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Research Article

Anadara kagoshimensis (Mollusca: Bivalvia: Arcidae) in the Adriatic Sea: morphological analysis, molecular taxonomy, spatial distribution, and prediction

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Abstract

Morphological analysis, molecular characterization, and a study of the distribution and density of *Anadara kagoshimensis* (Tokunaga, 1906) specimens collected in the Adriatic Sea were carried out using materials and data collected in the course of 329 bottom trawl hauls conducted in five yearly surveys, from 2010 to 2014. Morphological and molecular analysis allowed clarifying the confused taxonomy of the largest alien ark clam species invading Italian waters and the Mediterranean Sea. Analysis of the distribution and density data demonstrated that, along the Italian coast, *A. kagoshimensis* is mostly found at depths of 8 to 50 m, with a catch frequency of more than 98% in the hauls involving silty-clay sediment at a depth of 8-30 m. The hotspot map clearly shows a reduction in the distribution area of the species from 2010 to 2012.

Keywords: Alien species, Invasive species, DNA barcoding, Ark clam, GIS.

Introduction

The bivalve *Anadara kagoshimensis* (Tokunaga, 1906) (family Arcidae Lamarck, 1809) is endemic in several areas from the central Indian Ocean to the western Pacific (India, Sri Lanka, Indonesia, Korea, China, Japan, northern Australia). Recently, it has also been recorded in the Mediterranean Sea (Catalan, Ligurian, Tyrrhenian, Adriatic, Aegean), the Marmara Sea, and along the coast of the Black Sea and the Azov Sea (Bulgaria, Romania, Ukraine, Russia, and Georgia) (Bañón *et al*., 2015). *A. kagoshimensis* is an important food resource in East Asia, where it inhabits muddy sediments in shallow coastal waters. In 1945 it was introduced in several Asian coastal areas as an additional source of food (Tanaka & Aranishi, 2014). It has also, unintentionally, been introduced in the Mediterranean Sea from the Indo-Pacific area, probably through shipping and maritime transport (Crocetta, 2012).

A. kagoshimensis is among the 100 most invasive species living in the Mediterranean (Streftaris & Zenetos, 2006 as *A. inaequivalvis*). Its ability to bind oxygen in hypoxic conditions and to attach itself to various types of hard substrata by its byssus enables it to grow also in the Adriatic Sea, where it has been recorded since 1960 (Ghisotti & Rinaldi, 1976; Morello *et al*., 2004; Crocetta, 2012) and where it now forms large banks along the northeastern Italian coast, from inshore brackish waters to a depth of 30 m, mostly on silty-clay sediment but also on sandy and rocky bottoms (Rinaldi, 1985; Zenetos *et al*.,

2004; Crocetta, 2011). More recently, *A. kagoshimensis* has been recorded in the deepest bottoms of the northern Adriatic basin (Despalatović *et al*., 2013).

The species has a troubled taxonomic history in the areas it has invaded: in Italian waters it was initially identified by Ghisotti (1973) as *Scapharca* (cf.) *cornea* (Reeve, 1844), but a few years later Ghisotti and other researchers re-identified it as *Anadara inaequivalvis* (Bruguière, 1789) (ex *Scapharca inaequivalvis*) (Ghisotti & Rinaldi, 1976; Rinaldi, 1977; Lazzari & Rinaldi, 1994). Comparison of *A. inaequivalvis* specimens from India and from the Adriatic Sea led Lutaenko (2006) to describe several morphological differences, particularly in rib number and shell shape (33-35 ribs and a slightly larger shell in the specimens from southern India vs. 30-32 ribs in those from the Adriatic and the Black Sea). Finally, Huber (2010) suggested that the Mediterranean alien species matched *A. kagoshimensis* from Japan. Subsequently, some scientists have begun to identify these ark clams as *A. kagoshimensis* (Zenetos *et al*., 2010; Crocetta, 2011, 2012; Lipej *et al*., 2012; Crocetta *et al*., 2013; Despalatović *et al*., 2013; Grati *et al*., 2013), whereas others still consider the species as *A. inaequivalvis* or *S. inaequivalvis* (Andreani *et al*., 2011; Foschi *et al*., 2011; Occhipinti-Ambrogi *et al*., 2011; Mistri & Munari, 2013; Huntley & Scarponi, 2015), although it is unclear whether they have failed to accept or have merely ignored the taxonomic change.

The present study provides the correct identification of this alien ark clam species found in the Adriatic

Sea using a molecular approach to resolve the problems posed by the slight morphological differences between *A. kagoshimensis* and *A. inaequivalvis* and by the confused taxonomic information on Italian and Mediterranean specimens.

The question is here ultimately resolved through DNA barcoding, a molecular tool that, coupled with a robust taxonomic validation, has already been applied to distinguish between different bivalve species (Mikkelsen *et al.*, 2007; Chen *et al.*, 2011). Moreover, even though this ark clam has been recorded in the Adriatic Sea by several authors, the present study provides for the first time data on its density and a description of its distribution in the Adriatic basin.

Materials and Methods

Sampling

The study was carried out in GFCM-FAO Geographical Sub-Area 17 (GSA 17: northern and central Adriatic). It involved an area of about 36,700 km² spanning from the Italian coast to the 12 nm limit of Croatian territorial waters. Depth ranged from 8 m up to 100 m (for additional details see Scarcella *et al.*, 2014). Megazoobenthos samples were collected using the rapido trawl, a modified beam trawl commonly used by Italian fishermen to catch flat fish and other benthic species. Five yearly surveys were conducted in the autumn, from 2010 to 2014, in the framework of the Solemon project. A total number of 67 stations were sampled in 2010, 2011, and 2014, 65 in 2013, and 63 in 2012 (Fig. 1). Density was calculated in the 5-year dataset (2010-2014), whereas a 3-year dataset (2010-2012) was used for spatial modelling. Twelve specimens of the genus *Anadara* collected along the Italian coast in autumn 2014 were subjected to molecular and morphological analysis.

