able to fully identify the two species, with correct classification rates between 61.1% and 94.4%, confirming their high morphological plasticity. The results of this study suggest that species records of dreissenid mussels should generally be genetically verified.

Key words: aquatic invasive species, species identification, RFLP, morphometry, invasional replacement, morphological plasticity, upper Danube River

Introduction

The Ponto-Caspian region is a major source of species that became invasive in many parts of the world (e.g. Lowe et al. 2000). The zebra mussel *Dreissena polymorpha* (Pallas, 1771) is thereby one of the most successful invaders in terms of abundance (Lowe et al. 2000; Hallstan et al. 2010; Naddafi et al. 2011). After introduction it is known to quickly spread into inter-connected aquatic ecosystems, forming high densities and biomass, consequently creating great financial and ecological damage (Strayer 2009; Keller et al. 2011; Sousa et al. 2014; Nakano and Strayer 2014). Another dreissenid species, *Dreissena bugensis* Andrusov, 1897 is known to have a similar invasive potential and frequently appears in geographic regions that were initially invaded by *D. polymorpha*. According to model predictions, 75% of the potential *D. bugensis* distribution overlaps with *D. polymorpha* distribution on a global scale, although only 43% of the predicted *D. polymorpha* distribution areas are expected to contain *D. bugensis* (Quinn et al. 2013).

The appearance of *D. bugensis* is often accompanied by a decline in *D. polymorpha* populations (Ram et al. 2012), implying strong interactions between these two species. In North America, *D. polymorpha* was first recorded in 1986 (Herbert et al. 1989), and *D. bugensis* in 1991 (May and Mardsen 1992). In this region, the replacement of *D. polymorpha* by *D. bugensis* populations is frequently reported in the literature, especially in the Laurentian Great Lakes area (Ram et al. 2012). Similar patterns are reported from Eastern Europe in the invaded Dniepr and Volga basins (Orlova 2005). In Western Europe, *D. polymorpha* has been present at least since 1827, but *D. bugensis* was more recently detected in 2006 in the lower River Rhine (Molloy et al. 2007). An understanding of the ecological niches and the species-interactions of dreissenid mussels requires a reliable determination of species. This is particularly important for morphologically highly similar species such as *D. bugensis* and *D. polymorpha* (e.g. Voroshilova et al. 2010). The species identification of freshwater bivalves in general, and dreissenids in particular, by morphological

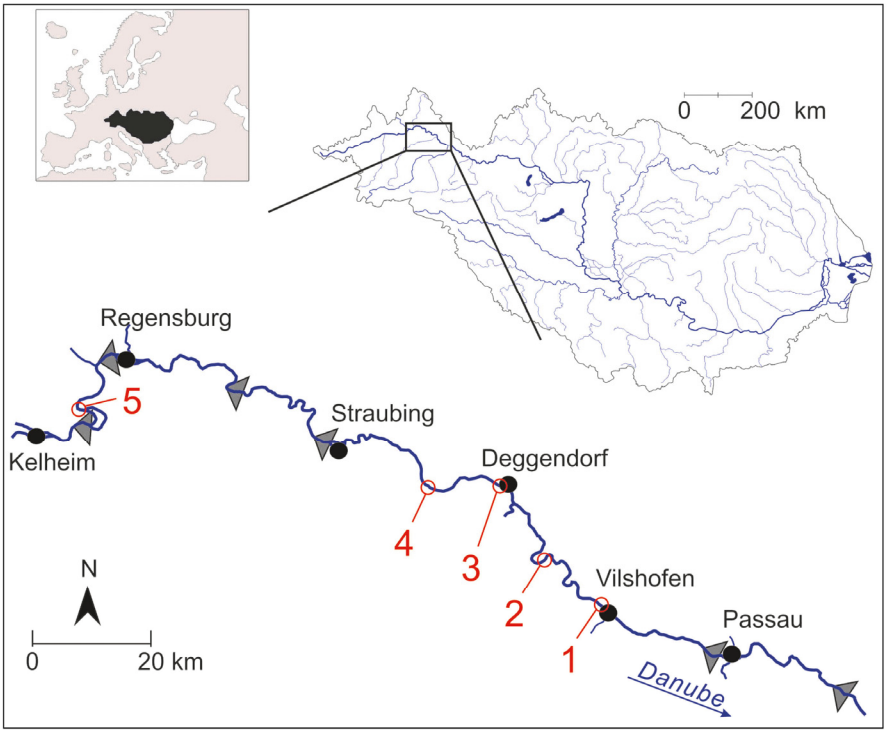

Figure 1. Sampling sites 1 to 5 in the study area of the upper Danube River (upper right part). Triangles represent dams.

characteristics alone is known to be often difficult or inconsistent (May and Marsden 1992; Zieritz et al. 2012). This is mostly due to habitat-specific growth patterns influenced by environmental parameters such as temperature, depth and flow conditions (Zieritz and Aldridge 2009).

