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MORPHOLOGICAL AND MOLECULAR DIFFERENCES BETWEEN THE INVASIVE BIVALVE *RUDITAPES PHILIPPINARUM* (ADAMS & REEVE, 1850) AND THE NATIVE SPECIES *RUDITAPES DECUSSATUS* (LINNAEUS, 1758) FROM THE NORTHEASTERN ADRIATIC SEA

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ABSTRACT The alien bivalve *Ruditapes philippinarum* (Manila clam) was intentionally introduced along the Italian Coast of the northwestern Adriatic Sea for aquaculture purposes in 1983. In February 2013, *R. philippinarum* was recorded at a site of the northeastern Adriatic Sea (Zelena Laguna, vicinity of city Poreč, west Istrian Coast, Croatia). This finding represents the first record of *R. philippinarum* in Croatian waters. The colonized site is located at a distance of approximately 100 km by a straight line in the west east direction from the site where the mollusc was firstly introduced. At Zelena Laguna, *R. philippinarum* colonized the intertidal sandy substrate together with the native species *Ruditapes decussatus*. The two sympatric species were initially differentiated based on the morphology of the siphons. Molecular analysis of 16S rRNA gene confirmed the morphological distinction between the two species. Although the two species are very similar in shell morphology, the relationships width/length and width/height were negative allometric for *R. decussatus* and isometric for *R. philippinarum*. The relationship height/length was isometric for both species. Additionally, the length of the pallial sinus was significantly different between the two species ($P < 0.001$).

KEY WORDS: clam, *Ruditapes philippinarum*, *Ruditapes decussatus*, invasive species, biometry, 16S rDNA, northern Adriatic Sea

INTRODUCTION

In the Mediterranean Sea, the family Veneridae (Bivalvia: Veneroida) is represented by 25 genera (Costello et al. 2001, CLEMAM 2008). Among these genera, the genus *Ruditapes* comprises three species: *Ruditapes corrugata* (Gmelin, 1791), *Ruditapes decussatus*, and *Ruditapes philippinarum* (Adams & Reeve, 1850) (Costello et al. 2001, Zenetos et al. 2004). All these three species are commercially important. The first two species are indigenous for the Mediterranean; the latter is a nonindigenous species.

The invasive species *Ruditapes philippinarum* (Manila clam or Japanese carpet shell) is an infaunal bivalve that lives in sand, muddy gravel, or stiff clay from mid-tide level to a few meters depth. It originates from the western Pacific, having natural populations distributed from the Philippines up to the southern Kuril Islands (Scarlato 1981). Depressed fisheries and aquaculture activities of the native European *Ruditapes decussatus* (crosscut carpet shell) led to imports of *R. philippinarum* into European waters. In 1972, the species was introduced into France for commercial hatchery, where the intense cultivation of the species initiated in the early 1980s (Goulletquer 1997). The high reproduction rate of *R. philippinarum* caused a geographical expansion of the species outside growing areas in France and Ireland (Goulletquer 1997). After that, demand in aquaculture resulted in its imports into Norway, Germany, Belgium, Israel, Tunisia, and Italy (Cesari & Pellizzato 1985, Shpigel & Fridman 1990). The *R. philippinarum* culture was preferred to that of *R. decussatus* due to high growth rate, ease in obtaining seed from controlled reproduction, and higher tolerance to variations in temperature, salinity, and substrate type (Gosling 2003). In 1983, *R. philippinarum* was introduced in the northern Adriatic (Venice Lagoon) to supplement the local fishery of the autochthonous *R. decussatus* (Cesari & Pellizzato 1985, Breber 2002). In a relatively short period of time *R. philippinarum* colonized the entire Venice Lagoon (Breber 2002), expanding also into other nearby location, such as the Marano Lagoon (Zentilin 1990) and the Po River Delta (Carrieri et al. 1992). So far, there has been no evidence of its presence on the eastern (Croatian) Adriatic Coast where the indigenous *R. decussatus* commonly inhabits suitable habitats (Peharda et al. 2010).

Distinguishing *Ruditapes philippinarum* from *Ruditapes decussatus* is usually difficult due to the similarity of specimen shell morphology as well as the fact that more confident discrimination can be done only when clams open their valves displaying the siphons (Holme 1961, Cesari & Pellizzato 1990). A genetic analysis of molecular markers certainly represents the most reliable approach in species differentiation; but this destructive procedure is costly and time consuming. For this reason, *R. philippinarum* is often subjected to food frauds at marketplaces being misidentified as *R. decussatus*. For instance, in Italy, both species are commercialized under the same name ''Vongola verace'' despite the fact that the commercial value of *R. decussatus* is significantly higher than the one of *R. philippinarum* because of the higher organoleptic characteristics of the former compared with those of the latter (Costa et al. 2010).