Morphological identification

The *Anadara* specimens were identified according to Okutani (2000), Huber (2010), and Bañón *et al.* (2015) and compared to 6 specimens of *A. inaequivalvis* from the Philippines. The morphological description was based on taxonomic characters and morphometric parameters: length, width (top umbo to ventral margin), and number of ribs in the left valve were measured to the nearest 0.1 mm using a calliper. Specimens were then placed in the macrozoobenthos collection of CNR-ISMAR (Ancona, Italy).

DNA amplification and sequencing

Foot muscle tissue cut from each individual with sterile tweezers and clippers was placed into a clean tube with 96% ethanol for subsequent DNA analysis. Total genomic DNA (gDNA) was extracted from 20 mg of each foot muscle sample according to the Invisorb® Spin Tissue Mini Kit (Stratec® Molecular GmbH, Berlin, Germany) protocol. A fragment of the mitochondrial *cox-I* gene (about 650 bp) was amplified using the universal primer set LCO 1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO 2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer *et al.* 1994). The PCR reactions were performed in a total volume of 50 μL containing 2 μL gDNA, corresponding to ~ 20 ng, 10 μL PCR Buffer (1 X), 3 μL MgCl$_2$ (1.5 mM), 1.5 μL of each primer (0.3 μM), 1.5 μL of dNTP mix (0.075 mM each), and 1.25 U Recombinant Taq DNA Polymerase (Promega, Bologna, Italy). DNA amplifications were run on a Biometra T-Gradient Thermocycler (Biometra GmbH, Göttingen, Germany) as follows: after an initial denaturation at 94°C for 5 min, amplification was performed with 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Amplicons were checked on 1.5% agarose gel; successfully amplified fragments were treated with PureLink® PCR Purification Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20ºC until they were shipped to Macrogen Europe (Amsterdam, The Netherlands), a commercial sequencing service provider. Sequencing was performed on an ABI3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) with the same primers used for amplification.

Sequence analysis

The forward and reverse strand electropherograms were manually edited using MEGA v.5.2.2 software (Tamura *et al.*, 2013), aligned with CLUSTAL W software (Thompson *et al.*, 1994), and incorporated into MEGA v.5.2.2 software (Tamura *et al.*, 2013). The accuracy of amino acid translation was checked to exclude the presence of stop codons and sequencing errors (Moulton *et al.*, 2010). Each consensus *cox-I* sequence was first compared to published sequences from the Barcode of Life Data System (BOLD; http://www.boldsystems.org) and the NCBI online database (http://www.ncbi.nlm.nih.gov/genbank/) using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to rule out any error due to mishandling of samples on board or in the laboratory. Then, available sequences for Arcidae taxa were retrieved from the two databases; where possible, records from different geographical areas were selected: Australia, Japan, China, USA, Panama, Black Sea, and Spain. The ark clam nominal species are reported as they are originally cited in the databases, although all *Scapharca kagoshimensis* could probably be renamed *Anadara kagoshimensis*. The sequences thus retrieved were merged with those of the Adriatic *Anadara* specimens. *Ostrea edulis* (Linnaeus, 1758) was used as the outgroup for the whole analysis. The most appropriate evolutionary substitution model was assessed using MEGA v.5.2.2 software based on the corrected Akaike Information Criterion (AICc, Akaike, 1977). Where possible, phylogenetic reconstruction was carried out using the Hasegawa-Kishi-
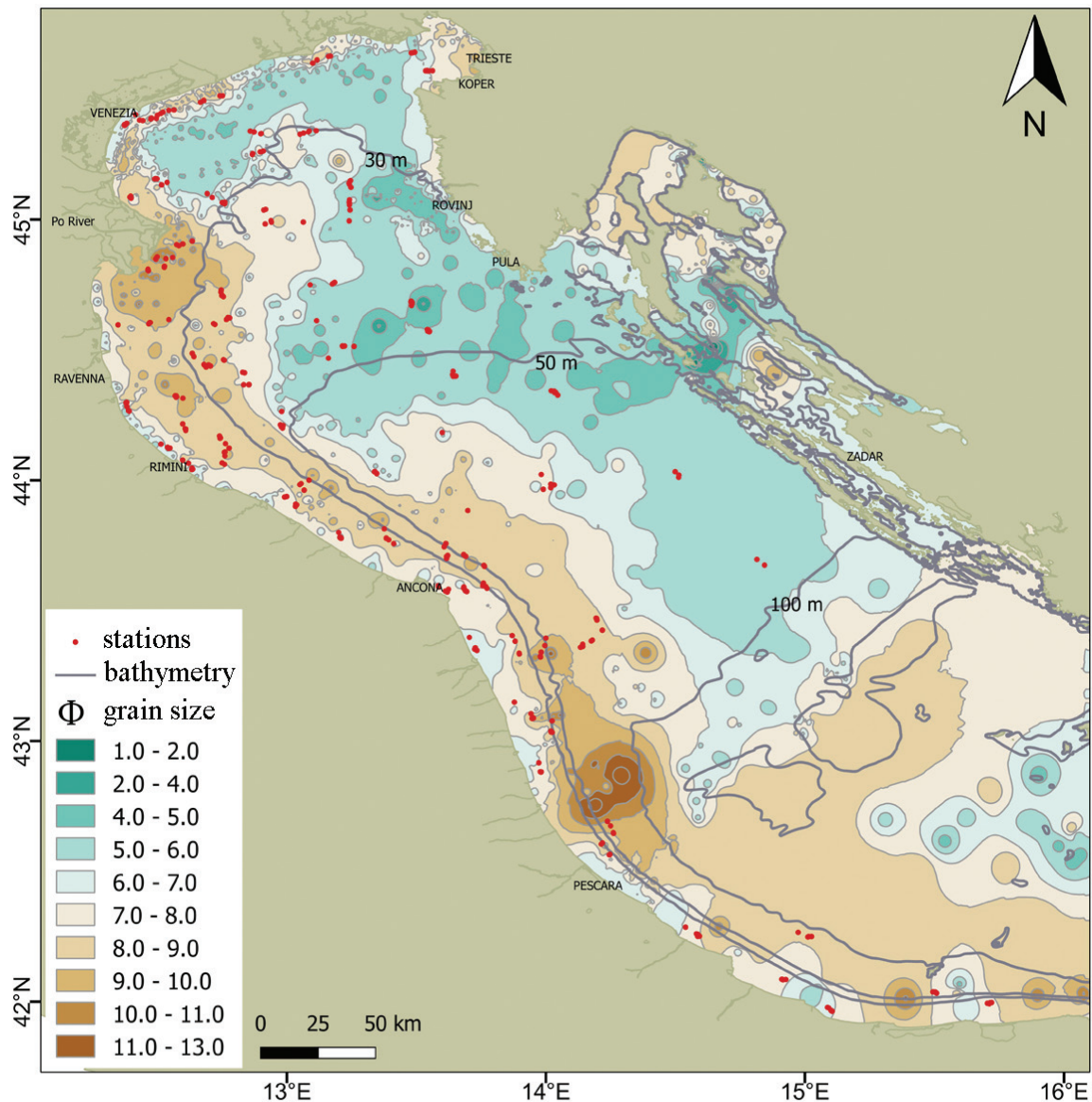
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Fig. 1: Geographical view of the Adriatic Sea with details of the stations (red dots), bathymetry, and grain size (Φ).