The aims of this study were to (i) identify, verify and map *D. bugensis* at a very early stage of the invasion process in the upper Danube River, using molecular genetic methods (RFLP) and to (ii) compare species assignments of *D. bugensis* and *D. polymorpha* based on molecular genetic vs. shell morphological and morphometric classifications.

Material and methods

Mussel sampling

Mussel samples originated from two sampling surveys conducted in 2010 and 2014 within the upper Danube River (Figure 1). All mussels were randomly sampled in bank areas accessible by wading (depth < 1 m) and were immediately snap-frozen on dry ice after collection. The sampling sites were at Vilshofen (E 13°10'44", N 48°38'24"), Winzer (E 13°03'08", N 48°43'37"), Deggendorf (E 12°59'50", N 48°47'31"), Mariaposching (E 12°52'12", N 48°50'28") and Bad Abbach (E 12°00'13", N 48°57'57") as shown in Figure 1. A total of 60 specimens were used for further analysis.

Species identification and data analysis

Species identification was carried out by three different methods: genetic, morphological and morphometric analyses. Genetic species identification was carried out by restriction fragment length polymorphism (RFLP) of the cytochrome C oxidase subunit I (COI). Genomic DNA was extracted using the phenol–chloroform method following Sambrook et al. (1989). Primers used for amplification of a 608 bp long portion of the COI mitochondrial gene were chosen according to Claxton and Boulding (1998) and are specified in Table 1. For each PCR reaction, a total volume of 15 µl was used, containing 0.04 U/µl Taq polymerase, 0.2 pmol/µl of each primer, 0.2 mM dNTPs (Biomers, Germany), and 3.0 mM $MgCl_2$ and 10x buffer (Solis Biodyne, Estonia). PCR was run for 40 cycles (denaturation: 95°C, 30 s; annealing: 50°C, 30 s; extension: 72°C for 30 s), using 2 µl template DNA and 3.0 mM of $MgCl_2$.

Table 1. Primers used for amplification of the COI gene fragment in dreissenids as reported by Claxton and Boulding (1998).

Primer Name	Direction	Primer Sequence 5' – 3'	Length (bp)	Tm (°C)	GC (%)
Dreissenid A	Forward	SCTTGTKGGMACRGGTTTTAGTG	23	54.2 – 61.0	50
Dreissenid B	Reverse	GGATCTCCTAACCCTGTWGGATCAA	25	57.3 – 58.1	48

Restriction analysis of the COI gene region was carried out using the *ScrFI* restriction enzyme (Thermo Fisher Scientific Inc.) as suggested by Claxton and Boulding (1998). The reaction mixture consisted of 2 µl sterile distilled water, 1 µl of restriction buffer (50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl, and 0.1 mg/ml BSA), 1 µl restriction enzyme (10 U/µl) and 6 µl of PCR products. Digests were incubated for 6 h at 37°C and loaded onto 2 % (w/v) agarose gels stained with ethidium bromide. Electrophoresis was carried out in a standard TBE buffer for 30 min at 100 mV. Fragment patterns were visualized under UV light and photographed.

A subset of 36 specimen were independently examined by seven researchers (PhD students and Postdocs in Aquatic Ecology with taxonomic identification and zoological background) and distinguished according to common morphological features as described in Ram et al. (2012); e.g. *D. polymorpha*: sharp angled transition of dorsal and ventral surface, flat or concave bottom, striped color patterns; *D. bugensis*: rounded angle of transition between dorsal and ventral surface, convex or round bottom.

A multivariate, morphometric analysis was performed to distinguish species by statistical means. For this purpose, measurements of the three shell dimensions, length (the longest antero-posterior distance), height (the longest dorso-ventral distance) and width (the longest distance between left and right valve), were taken (to the nearest 1 mm). The shell angle in a subset of 36 specimens was quantified according to Claxton et al. (1997). It was measured at the hinge between the line connecting the anterior to the posterior part of the shell and the line from the dorsal curvature inflexion point at the dorso-posterior shell outline (Figure 2).