In general, most bivalves such as clams and scallops are suitable for morphological analysis as they have solid, hard shells showing no deformation of shape during manipulations (Speiser & Johnsen 2008, Leyva-Valencia et al. 2012, Gonzalez-Pelaez et al. 2013). A number of studies deal with geometric morphometric analyses within species (Zelditch & Fink 1995, Rosas & Bastir 2002, Márquez et al. 2010) and among species (Penin & Berge 2001, Rosenberg 2002). Morphometric techniques have been used to discriminate species on the basis of shell form variation (Marko & Jackson 2001,

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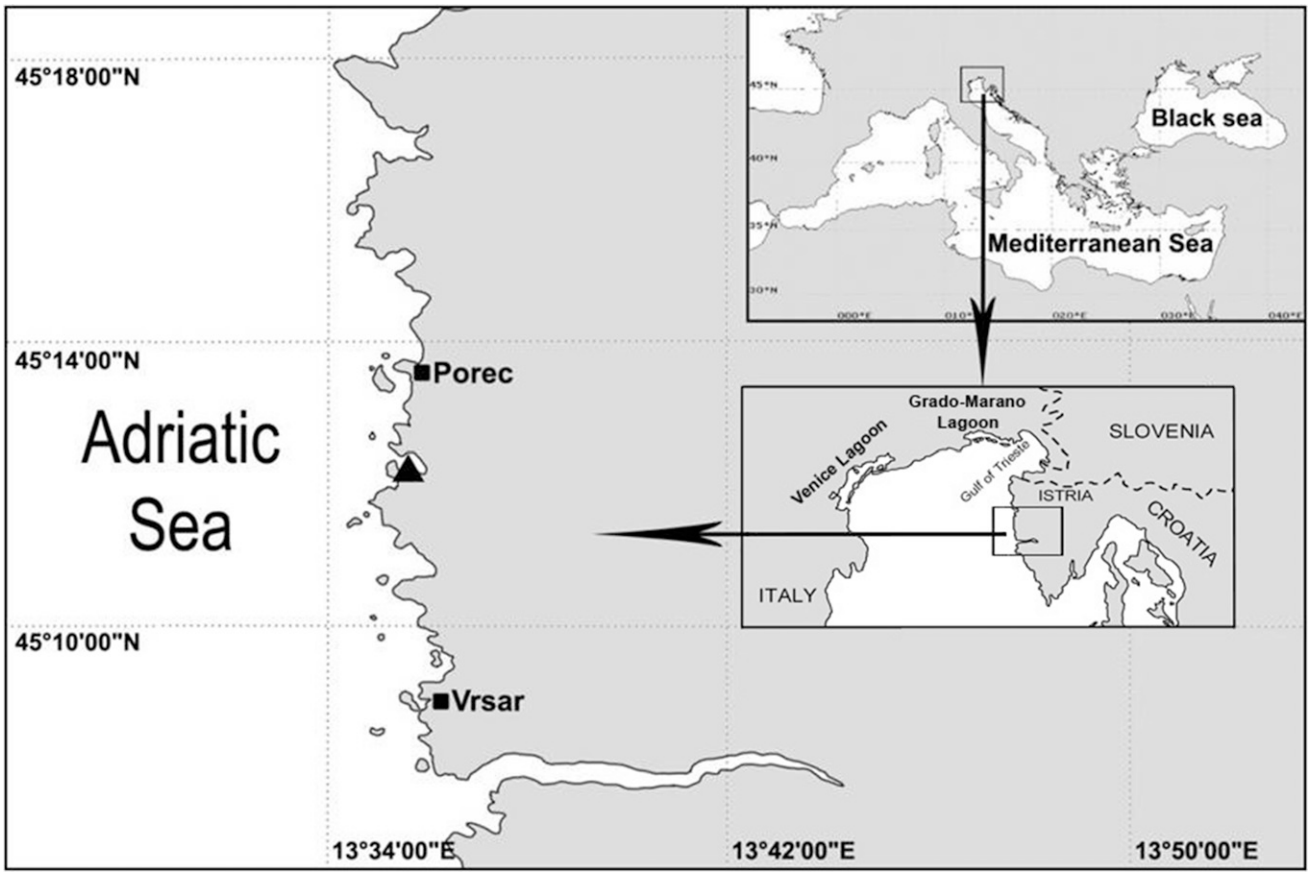

Figure 1. A map showing the investigated area. The triangle indicates the location of Zelena Laguna near the city of Poreč where *Ruditapes* spp. specimens were sampled.

Anderson & Roopnarine 2005, Kosnik et al. 2006, Ampili & Shiny Sreedhar 2015) and to examine pattern of shell development (Roopnarine 2001, Tang & Pantel 2005). To facilitate the discrimination between *Ruditapes philippinarum* and *Ruditapes decussatus*, a non-invasive morphometric method based on the elliptic Fourier analyses of the external shell shape was recently suggested (Costa et al. 2008, 2010). The basic morphometric analysis of the shell length, height, and width has not yet been used in *R. philippinarum*/*R. decussatus* differentiation.

The aim of this study was to report the first record of *Ruditapes philippinarum* in Croatian waters and to compare the morphology and the morphometric relationships of *R. philippinarum* and *Ruditapes decussatus* sympatric populations. In parallel with the anatomy of the siphons and pallial sinus shape, the identity of the species was corroborated by a mitochondrial 16S rRNA gene analysis.

MATERIALS AND METHODS

Sampling

The specimens *Ruditapes* spp. were collected from an intertidal sandy substrate at Zelena Laguna (45° 12′ 10.27″ N; 13° 35′ 22.78″ E) in the vicinity of the city of Poreč (northern Adriatic—central part of the west Istrian Coast, Croatia) during February 2013 (Fig. 1). The colonized site is located at a distance of approximately 100 km by a straight line in the west-east direction from the Italian site where the mollusc was firstly introduced. As the aim of this study was to compare the morphology and morphometry of commercial-sized clams, juveniles were not collected. The sampling was performed using a rake to dig the sediment during the period of maximum low tide. In total, 140 *Ruditapes* spp. were collected.