no-Yano (HKY+G+I) model (Hasegawa *et al.*, 1985). Intra- and inter-specific genetic p-distances were calculated on the complete dataset using MEGA. The comparison of *cox-I* sequences based on p-distance metric with pairwise deletion was summarized using the Neighbour-Joining (NJ) Tree method (Saitou & Nei, 1987). Statistical support for NJ was calculated using the bootstrap method (Felsenstein, 1985) with 1,000 replicates. Single-node Bootstrap (BS) values ≥ 50% were considered as the lower threshold for statistical support; BS values > 50% were not reported in the NJ reconstruction. Clade relationships were further estimated using the Maximum Likelihood (ML) method based on the HKY+G+I model of nucleotide substitution using a BS with 100 replicates. The model was also used for Bayesian Inference (BI) analysis, which was unraveled with Beast v.1.8.0 (Drummond *et al.*, 2012). A Markov Chain Monte Carlo (MCMC) run of 80,000,000 generations sampled every 1,000 generations with the first 25%

of the sampled points removed as burn-in was performed to ensure convergence of the posterior distributions. Run convergence was checked by inspecting the Estimated Sample Size (ESS) values in TRACER v1.6 (Rambaut & Drummond 2007). The resulting 80,000 trees were processed and summarized using TREEANNOTATOR v1.7.5 (Drummond *et al.* 2012); supported nodes were tested using the 95% Posterior Probability (PP) criterion. The results of NJ and ML (newick files from MEGA) and BI (tree file from TREEANNOTATOR) were visualized and edited with FIGTREE v.1.4.2 (Rambaut, 2009).

Density and spatial modelling

Data on species abundance and haul duration and location were stored in the ATrIS database (Gramolini *et al.* 2005), which allowed standardizing the number of specimens to swept area (N/km^2). Due to the limited availability of grain size data, which are critical for this analysis, the

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3-year dataset (2010-2012) was used for the prediction of *A. kagoshimensis* spatial distribution in the Adriatic Sea. Explanatory analyses were first conducted using GLM/GLMM and GAM models (not shown). Anyhow, the percentage of total variance explained for the species was not satisfactory, likely due to the large amount of zero catches in the data matrix. Therefore, a Zero-Inflated Generalized Additive Model (ZIGAM; Liu & Chan, 2010; Yu *et al.*, 2012) was applied to define the spatial distribution in the previous analyses. The model assumes that the response variable follows a probabilistic mixture distribution of a zero atom and a continuous distribution belonging to the exponential family (Lambert, 1992; Hall, 2000; Yu *et al.*, 2012), thus providing a better performance when zero-inflated data are involved. Data modelling involved two stages. First, the probability (p) of observing a density > 0 was modelled with a GAM, where the dependent variable had a binomial distribution and the link function was the logit (logarithm of the probability to observe a positive catch on the probability to observe a zero):

$$logit\big(p(v,\,t)\big) = \beta_0 + \sum_{j=1}^{p} s\,\big(x_j(v,\,t)\big)$$

Then, a GAM model was estimated only on the positive catches using a Gaussian family model and the identity link function. The dependent variable, distributed as a normal random variable, was $\log\big(y(v,\,t)\big)$. Log-transformed data (N/km²) were assumed to be independent; accordingly, the outcomes of both models were crossed to obtain density predictions using the Year as a factor and Latitude, Longitude, and Grain size (Φ) as covariates. Spatial autocorrelation in the residuals was checked as in the GLM and GAM exploratory analyses. Annual abundance hotspots were identified using local methods in Getis-Ord Gi* statistics (Getis & Ord, 1992), with a radius of 5.0 km and a significance level of 0.95. This approach allowed testing whether the high density clusters of *A. kagoshimensis* were significantly different. Results were plotted in the hotspot map reporting the 0.81-1 class.

All analyses were performed using R software libraries, which also produced shape files. The results were visualized on spatial georeferenced maps using GIS software (QGIS 2.12.3). A georeferenced map was produced to show grain size characteristics (from Jenkins, 2008) of the whole study area.

Results

Morphological identification

All specimens belonged to *A. kagoshimensis* (Tokunaga, 1906). Their morphological characters compared to those of the 6 specimens of *A. inaequivalvis* (Bruguière, 1789) from the Philippines are shown in Table 1 and Figure 2. The two congeneric species show no marked differences in shell shape, ligament, or inequivalvity; their shells are thick and solid, although the shell of *A. kagoshimensis* is larger and the periostracum provides a continuous cover only along the margin, whereas the periostracum of *A. inaequivalvis* covers a wider area of the shell and is thicker along the margin. The ribs are on average more numerous in *A. inaequivalvis*, whose external colour is brighter. The Indo-Pacific and the Italian *A. kagoshimensis* show no differences in shell morphology (Fig. 2).