Principal component analysis (PCA) was used to extract main factors of shell morphometric measurements by using shell characteristics "shell elongation", "shell inflation" and "shell angle" as input variables. Morphological characteristics "shell elongation" and "shell inflation" were calculated

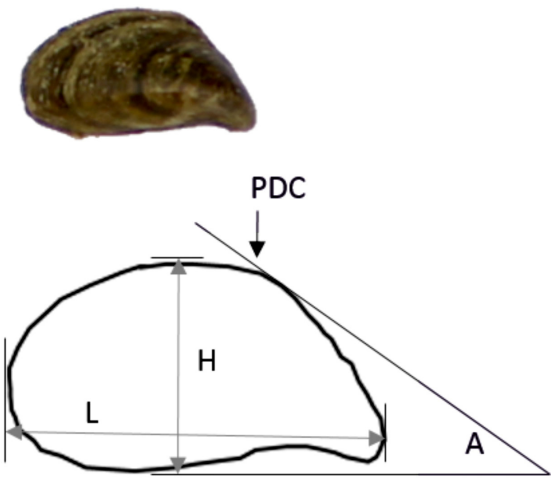

Figure 2. Morphometric measurements taken on mussel shells. **L**: length (the longest antero-posterior distance), **H**: height (the longest dorso-ventral distance), **A**: shell angle between the line connecting the anterior to the posterior part of the shell and the line from the point of dorsal curvature (PDC) at the dorso-posterior shell outline.

from the three measurements of shell dimensions (length vs. height and width vs (length+height)/2, respectively). Square root transformation was used to normalize the morphometry data. We tested for normal distribution and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively. Comparisons of morphometric shell characteristics "elongation", inflation" and "angle" between mussel species were performed by analysis of variance (ANOVA). Differences in principal component factor scores were compared using the Kruskal-Wallis test. Subsequently, these characteristics were used as predictor variables to perform the discriminant function analysis for each individual measurement and for a combination of all variables using principal component factor scores. Mussels were grouped by species as determined by the RFLP method to compare the respective species classification of the discriminant function. All statistical anlyses were performed using SPSS 21 (IBM, USA).

Results

Species identification by three different approaches, i.e. using genetic RFLP analysis, common identification keys and morphometric shell measurements, lead to inconsistent results in species assignment. RFLPs of the COI mitochondrial gene region clearly separated the tested samples into two distinct groups by restriction band-patterns, with gene fragments of approximately 50, 150 and 400 bp and 50, 175, 200 and 250 bp, respectively. These results match the restriction fragment sizes and numbers reported by Claxton and Boulding (1998) for *D. polymorpha* (3 fragments) and *D. bugensis* (4 fragments). No variation in RFLP patterns within species was found. Accordingly, 34 of the 60 mussels were identified as *D. polymorpha* and 26 as *D. bugensis*. Exterior shell features, especially the striped color patterns did not allow a distinct separation between species. The typical features of *D. polymorpha*, i.e. the arched and flattened ventral surface of the shell and the sharp angled transition of the ventral and dorsal surface were highly variable among the examined specimens. Morphological variation in shell appearance is exemplarily shown in Figure 3. Species assignments by seven researchers based on morphological identification keys thereby resulted on average in 17.5% misidentification (min. 6%, max. 25%) of invasive dreissenid species using genetic results as a reference for the "true" species. On average, *D. polymorpha* was more frequently identified correctly (98%) compared to *D. bugensis* (74%).

Principal component analysis of morphometric measurements extracted two principal components (PCs) explaining 87.7% of variability in the dataset (Figure 4). PC1 based mainly on "shell inflation" and "shell elongation" and explained 55.9% of variability and PC2 based mainly on "shell angle" and explained another 31.8%. The Kruskal-Wallis-test showed significant differences between genetically identified mussel species based on PCs (PC1, $p < 0.001$; PC2, $p < 0.05$), however individual data points were highly overlapping on both axes. Morphometric analysis yielded a significant difference in shell elongation ($F(1,58)=18.214$, $p<0.001$), shell inflation ($F(1,57)=33.650$, $p<0.001$), and to lower extend, in shell angle ($F(1,34)=7.534$, $p<0.05$) between genetically identified *D. bugensis* and *D. polymorpha* specimens (Figure 5). Despite these significant differences, the overlap in shell morphology characteristics of the two species did not allow a clear separation of species.