Morphological Analyses

The two species were differentiated according to six morphological criteria.

1. The morphology of the siphons was compared in the live specimens kept in sea water when they had opened their valves, according to Gosling (2003). The siphons of *Ruditapes philippinarum* are fused along most of their length being separated only at the tips, whereas the siphons of *Ruditapes decussatus* are completely separated and longer than that of *R. philippinarum* (Fig. 2A).
2. Differences in radial sculpture and radial line morphology also occur between the two species (Geri et al. 1996, Hurtado et al. 2011). The radial sculpture of *Ruditapes philippinarum* is more distinct than the *Ruditapes decussatus*. In contrast to *R. decussatus*, radial lines of *R. philippinarum* are tick forming and shaping squares (Fig. 2B).

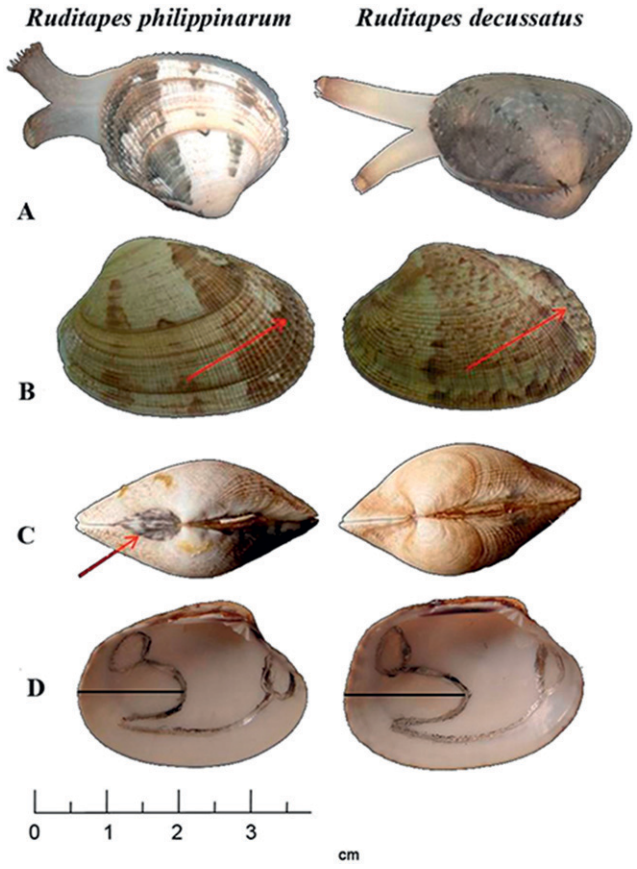

Figure 2. Differences in morphology between *Ruditapes philippinarum* and *Ruditapes decussatus*. (A) Siphons, (B) shape and sculpture of the posterior region, (C) lunule, and (D) the length of pallial sinus with a line parallel to the shell length (line from the depart point of the sinus to the shell edge length of the pallial sinus), inner of the left shell valves.

3. In terms of shell morphology, *Ruditapes phillippinarum* is broadly oval in outline, whereas in the posterior region *Ruditapes decusstaus* is more triangular (Fig. 2B).
4. The lunulae on the external side of the hinge area are dark brown, presented with fine radiating ridges in *Ruditapes philippinarum*, whereas the same area in *Ruditapes decussatus* is colorless (Fig. 2C).
5. The internal shell coloration of *Ruditapes philippinarum* is mainly characterized by purple-colored spots on the muscles and pallial sinus, as well as pallial sinus impressions and ventral margin of valves (Quéro & Vayne 1998). In contrast, the same areas in *Ruditapes decussatus* are either whitish or light yellow without any purple coloring (Gofás et al. 2011).
6. The depth of pallial sinus (Fig. 2D) is a reliable indicator of the length of the siphons (Gosling 2003), and in *Ruditapes Philippinarum*, it is less pointed to the ventral margin of the shell, whereas in *Ruditapes decussatus*, it is pointed deeper (Quéro & Vayne 1998).

Morphometric Analyses

The maximum dimension (distance) of the anterior-posterior axis was recorded as shell length representing the greatest distance from the anterior to the posterior end of the shell

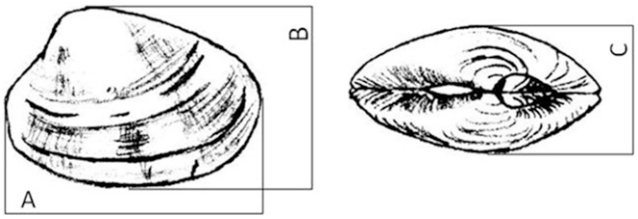

Figure 3. Used shell morphometric measures: (A) shell length, (B) shell height, and (C) shell width.