Genetic analysis

Total gDNA was successfully extracted from all 12 individuals, although *cox-1* amplification was unsuccessful in one specimens. Reliable, high-quality sequences were obtained from 10 specimens (Genbank accession numbers: MF426975, MF426976, MF426977, MF426978, MF426979, MF426980, MF426981, MF426982, MF426983, MF426984). The incomplete sequencing success may be related to the presence of mucopolysaccharides, which in bivalves may inhibit amplification and sequencing (Layton *et al.*, 2014; 2016). Amino acid translation showed no stop codons, and comparison of the sequences obtained in the study to those found in public repositories confirmed their assignment to the Arcidae family.

A total number of 39 sequences were retrieved from the BOLD systems and NCBI databases (12 for *Anadara*

Table 1: *A. kagoshimensis* (Adriatic Sea) versus *A. inaequivalvis* (Philippine) morphological characteristics comparison.

| Morphological features	*Anadara kagoshimensis*	*Anadara inaequivalvis*
Shell	thick – solid	thick - solid
Shape	inequilateral - subquadrate - slightly larger - strongly inflated	inequilateral - subquadrate or oval - strongly inflated
Valves	slightly inequivalve - left larger than right	slightly inequivalve - left larger than right
Umbo position	slightly shifted towards anterior half of valve	slightly shifted towards anterior half of valve
Surface	ribbed	ribbed
Periostracum	thin - continuous cover only along margins	thick - continuous cover over 1/3 of outer surface - thicker along margins
Ribbing	radial	radial
Ribs	32-34 - rounded section - nodules on right valve	33-37 - rounded section - nodules on right valve
Teeth	taxodont	taxodont
External colour (from umbo to margins)	light brown - yellow - orange - light pink – white	(clearer) - light pink - cream - green highlights

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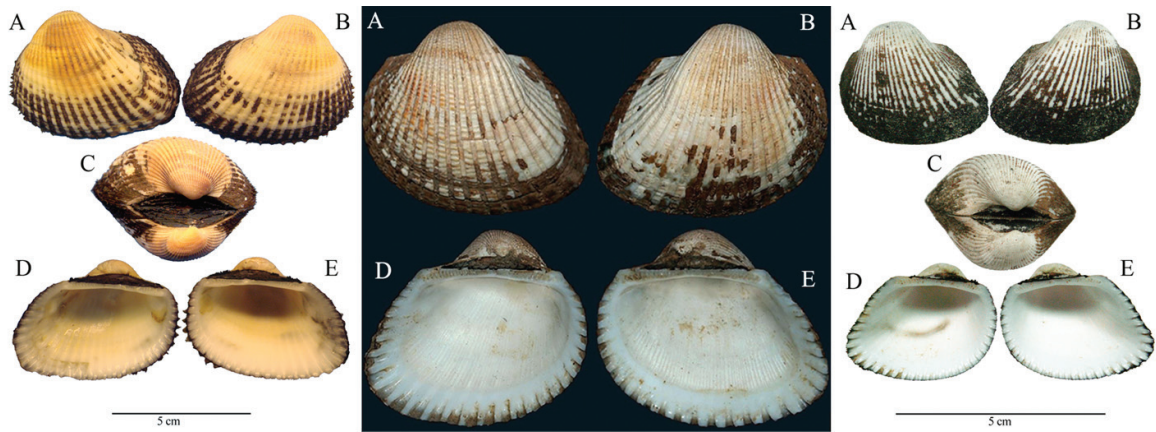
Fig. 2: *Anadara kagoshimensis* from the Adriatic Sea (left), *A. kagoshimensis* from the Indo-Pacific region (from Lutaenko, 2015) (centre), and *Anadara inaequivalvis* from the Philippines (right): (A, B) external view of left and right valve; (C) umbo; (D, E) internal view of left and right valve.

[*Scapharca*] *kagoshimensis*, 5 for *A. brasiliana*, 4 for *A. sativa*, 4 for *A. antiquata*, 2 for *S. cornea*, 2 for *S. inaequivalvis*, 2 for *S. satowi*, 1 each for *S. globosa*, *A. ovalis*, *S. gubernaculum*, *A. grandis*, *A. pilula*, *A. trapezia*, and *A. vellicata*, and 1 for *O. edulis*, the outgroup) and were added to the 10 newly obtained sequences (Table 2). The final dataset for downstream analyses consisted of 49 sequences (of 548 bp), where each individual was identified with the corresponding GenBank or BOLD Process ID and geographical origin.

The *cox-I* sequences of the 15 species considered in the final alignment showed the following average nucleotide composition: T=39.9%, C=15.6%, A=22 %, and G=22.5%. Based on the Tamura-Nei model (Tamura & Nei, 1993), the genetic p-distance within species ranged from 0.16% in *A. kagoshimensis* to 12.50% in the Japanese *S. satowi* (Table 2). The genetic distance between species was very low in closely related taxa such as *A. kagoshimensis* and *S. sativa* (1.43%) and *S. satowi* and *S. inaequivalvis* (15.63%) and was highest between *S. inaequivalvis* and *A. brasiliana* (42.16%).

The three reconstruction methods provided highly similar topologies; for this reason, only the NJ reconstruction is shown here (Fig. 3). The 10 newly obtained sequences of the *Anadara* specimens collected in the Adriatic Sea showed a BS=98% (NJ), a BS=53% (ML), and a PP=1 (BI). They clustered with *A. kagoshimensis* and *S. kagoshimensis* from the public databases regardless of the geographical origin of the records (i.e. Japan, Spain and Black Sea), and disclosed a degree of haplotype diversity within the Japanese *S. kagoshimensis* (Fig. 3). *S. inaequivalvis* and *S. cornea*, the two species that often contribute to morphological misidentification, formed two species-specific clusters with high statistical support (BS=100% for both NJ and ML and PP=1 for BI; Fig. 3) and a high genetic distance from *A. kagoshimensis* (16.6% and 21.1%, respectively). All the other species, which were represented by two or more specimens, were grouped in supported clusters except for *S. satowi*, which

revealed two different lineages. As concerns BI analysis, all effective sample size (ESS) values exceeded 200, indicating a convergence of the MCMC algorithms. The *A. kagoshimensis* and *S. kagoshimensis* cluster, which contained the 10 sequences of the Adriatic specimens, was clearly separated from all the other species.