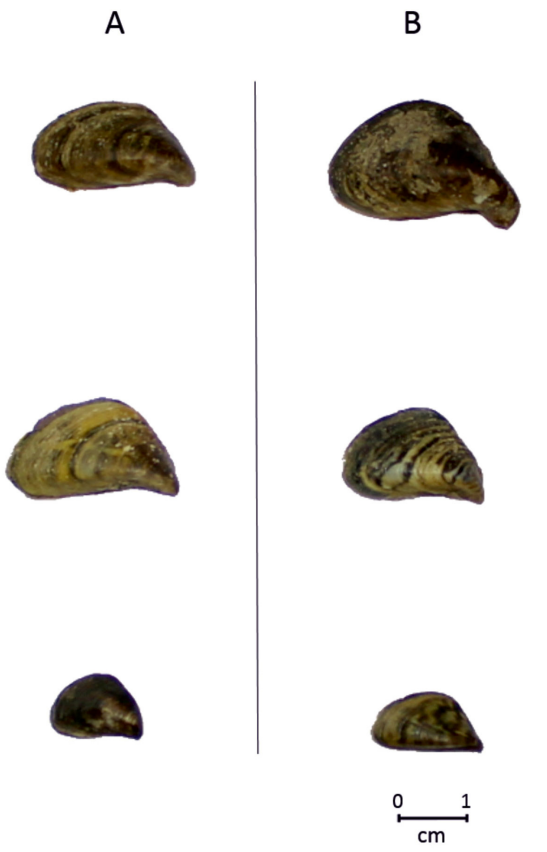

Figure 3. Lateral views of genetically determined *Dreissena polymorpha* (**A**) and *Dreissena bugensis* (**B**) shells collected in the Danube River (Germany). Both species showed pronounced intraspecific variation in shell morphology and color patterns.

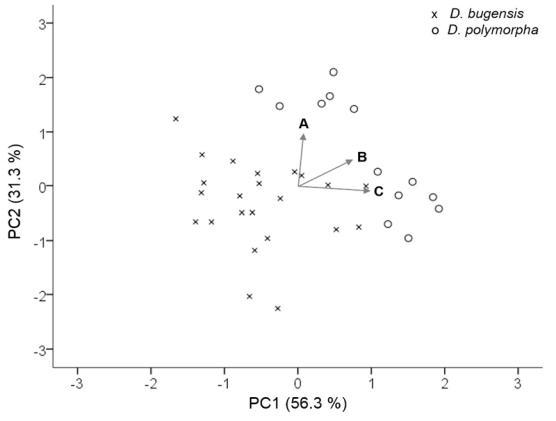

Figure 4. Principal component factor scores and factor loadings of individual measurements of shell characteristics **A**: "shell angle", **B**: „shell inflation"(ratio width to (length+height)/2) and **C**:"shell elongation" (shell length to height ratio). Genetically determined species identity is indicated by (x) for *D. bugensis* and (○) for *D. polymorpha*. Note the overlap between species which would result in misidentification based on the use of these morphological characteristics.

Table 2. Results from discriminant analyses using predictor variables elongation (A), inflation (B), angle (C), variables A,B,C combined (D) and principal components (E) to classify *D. polymorpha* and *D. bugensis*.

		Discriminat function analysis				
		A	B	C	D	E
Genetic classification						
D. bugensis	Total N	26	26	23	23	23
	N misidentified	6	4	8	2	3
	% misidentified	**23.1**	**15.4**	**34.8**	**8.7**	**13.0**
D. polymorpha	Total N	34	34	13	13	13
	N misidentified	8	4	6	0	0
	% misidentified	**23.5**	**11.8**	**46.1**	**0**	**0**

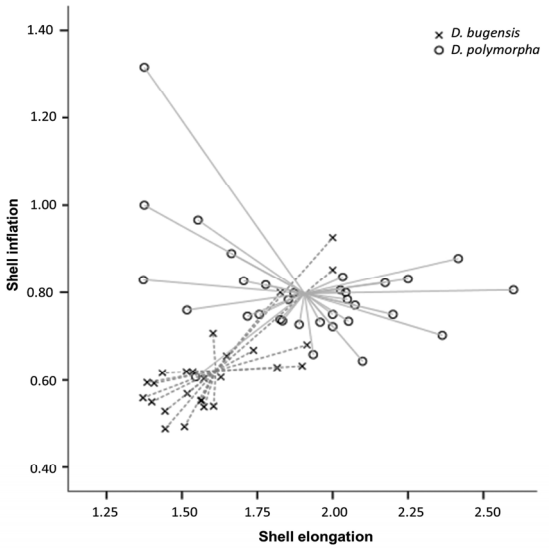

Figure 5. Centroid view of individual measurements of shell characteristics „shell elongation“ (shell length to height ratio) and „shell inflation“ (ratio width to (length+height)/2). Genetically determined species identity is indicated by (x) for *D. bugensis* and (○) for *D. polymorpha*. Note the overlap between species which would result in misidentification based on the use of these morphological characteristics.