(Fig. 3A). The maximum distance between the dorsal and ventral edges of the shell was measured as the shell height (Fig. 3B). The shell width was determined as the distance between the furthest expansion of the left and right valves in the closed shell (Fig. 3C). All measurements were made to the nearest 0.01 cm using Vernier calipers. The shell morphometric relationships, height/length, width/length, and width/height, were investigated by linear regression analysis for both species separately. Differences between regressions slopes were ascertained by analysis of covariance (ANCOVA) after log transformation of data. Patterns of morphometric relationships (isometric: slope = 1, negative allometric: slope < 1, positive allometric: slope > 1) were assessed according to Gaspar et al. (2002). F3

A *t*-test was used to compare the length of the pallial sinus between the two species. Sinus length was measured along a line parallel to the shell length line from the depart point of the sinus to the shell edge (2D). Before testing, the length of the pallial sinus was expressed as percent of the shell length. All statistical analyses were performed using a software package SYSTAT v. 12.

Genetic Analyses

Total genomic DNA was extracted from 25 mg of the adductor muscle tissue by using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. We avoided DNA isolation from gonads or from a complete specimen to prevent the potential delusive results caused by doubly uniparental inheritance of mitochondria (Plazzi & Passamonti 2010). An approximately 500-bp long fragment of the 16S rRNA gene was amplified using the primers 16Sar (5′- CGC CTG TTT ATC AAA AAC AT -3′) and 16Sbr (5′- CCG GTC TGA ACT CAG ATC ACG T -3′) (Palumbi et al. 2002). All polymerase chain reaction (PCR) amplification steps were carried out in 40 µl reaction volumes and contained 50 ng DNA, 1 × PCR buffer, 2.5 mM $MgCl_2$, 50 µM each dNTP, 1 µM each primer, and 1 U GoTaq DNA polymerase (Promega). Thermal cycling parameters in all cases included initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 20 sec, 55°C for 20 sec, and 72°C for 20 sec, and ended with a 10-min extension at 72°C. Double-stranded PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), and sequenced by Macrogen Europe Laboratory (Amsterdam, The Netherlands) with the same primers used in PCR amplifications; both strands were sequenced to ensure accuracy. Primer trimming and initial sequence editing were done with the BioEdit software package v.7.2.5 (Hall 1999), whereas sequence alignments were constructed with ClustalX v.2.1 (Larkin et al. 2007). The sequences were deposited in the NCBI GenBank database under accession numbers KP055816-KP055817. The consensus sequences were subjected to BLAST

analysis (Altschul et al. 1990), using a MegaBLAST algorithm optimized for highly similar sequences. The BLAST matches with complete query coverage, the E-value = 0.0, and maximum identity >99% were treated as significant.

We compared our *Ruditapes* haplotypes to 12 *Ruditapes philippinarum* and 4 *Ruditapes decussatus* haplotypes characterized for European populations from different Adriatic and Atlantic sampling sites (Chiesa et al. 2014). The phylogenetic analysis was done in MEGA6 (Tamura et al. 2013), and also included *Ruditapes* GenBank records under the accession numbers: KF736199-KF736211, DQ356383, AJ548764, HQ634141, AF038999, and DQ184754. The 16S rDNA sequences from *Corbicula fluminea* (AF038999) and *Glaucomone rugosa* (DQ184754), both belonging to the order Veneroida, were used as outgroups following the recent phylogenetic studies of Venus clams (Chen et al. 2011, Chiesa et al. 2014). The maximum likelihood tree was reconstructed based on the T92 + G (Tamura-3-parameter with gamma distribution) model selected as the best-fit model with the lowest Bayesian information criterion score. Initial trees for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 0.6699)]. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. The analysis involved 20 nucleotide sequences. There were a total of 442 positions in the final dataset.

RESULTS

Morphological Comparison

All considered morphological characteristics (Fig. 2) resulted as very useful in differentiating *Ruditapes philippinarum* from *Ruditapes decussatus* sampled at the same site (Zelena Laguna) along the west Istrian Coast (northern Adriatic, Croatia) except internal shell coloration (Fig. 2D). In all the *R. philippinarum* specimens collected in this study, no purple spots were observed on the inside parts of the shell. Based on morphological observations, 17 specimens were identified as *R. philippinarum*, whereas the remaining 123 individuals were classified as *R. decussatus*. The length of *R. decussatus* ranged from 24.22 to 45.43 mm (Fig. 4). The distribution of the lengths of *R. decussatus* was normal (Lilliefors 2-tail Kolmogorov-Smirnov test for normality $P = 0.251$). Specimens *Ruditapes phillipinarum*, ranging from 26.10 to 38.71, were present in nearly all length classes.

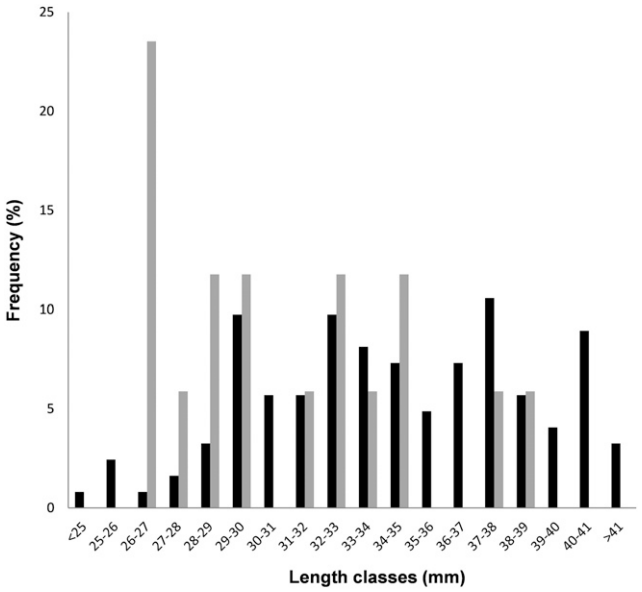

Figure 4. Shell length frequency for *Ruditapes philippinarum* (grey bars) and *Ruditapes decussatus* (black bars) specimens.