Density and distribution

A. kagoshimensis was caught in 50.7% of hauls in 2010, in 40.3% of hauls in 2011, in 34.9% of hauls in 2012, in 40.0% of hauls in 2013, and in 31.3% of hauls in 2014. Mean abundance (12476.17 ± 9768.43 N km^{-2}) was highest on silty-clay sediment at depths ranging from 8 to 30 m, whereas values were markedly lower (158.16 ± 51.52 N km^{-2} from 30 to 50 m and 11.95 ± 17.58 N km^{-2} from 50 to 100 m) in deeper waters and on fine sand (Fig. 1). Abundance was always highest at a depth of 0-30 m, where it ranged from 97.10% of all hauls in 2012 (3101.98 ± 10772.32 N km^{-2}) to 99.61% of all hauls in 2010 and 2013 (26456.48 ± 94310.84 N km^{-2} and 18670.32 ± 100037.51 N km^{-2}, respectively) (Table 3). The main distribution areas of *A. kagoshimensis* were the western coastal region of GSA 17 (an open water area off the Po river delta), the north-eastern side of the basin in front of the Slovenian coast, and the north Croatian coast (Fig. 4). A single hotspot was detected every year: its maximum depth was 30-50 m and its extension declined from 2010 to 2012, shifting towards the shallower coastal area (Fig. 5).

Discussions and Conclusions

Morphological and molecular analysis confirmed that *A. kagoshimensis* (Tokunaga, 1906) is the correct binomial name for the ark clam species from the Indo-Pacific that has been invading the Atlantic, the Mediterranean, and the Black Sea since the 1960s (Molnar *et al.*, 2008; Occhipinti-Ambrogi *et al.*, 2011; Crocetta, 2012). The *A. kagoshimensis* versus *A. inaequivalvis* taxonomic issue has been already discussed (Lutaenko, 2006; Huber, 2010) and has

Table 2: Mean intra- and interspecific genetic p-distance of the ark clam species examined in the study. Associated Standard Error (SE) reported above the diagonal for all comparisons. Accepted names: [1] *Anadara globosa*; [2] *A. satowi*; [3] *A. cornea*; [4] *A. inaequivalvis*; [5] *A. gubernaculum*; [6] *Lunarca ovalis*.

Ark clam species	**N**	**Intra-species**	**SE**	**Inter-species**													
Anadara (Scapharca) kagoshimensis (Tokunaga, 1906)	22	0.0016	0.0007		0.0158	0.0252	0.0333	0.0320	0.0319	0.0270	0.0218	0.0296	0.0341	0.0303	0.0412	0.0355	0.0053
Scapharca satowi[2] Dunker, 1882	2	0.1250	0.0184	0.1293		0.0248	0.0334	0.0314	0.0292	0.0218	0.0192	0.0274	0.0326	0.0281	0.0422	0.0359	0.0158
Scapharca globosus[1] Reeve, 1844	1	n/c	n/c	0.2245	0.2331		0.0311	0.0282	0.0320	0.0282	0.0273	0.0297	0.0325	0.0255	0.0386	0.0328	0.0254
Anadara grandis (Broderip & G. B. Sowerby I, 1829)	1	n/c	n/c	0.3235	0.3439	0.2882		0.0349	0.0340	0.0386	0.0370	0.0393	0.0340	0.0350	0.0323	0.0330	0.0337
Anadara trapezia (Deshayes, 1839)	1	n/c	n/c	0.3203	0.3337	0.2666	0.3450		0.0323	0.0370	0.0310	0.0348	0.0390	0.0351	0.0398	0.0343	0.0336
Anadara pilula (Reeve, 1843)	1	n/c	n/c	0.2769	0.2616	0.2592	0.3043	0.2890		0.0342	0.0308	0.0316	0.0359	0.0301	0.0548	0.0379	0.0317
Scapharca cornea[3] (Reeve, 1844)	2	0.0026	0.0027	0.2107	0.1807	0.2119	0.3343	0.3399	0.2943		0.0247	0.0315	0.0358	0.0346	0.0479	0.0416	0.0269
Scapharca inaequivalvis[4] (Bruguière, 1789)	2	0.0026	0.0026	0.1667	0.1563	0.2252	0.3438	0.2809	0.2519	0.1828		0.0301	0.0362	0.0317	0.0483	0.0357	0.0213
Scapharca gubernaculum[5] (Reeve, 1844)	1	n/c	n/c	0.2631	0.2583	0.2427	0.3600	0.2968	0.2879	0.2713	0.2603		0.0319	0.0322	0.0484	0.0353	0.0308
Anadara antiquata (Linnaeus, 1758)	4	0.0036	0.0024	0.2929	0.3034	0.2563	0.3105	0.3458	0.3227	0.3193	0.3162	0.2879		0.0297	0.0474	0.0365	0.0350
Anadara vellicata (Reeve, 1844)	1	n/c	n/c	0.2504	0.2469	0.1750	0.2979	0.3169	0.2503	0.3000	0.2520	0.2815	0.2346		0.0452	0.0364	0.0315
Anadara brasiliana (Lamarck, 1819)	5	0.0035	0.0019	0.3721	0.3983	0.3255	0.2723	0.3503	0.4155	0.3777	0.4216	0.3779	0.3676	0.3379		0.0317	0.0434
Anadara ovalis[6] (Bruguière, 1789)	1	n/c	n/c	0.3511	0.3767	0.3171	0.3237	0.3434	0.3361	0.3745	0.3514	0.3140	0.3290	0.3121	0.2359		0.0356
Anadara sativa (Bernard, Cai & Morton, 1993)	4	0.0000	0.0000	0.0143	0.1243	0.2137	0.3195	0.3300	0.2765	0.2063	0.1570	0.2713	0.3009	0.2584	0.3772	0.3476	