Discriminant function analysis provided species group classifications with different error rates depending on the morphological predictor variables used. The overall wrong classification was highest for the measurement of “shell angle” (38.9%, canonical correlation 0.419), followed by “shell elongation” (26.7%, canonical correlation 0.489). A better discrimination was achieved for “shell inflation”, with 13.6% wrong classification of both species (canonical correlation 0.636). Using the PCA factor scores as input variable for the discriminant analysis did result in a better classification rate with 8.3 % wrong classification (canonical correlation 0.828). In comparison, discriminant function analysis using the three morphometric measurements as input variables in combination (“shell angle”, “shell elongation” and “shell inflation”) resulted in 5.6% wrong classification of species identity (canonical correlation 0.855). *Dreissena bugensis* was more frequently misidentified compared to *D. polymorpha*. Species specific classification rates are summarized in Table 2. In contrast to the genetic species assignment, none of the species identification methods relying on morphology or morphometry was precise, suggesting that morphological species identification is not reliable, especially for low sample sizes.

Using morphological keys, *D. bugensis* was found at sites 2 (Winzer), 3 (Deggendorf), 4 (Mariaposching) and 5 (Bad Abbach) (Figure 1). The distribution range of *D. bugensis* was over-estimated by focusing solely on morphological species identification features. Genetically determined *D. bugensis* only originated from sites 1, 2, 3 and 4. The occurrence of *D. bugensis* at the most upstream site 5 (Bad Abbach) could not be confirmed.

Discussion

This study provides the first genetic evidence for the arrival and establishment of *Dreissena bugensis* in the middle-European parts of the Danube River starting from the year 2011. However, estimates on frequency and distribution of this species could be biased when only shell morphology or morpho-metry is used for species identification. This is in line with previous observations that freshwater mussels show a high intraspecific variability in morphological characteristics and color patterns (Voroshilova et al. 2010; Zieritz et al. 2012). For instance, observed differences in the zebra banding and the transition angle between the dorsal and ventral side did not match the genetic assignments.

Both characteristics are highly subjective, as evident from the wrong species assignment by experts varying between 6% and 25%. Within this study, the differentiation into two distinct groups was only clear using the molecular method. We also observed no evidence for possible hybrids of both species, which would have been a possible explanation for the high morphological variation. The comparison of shell dimension morphometry seems to allow distinguishing between species in principal, with misidentification rates below 10%, but there is a high variability within the dataset in contrast to the RFLP method. Species assignment based on shell morphological features can be particularly problematic if only few specimens of both species are available, or if only one of the two species is present. Both scenarios are likely especially in early stages of dreissenid invasions as evident from our sampling sites in the upper Danube River.

A reconstruction of distribution pathways requires thorough mapping and species validation. Recent studies reported the first introduction of *D. bugensis* into the Main River through the Main-Danube Canal in 2004 via inland water transport (Molloy et al. 2007), also referred to as *jump dispersal* (Heiler et al. 2012), and a subsequent appearance in the Dutch Rhine Delta in 2006 (Molloy et al. 2007, van der Velde and Platvoet 2007). Heiler et al. (2012) reported *D. bugensis* in the upper Danube River in 2009 at sampling sites between Straubing and Regensburg and in the Main-Danube Canal in Kelheim, but species identities were not confirmed genetically. In 2011, the species was also detected in this river section, but without reporting the species identification method (Schoell et al. 2012). Based on our study, the presence of *D. bugensis* was only confirmed for three adjacent sampling sites of the upper Danube River, suggesting that a validation of other reported areas of distribution should be carried out.

The accurate validation of species identities is required for the investigation of population establishment, spreading and possible interactions with already established invasive species, particularly the closely related *D. polymorpha*. Fast shifts in species-densities or even displacement of *D. polymorpha* by *D. bugensis* are a known and often common phenomenon (Molloy et al. 2007). *Dreissena bugensis* is known to have a wider range of temperature tolerance (Mills et al. 1996), but other mechanisms contributing to its success, e.g. competition or hybridization are not fully understood (Sousa et al. 2014; Sanz-Ronda et al. 2014). The contemporary arrival of *D. bugensis* in the upper Danube section provides the ideal time slot for the investigation of introduction, spreading mechanism and species interactions, including interactions with already established (invasive) species, especially since comparable information on the invasion front are already available for invasive Ponto-Caspian neogobiid fishes in the same area (Brandner et al. 2013). The investigation of invasion histories is usually limited to indirect methods relying on *back-dating* (Heiler et al. 2012) and thus the detection of *D. bugensis* at an early stage of the invasion process is of particular importance.

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