Morphometric Comparison

In morphometric comparisons, we included all the 17 *Ruditapes philippinarum* specimens, and, to balance the analyses, 17 *Ruditapes decussatus*, randomly selected among the 123 collected specimens. For both, *R. philippinarum* and *R. decussatus*, the assessed morphometric variables (shell length, height, and width) were significantly linearly interrelated (Fig. 5). Analysis of covariance comparing the slopes of regression lines between the two species (Table 1) revealed a significant difference in the case of log shell width to log shell length ($P = 0.004$) and log shell width to log shell height ($P = 0.010$) relationships. Concerning the relationship of log shell height to log shell length, the regression slopes for two species can be considered similar ($P = 0.551$). Hence, for the latter case, a linear regression was fitted using jointly data for both species (Fig. 5A). This linear regression explained a relevant portion of total variation ($r^2 = 0.708$) and the slope was highly significant ($P < 0.001$). The linear regressions for log width on log length and for log width on log height were conducted separately for each species (Fig. 5A, B). The linear regression lines explained a relevant portion of total variation: the determination coefficient (r^2) of linear regression analyses ranged from 0.714 (*R. decussatus*, for log width on log height) to 0.876 (*R. philippinarum*, for log width on log height). All slopes were significant ($P < 0.001$).

For *Ruditapes philippinarum* and *Ruditapes decussatus* pooled data, the relationship height/length indicated an isometric growth (95% confidence interval from 0.635 to 1.018, DF = 32, $t = 1.840$, $P > 0.05$). The relationship width/length was isometric for *R. philippinarum* (95% confidence interval from 0.912 to 1.410, DF = 15, $t = 1.316$, $P > 0.05$) and negative allometric for *R. decussatus* (95% confidence interval from 0.565 to 0.876, DF = 15, $t = 3.836$, $P < 0.05$). The relationship width/height was isometric for *R. philippinarum* (95% confidence interval from 0.882 to 1.343, DF = 15, $t = 1.046$, $P > 0.05$) and negative allometric for *R. decussatus* (95% confidence interval from 0.444 to 0.920, DF = 15, $t = 2.839$, $P < 0.05$).

Visual observations suggested that the depth of pallial sinus differs between the two species (Fig. 2D). The length of the pallial sinus of *Ruditapes decussatus* amounted to 49.87% ± 3.64% (mean ± SD, $n = 17$) of the shell length, whereas that of *Ruditapes phillipinarum* was 42.20% ± 2.29% (mean ± SD, $n = 17$). A two-sample *t*-test revealed that the length of the pallial sinus significantly differed between the two species ($t = 7.348$, DF = 32, $P < 0.001$).