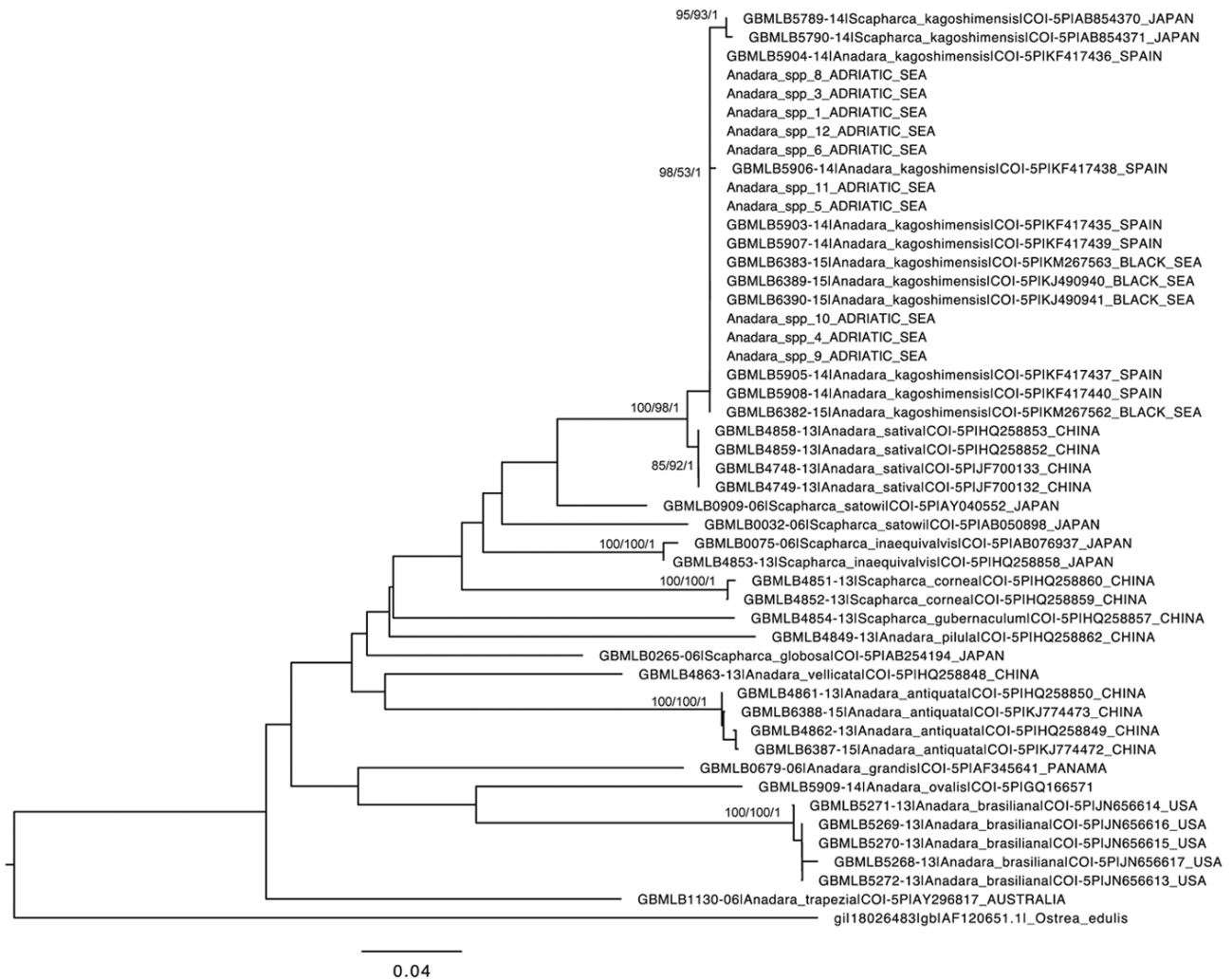
Fig. 3: NJ tree rooted using the sequence of *Ostrea edulis* (GenBank code gi|18026483|gb|AF120651.1). Numbers near nodes are BS and PP values for NJ, ML, and BI, respectively. Only BS values ≥ 50 and PP values ≥ 0.95 are reported for species-specific clusters (Genbank accession numbers of the 10 newly obtained sequences: MF426975, MF426976, MF426977, MF426978, MF426979, MF426980, MF426981, MF426982, MF426983, MF426984).

Table 3: *A. kagoshimensis* density during the 5-year study: h = number of hauls, % h = percentage of hauls with positive catchs, N = average abundance per km^{-2}, SD = standard deviation, % N = percentage of abundance.