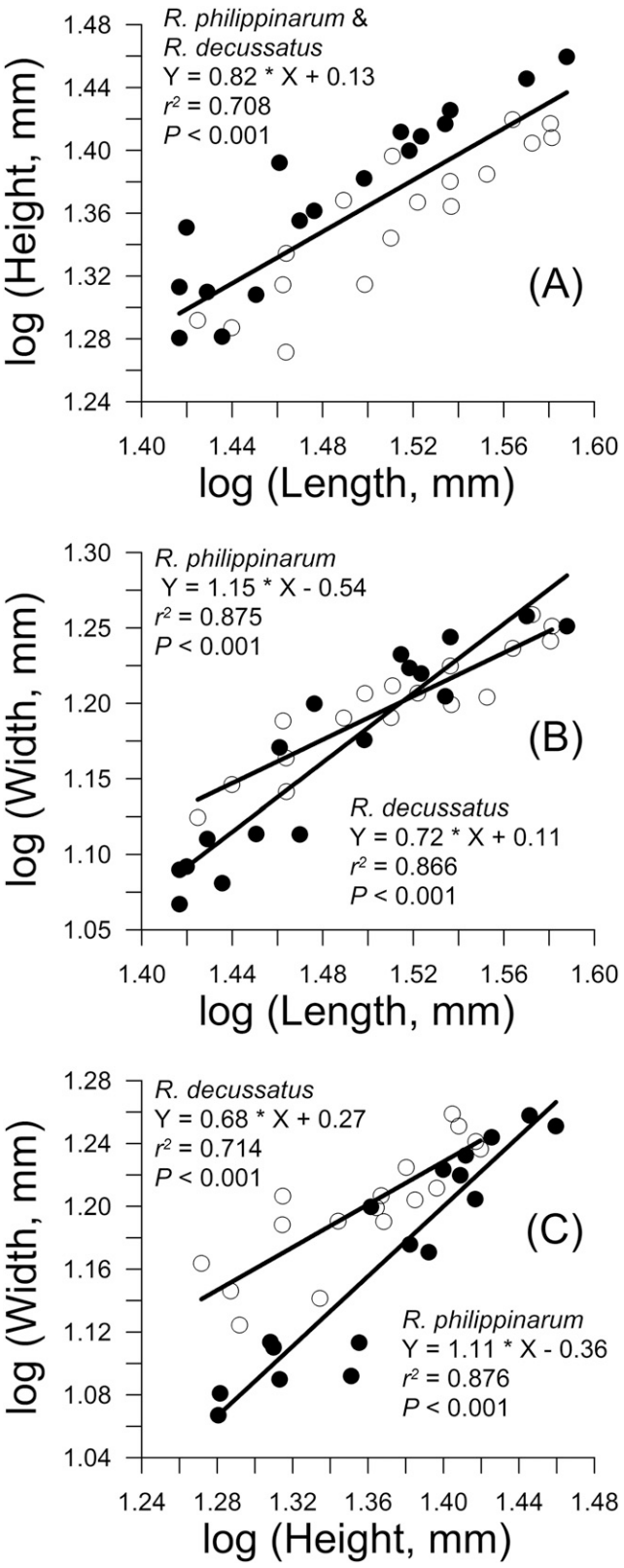

Figure 5. Linear regression of morphometric variables: (A) height length, (B) width length, and (C) width height for *Ruditapes philippinarum* (filled circles) and *Ruditapes decussatus* (open circles). Before analyses data were log transformed.

16S rDNA Analysis

To check whether the 17 Venus clams, classified as Manila clams according to morphometric characteristics, belong indeed to *Ruditapes philippinarum* species, we analyzed the mitochondrial 16S rRNA gene fragment. In addition to 17 *R. philippinarum* suspected specimens; five randomly chosen *Ruditapes decussatus* individuals were included in molecular identification as a control. The amplification of the 16S rRNA gene sequence revealed fragments of two different lengths, which totally corresponded to the morphological *philippinarum*/*decussatus* classification. Fragments 553-bp long were amplified in all 17 specimens that were morphologically identified as *R. philippinarum*, whereas 522-bp long PCR products were generated in the remaining five individuals classified as *R. decussatus*. The differences in DNA fragment lengths were initially evidenced by agarose gel electrophoresis, and additionally confirmed by an alignment of the 22 sequenced fragments (not shown). Aside from clustering the sequences into two distinct groups (named *philippinarum* and *decussatus*), the alignment also revealed the extreme intraspecific homogeneity of 100% sequence identity within each of the groups. Therefore, the consensus sequences were generated and used in the further analysis. A pairwise alignment of the *philippinarum* and *decussatus* consensus sequences (Fig. 6A) showed nucleotide divergence of 24.5%, based on 91 nucleotide substitutions (72.3%) and 34 insertion/deletion events (27.7%). F6

The *philippinarum* and *decussatus* consensus sequences were used as the queries in NCBI GenBank database search. The BLAST best matches clearly related the *philippinarum* consensus to *Ruditapes* (= *Tapes* = *Venerupis*) *philippinarum* entries, whereas the *decussatus* consensus aligned best with *Ruditapes* (= *Tapes* = *Venerupis*) *decussatus* entries. In other words, the 16S rRNA gene analysis confirmed that 17 specimens collected in Zelena Laguna belong to the species *R. philippinarum* indeed. All *R. philippinarum* specimens analyzed in this work are characterized by the 16S rDNA haplotype that shows the perfect BLAST match (100% covered query in its total length with 100% identity, E-value = 0.0) with the specimens collected in the Venice Lagoon (GenBank Accession AF484289, Passamonti et al. 2003) as well as with the specimens sampled at San Simon Bay at NW Spain (GenBank Accession HQ634142, Hurtado et al. 2011). Comparison with *R. philippinarum* and *R. decussatus* haplotypes characterized for European populations also revealed that *R. philippinarum* specimens from Zelena Laguna belong to the Rphap1 (Fig. 6B), the most frequent *R. philippinarum* haplotype in Italian, Spanish, and Portuguese populations (according to Chiesa et al. 2014).

DISCUSSION

The alien and invasive *Ruditapes philippinarum* was recorded for the first time in eastern Adriatic Croatian waters in February 2013 in the intertidal sandy substrate of a station located near the city of Poreč (west Istrian Coast). The sampled *R. philippinarum* was sympatric with the autochthonous *Ruditapes decussatus*. Of the total collected *Ruditapes* spp., *R. philippinarum* amounted to the 12%. The two species were differentiated according to morphological differences of the shell (radial sculpture and radial line, outline of the posterior region, external side of the

TABLE 1.

Analysis of covariance and homogeneity of slopes for length, height, and width interrelationships between *Ruditapes philippinarum* and *Ruditapes decussatus*.

		Height[a]/Length[b]			Width[a]/Length[b]			Width[a]/Height[b]		
Source	**DF**	**MS**	***F***	***P***	**MS**	***F***	***P***	**MS**	***F***	***P***
C	1	0.379	150.406	<0.001	0.400	185.727	<0.001	0.362	130.467	<0.001
S	1	0.001	0.186	0.669	0.021	–	–	0.024	–	–
C × S	1	0.001	1.363	0.551	0.021	9.757	0.004	0.021	7.513	0.010
Residual	30	0.003	–	–	0.002	–	–	0.003	–	–

C, covariable; S, species: fixed factor with two levels, *Ruditapes philippinarum* and *Ruditapes decussatus*.
Analysis of covariance assumptions of linear trend (Fig. 5), homogeneity of variances (Levene's test $P > 0.05$), and normality of residuals (Shapiro–Wilk $P > 0.05$) tests were satisfied. Before analyses, data were log transformed.
[a] Dependent variables used in each analysis.
[b] Variables used as covariable.

hinge area) and morphology of the siphons. Morphometric differences of the shell (width to length ratio and width to height ratio) and the length of the pallial sinus significantly differed between two species. The two species were also genetically differentiated according to mitochondrial 16S rRNA.

The morphological characteristics (Fig. 2) could be subdivided in two groups: external and internal. Experts for a rapid differentiation between the two species can use external characteristics (radial sculpture and radial line morphology, general form, and the color of the lunulae). External shell coloring cannot be used as a criterion of differentiation between *Ruditapes philippinarum* and *Ruditapes decussatus* (Quéro & Vayne 1998). For the samples collected in this study, the external coloring of both species was quite variable with yellow or light brown to white shells and a visible pattern of darker zigzag lines that ranged from pale to dark brown, or were decorated with ray lines.

Internal characteristics, such as the morphology of siphons and the shape of the pallial sinus, provide sound results. On the other hand, these methodologies are time consuming and, therefore, impractical for observational monitoring surveys. Even though the internal shell coloration is widely recognized as a valid criterion for differentiation (Quéro & Vayne 1998, Gofás et al. 2011), in all the *Ruditapes philippinarum* specimens collected in this study, no purple spots were observed on the inside parts of the shell. Thus, we suggest that, like external coloration, the inside coloration patterns should also not be considered to be a reliable criterion of differentiation between the two species.

Width/length and width/height relationship ratios significantly differed in slope between the two species sampled at the same site (Table 1). In the scatterplots (Fig. 5), some points for the two species overlapped suggesting that differences in slopes cannot be used as a valid character for species differentiation. Further analyses revealed that width/length and width/height relationships were isometric in *Ruditapes philippinarum* and negative allometric in *Ruditapes decussatus*. This finding provides additional information on differences in morphometric relationships between the two sympatric *Ruditapes* populations.

Because of its high reproduction and growth rates, *Ruditapes philippinarum* has been able to colonize different areas around the world outside its original distribution range (Quayle 1964, Bourne 1982, Chew 1989, Flassch & Leborgne 1992, Sbrenna & Campioni 1994, Breber 2002). Concerning *Ruditapes philippinarum*, it was intentionally imported into the Venice Lagoon (northwestern Adriatic, Italy) in 1983 as an aquaculture species (Pranovi et al. 2006). In general, rapid globalization and increasing trends of trade, travel, and transport over the course of recent decades have accelerated marine biological invasions by increasing the rates of new introductions through various pathways, such as shipping, navigational canals, aquarium trade, and aquaculture (Hulme 2009, Katsanevakis et al. 2013).

The conspicuous commercial production (50,000 tons/y) of *Ruditapes decussatus* in Italy is mostly concentrated in the lagoons of the northern Adriatic, which contributes up to 95% of the overall Italian production (Orel et al. 2000, Zentilin et al. 2008). After the initial introduction, *Ruditapes philippinarum* successfully colonized the entire Venice Lagoon by settling in the same habitats that harbor. The invasive *R. philippinarum* as well expanding into the surrounding lagoons of the Italian Coast (Cesari & Pellizzato 1985, Zentilin 1990, Breber 2002, Chiesa et al. 2014). Afterward, populations of *R. philippinarum* extended clockwise along the northern Adriatic Coast. It was subsequently recorded in the Gulf of Trieste (Grado Lagoon, Fig. 1) and in the Bay of Piran in Slovenia (Lipej et al. 2012). The natural spread of *R. philippinarum* is usually due to larval movement, which is driven by wind and tidal currents (Ponurovski 2008).

Because of the scarcity of suitable habitats, the Croatian annual production of *Ruditapes decussatus* is relatively low, amounting to only several tons (Vrgoč et al. 2009). As the demand for this clam is particularly high during the tourist season, it is possible that fishermen will intentionally introduce *Ruditapes philippinarum* with the aim to augment productivity of the isolated sandy-muddy sites along the Croatian Coast.

The introduction of foreign species can have a significant impact on biodiversity, ecosystem function, and socioeconomics (Carlton & Geller 1993, Grosholz 2002, Stachowicz & Byrnes 2006). Among invertebrates, bivalves are one of the most invasive groups and many of them become successful invaders that establish thriving populations, being deleterious to the native species (Sousa et al. 2009). Accordingly, the planned import of *Ruditapes philippinarum* into the Venice Lagoon, coupled with its intensive harvesting, negatively