| Year	Depth (m)	h	% h	N	SD	% N
	0-30	38	63.16	26456.48	94310.84	99.61
2010	30-50	18	55.56	219.45	387.40	0.39
	50-100	11	0.00	0.00	0.00	0.00
	0-30	38	57.89	6161.18	21309.37	99.31
2011	30-50	18	27.78	90.96	169.34	0.69
	50-100	11	0.00	0.00	0.00	0.00
	0-30	36	50.00	3101.98	10772.32	97.10
2012	30-50	17	23.53	196.15	505.79	2.90
	50-100	10	0.00	0.00	0.00	0.00
	0-30	38	52.63	18670.32	100037.51	99.57
2013	30-50	17	29.41	156.01	349.27	0.37
	50-100	10	10.00	38.94	123.14	0.05
	0-30	39	43.59	7990.87	20200.18	99.23
2014	30-50	17	17.65	128.22	352.08	0.69
	50-100	11	9.09	20.82	69.07	0.07
	0-30		98.65			
Mean	30-50		1.25			
	50-100		0.10			
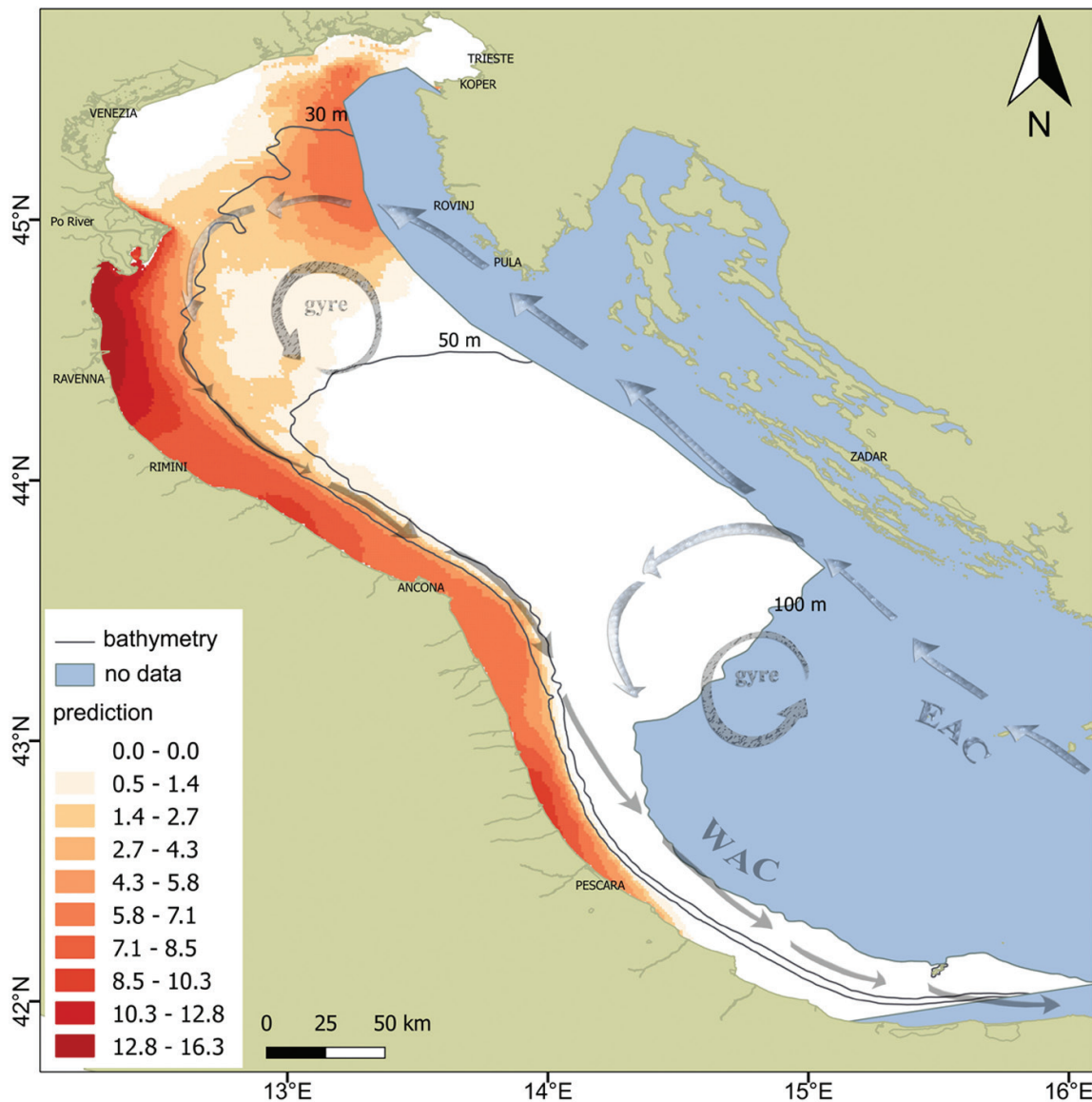
Fig. 4: Prediction map of the spatial distribution of *A. kagoshimensis*. The value is log-transformed (N/km^2). Arrows show the direction of the main currents.

provided a valuable contribution to the morphological investigation and detailed description of the two species. *A. kagoshimensis* from the Adriatic Sea differs from *A. inaequivalvis* from the Philippines by a very small number of taxonomic features that include a slightly more elongated shape, a less convex shell, a slightly larger number of ribs, and a different shell thickness (see also Lutaenko, 2006), whereas the two species do not differ morphologically from the Indo-Pacific *A. kagoshimensis* described and shown by Lutaenko (2015). However, these characters do not seem to be sufficient for "non-specialized" taxonomists to distinguish between them.

Nonetheless, little is known about the genetic traits of these species. Krapal *et al.* (2014) were the first to compare multiple *cox-I* gene sequences from Black Sea ark clams; their genetic results confirmed that the ark clam species invading the Black Sea belonged to *A. kagoshimensis* (Tokunaga, 1906), with a similarity level

ranging from 99.8% to 100%. As regards the specimens collected along the Atlantic coasts of Spain, the genetic distance among the *A. kagoshimensis* samples analysed by Bañón *et al.* (2015) ranged from 0% to 1.6%. The 0.16% genetic distance, found within the *A. kagoshimensis* cluster in the present study, suggests a limited role of geographical origin and highlights the invasion power of this species.