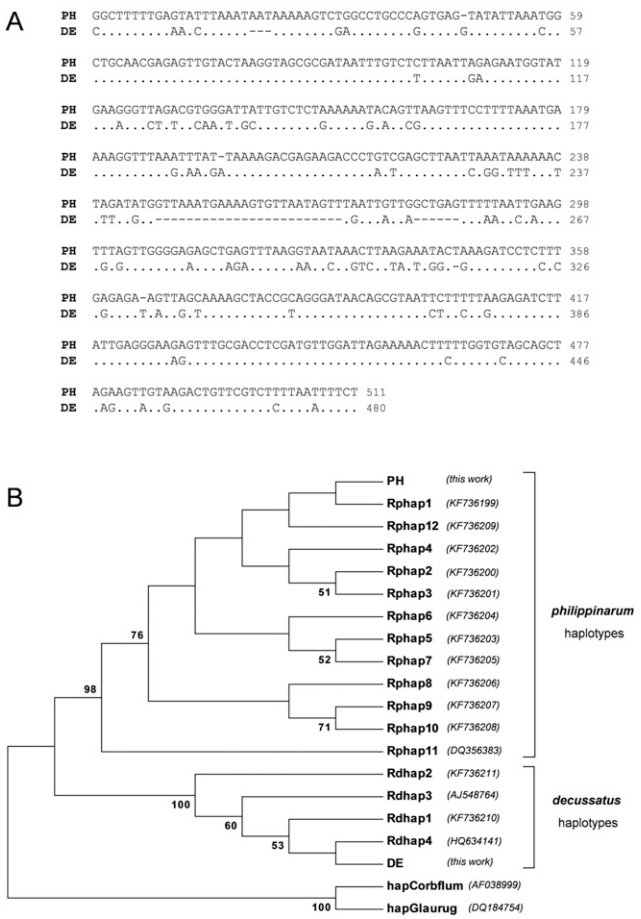

Figure 6. (A) Pairwise alignment of the 16S rRNA gene fragment consensus sequences from *Ruditapes philippinarum* (PH) and *Ruditapes decussatus* (DE). Identical positions are indicated by dots, and deletions are marked by dashed lines. (B) Phylogenetic tree of 16S rDNA *Ruditapes* haplotypes. Maximum likelihood tree was generated using the Timura-3-parameter model with a gamma distribution, and numbers above the branches represent bootstrap support values based on 1,000 replicates (only the support values above 50 are shown). The accession numbers of the sequences retrieved from the GenBank database are indicated in the parenthesis.

affected not only the native clam, *Ruditapes decussatus,* but also the whole lagoon ecosystem (Pranovi et al. 2006). There are various kinds of interactions between invaders and native species, including competition for resources, predation, release of toxins, disease transmission, ecosystem engineering, and hybridization between alien and native species (Hurtado et al. 2011). In particular, *R. philippinarum* is prone to hybridize with native congeneric species (Kitada et al. 2013). Hybrids between the allochthonous *R. philippinarum* and the autochthonous *R. decussatus* have been found in the north west and north of Spain (Hurtado et al. 2011, Habtemariam et al. 2015). As hybridization might lead to the local extinction of *R. decussatus* in areas where *R. philippinarum* is intentionally introduced to enhance commercial harvesting, Habtemariam et al. (2015) suggested prohibiting the release of the alien *R. philippinarum* into natural waters as well as selecting non-hybridized *R. decussatus* for repopulation of overexploited areas. Hybridization between the two species also imposes identification problems. For example, the introgressed genetic marker was found in individual specimens of apparent morphologically pure phenotypes, whereas individual specimens with intermediate morphology were genetically determined to be a pure species (Habtemariam et al. 2015).

Genetic analyses confirmed that the aforementioned morphological and morphometric characteristics can be soundly applied to differentiate between *Ruditapes philippinarum* and *Ruditapes decussatus*. Molecular data, hailing from both mitochondrial and nuclear DNA, have been frequently and successfully used in identification as well as in phylogenetic and population studies of Veneridae species, emphasizing the mitochondrial 16S rRNA gene as the most extensively used molecular marker (Canapa et al. 1996, Fernandez et al. 2002, Kappner & Bieler 2006, Hurtado et al. 2011, Chiesa et al. 2011, 2014). To positively prove the presence of *R. philippinarum* in the Croatian part of the Adriatic Sea, we tested all putatively determined specimens for the 16S rRNA gene sequence. Based on the 16S rRNA sequence data, all Croatian *R. philippinarum* specimens share the same haplotype with no genetic variability, implying a recent entrance of the species, most probably from a unique recruitment stock. Low genetic variability has also been noticed in other aquatic invaders at the first stage of their invasions, as it was recorded for the invasive mussel *Mytilus galloprovincialis* (Lamarck, 1819) (Zardi et al. 2007) and the European green crab *Carcinus maenas* (Linnaeus, 1758) (Darling et al. 2008). Although it is impossible to verify the exact origin of the Croatian *R. philippinarum* population, there is a strong possibility that it may have originated from founding populations in the Italian part of the northern Adriatic Sea, as identical 16S rDNA haplotypes have been evidenced in *R. philippinarum* specimens from the Venice Lagoon as well as the Marano Lagoon (Passamonti et al. 2003, Chiesa et al. 2014). It has to be stressed, however, that this haplotype (Rphap1, according to Chiesa et al. 2014) is the most dominant *R. philippinarum* haplotype among south European populations, and it is not strictly limited to the Mediterranean Sea (Hurtado et al. 2011, Chiesa et al. 2014).

As *Ruditapes philippinarum* represents a non-native species in the Adriatic Sea, with a strong capacity to impact indigenous populations of *Ruditapes decussatus*, it will be of great importance to follow its potential spread along the Croatian coastline. Despite the described morphological and morphometric differences between *R. philippinarum* and *R. decussatus*, which are very useful in monitoring surveys, in some instances, it still can be difficult or even impossible to correctly differentiate the two species (Hurtado et al. 2011). Similarity and plasticity of shell morphology between *R. philippinarum* and *R. decussatus* can complicate the identification process if it is only based on simple visual inspection (Geri et al. 1996, Hurtado et al. 2011).

Morphological, morphometric, and genetic identifications should be combined in planning monitoring strategies and in studies investigating the ecological consequences of the *Ruditapes philippinarum* invasion.

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