This work constitutes the first attempt at attributing 10 individuals ascribable to the species *A. kagoshimensis*, collected in the Adriatic Sea, using a molecular approach. In particular, the genetic analysis highlighted a clear separation of *A. kagoshimensis* from other ark clam species. The results of this study are in line with those of Krapal *et al.* (2014) and Bañón *et al.* (2015) as regards species identification and confirm the suitability of *cox-I* barcodes as molecular taxonomy markers for the attribution of specimens to one of multiple, closely related taxa.
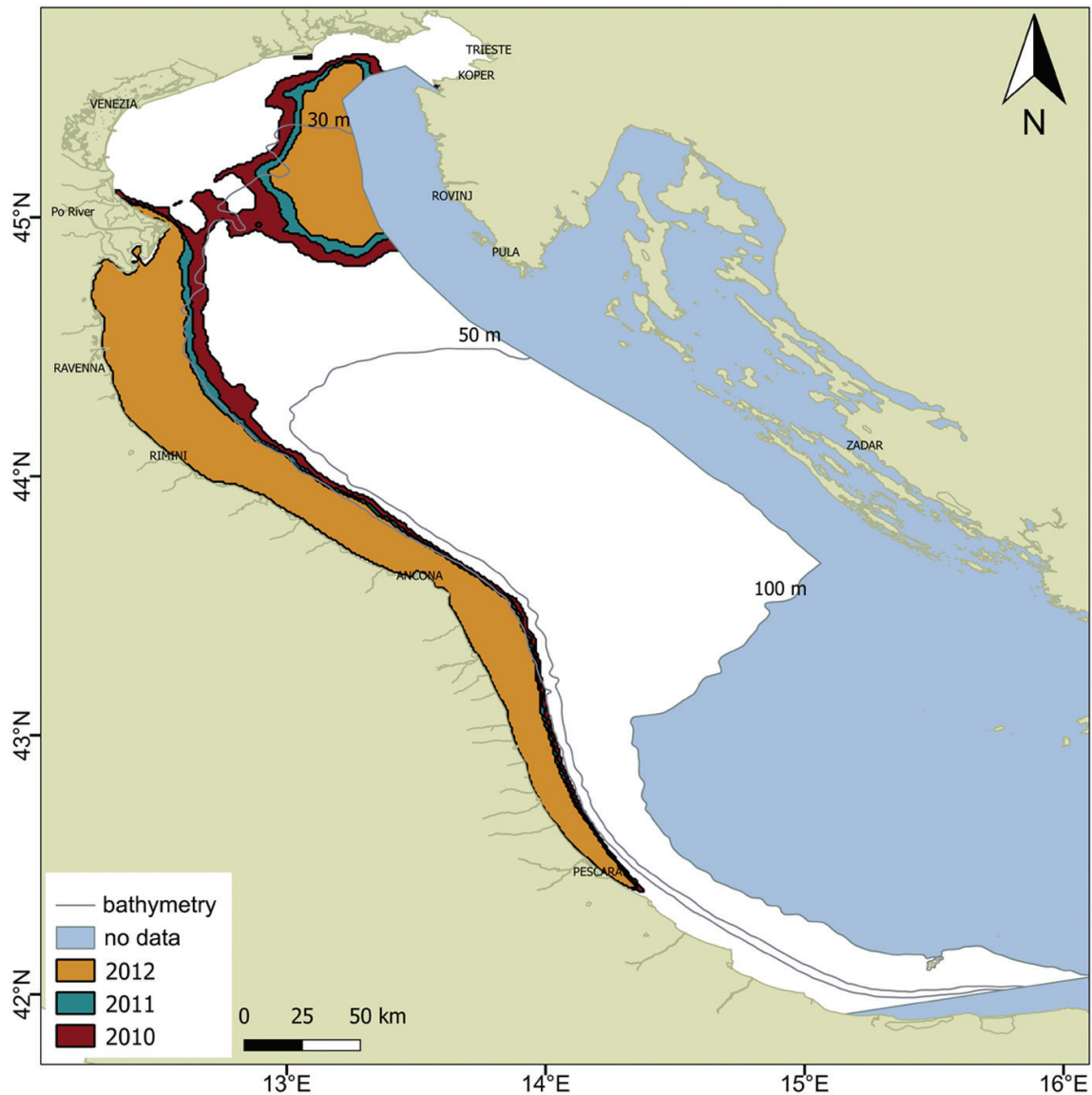
Fig. 5: *A. kagoshimensis* hotspot map: 2010 to 2012.

Moreover, the current paucity of data on the species of the genus *Anadara* suggests that the systematic data on this taxon should be improved by increasing the number of individuals analysed and using more performing molecular markers for phylogenetic reconstruction (i.e. NADH 1, NADH 2, cytochrome b), to enhance the robustness of topology results.

The mean density of *A. kagoshimensis* in the study area was much lower than in the Mediterranean and the Black Sea, where values of 476 N m^{-2} have been reported in the Romanian Black Sea (Micu & Micu, 2004) and where 3000 N m^{-2} and 8.73 N m^{-2} juveniles have been described respectively in the northern Caucasian coast of the Black Sea (Chikina & Kucheruk, 2004) and in Galician waters (north-western Spain; Bañón *et al.*, 2015). On the western coast of the middle Adriatic Sea, Ghisotti and Rinaldi (1976) found 120 N m^{-2}, a lower value than those found in the present study, although this may be as-

cribed to the sampling method, since the authors counted beached shells after the retreat of the tide.

Our findings confirm the presence of *A. kagoshimensis* at the Adriatic sites described by Froglia *et al.* (1998), Morello *et al.* (2004), and Despalatović *et al.* (2013). The prediction of its distribution (Fig. 4) may be explained with the cyclonic circulation characterizing the Adriatic basin, which consists of two main currents: the West Adriatic Current, flowing in south-eastern direction along the western coast, and the East Adriatic Current, flowing in north-eastern direction along the eastern coast. Additionally, two main cyclonic gyres have been described in the northern and southern part of the Adriatic Sea (Marini *et al.*, 2008). The current system may therefore be one of the reasons for the transport of larvae to the central area of the northern Adriatic Sea. Commercial fishing may be another factor in the spread of the species. The northern Adriatic is an important fishing ground (Piccinetti *et al.*, 2012), where numerous fishing vessels move across the

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basin from west to east and vice versa. The solid, heavy shell and the physiological characteristics of *A. kagoshimensis* allow it to survive the action of fishing gears and to be successfully transported towards the centre of the basin (Kaiser & Spencer, 1995).

Although *A. kagoshimensis* is considered as one of the 100 most invasive species in the Mediterranean (Streftaris & Zenetos, 2006), the hotspot map (Fig. 5) clearly shows a resizing of the distribution area. From 2010 to 2012 the area of the hotspots decreased as their borders shifted closer to the coast and completely disappeared from the north-central part of the basin. This trend seems to contrast with the invasive nature of this ark clam and probably depends on the invasion phase. After "introduction", "colonization", and "explosion", the species may have entered the "naturalization" stage, the final phase of invasion, where it may be affected by "boom and bust" cycles and pass through periods of sudden population decline or growth, as seen in several alien marine species in the early years of the invasion (Blackburn *et al.*, 2011). However, other factors might influence its distribution trend. Further, more detailed studies could provide useful data, including information on the impact of the species on Adriatic Sea biodiversity.

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