

Unraveling the complexity of *Corbicula* clams invasion in Lake Garda (Italy)

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ABSTRACT

Lake Garda, the largest Italian lake, is suffering from the introduction of several non-indigenous species during the last decades and can now be considered one of the main European freshwater hotspots of xenodiversity. Among the Bivalvia (Veneroidea, Cyrenidae), *Corbicula fluminea* and *Corbicula fluminalis* were first recorded in 2002 and 2008 respectively, and are now widespread in the southern part of the basin. Recent observation of specimens that did not resemble either of these taxa, suggested that the populations of invasive *Corbicula* of Lake Garda could include some other taxa not previously recorded. With this aim, a thoroughly characterization of *Corbicula* shells found at Lake Garda was made. By studying morphometric parameters and comparing them with specimens collected in Spain (Ebro and Ter rivers), the presence in Lake Garda of two other related species not previously recorded in Italy, namely *C. leana* and *C. largillierti*, has been confirmed. The syntopic presence of at least four species of this genus in a single environment is a singular occurrence both in Italy and Europe.

Key words: *Corbicula leana*; *Corbicula largillierti*; cryptic species; morphometrics; invasions; xenodiversity hotspot.

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INTRODUCTION

Corbicula fluminea (Müller, 1774) (Mollusca: Bivalvia: Veneroidea: Cyrenidae) is considered among the 100 worst invasive species in the world (Lowe *et al.*, 2000). It has spread over the last century in Europe and North and South America, endangering the biodiversity of most aquatic ecosystems in these continents and progressively increasing its range (McMahon, 1982; Araujo *et al.*, 1993; Marescaux *et al.*, 2010). This bivalve has a very complex life history, including clonal and androgenetic reproduction, which has complicated the interpretation by molecular analyses of invasive populations (Renard *et al.*, 2000; Hedtke *et al.*, 2008; Pigneur *et al.*, 2011). In addition, no useful monographs have been published dealing with the different taxa in this genus in their native area, which added to the apparent intraspecific variability of *C. fluminea*, further complicates the study of invasive populations of *Corbicula* clams.

Apart from *C. fluminea*, a number of other taxa have also been characterized as invasive in Europe and the Americas. The best-known species is *Corbicula fluminalis* (Müller, 1774), a taxon long considered either a synonym or a morphotype of *C. fluminea*. However, different studies have demonstrated that it is a valid species, both by molecular and anatomical characteristics (Renard *et al.*, 2000; Glaubrecht *et al.*, 2003; Mouthon and Parghentanian, 2004; Hedtke *et al.*, 2008). It is present in no less than ten European countries, including

Italy, Spain, France, Switzerland, Belgium, Netherlands, Germany, Poland, Hungary and Serbia (Csányi, 1999; Chevallier, 2003; Kornushin, 2004; Mouthon and Parghentanian, 2004; Cianfanelli *et al.*, 2007; Ciutti and Cappelletti, 2009; Bódis *et al.*, 2011; Quiñonero Salgado and López Soriano, 2014), and in South America (Martins *et al.*, 2006; Pereira *et al.*, 2014). Less known is *Corbicula largillierti* (Philippi, 1844), a species widely distributed in South America, where it usually lives syntopically with *C. fluminea* (Ituarte, 1994; Martins *et al.*, 2006; Reyna *et al.*, 2013; Torre and Reyna, 2013; Azevedo *et al.*, 2014; Pereira *et al.*, 2014), but also recently reported from Europe (Quiñonero Salgado and López Soriano, 2016a; Neseemann, 2018). Genetic studies suggest that *C. fluminea* could be mixed with other taxa in some populations (Pigneur *et al.*, 2011), and different invasion episodes in some places have been proposed (Chevallier, 2003; Peñarrubia *et al.*, 2016).

Recently, growing evidence suggests that invasive populations of *Corbicula* may hide cryptic or uncharacterized taxa. For example in North America, after more than 80 years with only a single described species, *C. fluminea*, a new study revealed at least three taxa, with distinct morphology and molecular markers, live syntopically in the Illinois River (Tiemann *et al.*, 2017). In South America, *C. fluminalis* and an unknown species have also been described in addition to *C. fluminea* and *C. largillierti* (Clavijo, 2014; Pereira *et al.*, 2014). The most complex situation has been described in western Europe, with up to seven distinctive taxa inhabiting

aquatic systems in NE Iberian Peninsula, all characterized by distinctive morphological characters and juveniles, with no apparent intermediate forms or hybridization (Quiñonero Salgado and López Soriano, 2016a; 2016b). One of these taxa has been characterized as *Corbicula leana* Prime, 1867, whose presence was previously postulated by molecular markers in France (Pigneur *et al.*, 2011), and later confirmed by morphologic traits both in Spain and France (Prié, 2017; Quiñonero Salgado and López Soriano, 2017). *C. largillierti* has also been reported for the first time in Europe, in Spain (Quiñonero Salgado and López Soriano, 2016a) and later in Germany (Nesemann, 2018), in populations where only *C. fluminea* had been described before.

In Lake Garda, the Asian clam *C. fluminea* was first recorded in 2002 in a very localized littoral area, and further studies backdated its introduction to 2000 (Nardi and Braccia, 2004; Ciutti *et al.*, 2007). Moreover *C. fluminalis* was found in July 2008 (Ciutti and Cappelletti, 2009). The observation of specimens found in the last years, which clearly do not resemble any of the previously described species in Lake Garda and in Italy, suggest that they could include some other not yet described taxa, and so the populations of invasive *Corbicula* should be thoroughly characterized in order to better know their composition. With this aim, we made a preliminary study on the morphometric distinctiveness of different *Corbicula* populations collected from the lake, and compared them with other European populations where similar morphs had been previously described.

METHODS

Lake Garda, the largest Italian lake (volume=49 km³, area=368 km² and maximum depth=350 m) is one of the deep Insubrian lakes south of the Alps, along with lakes Iseo, Como, Lugano and Maggiore. The lake is included in the ILTER network (Italian Long Term Ecological Research) and its trophic condition ranges from oligotrophy to mesotrophy (Salmaso, 2005; Salmaso *et al.*, 2009).

Shells of *Corbicula* were collected from Lake Garda between March and October 2017 in sampling stations along the lake shore and sediment samples by using a grab (maximum depth of 30 m) (Fig. 1). Shells of different *Corbicula* species collected by the authors in Ebro and Ter rivers (NE Spain) were used for comparison.

For the morphological analysis, a total of 879 collected shells were used (438 from Italy and 441 from Spain), divided into 4 size categories according to shell length in order to eliminate bias caused by allometric growth: 6-9 mm, 10-20 mm and 21-30 mm and 31-40 mm (Bódis *et al.*, 2011; Reyna *et al.*, 2013). The following morphometric variables were measured to the nearest 0.1

mm with digital caliper: shell length (L) (antero-posterior dimension) and shell height (H) (distance from the umbo to the ventral margin). The shell mass (W) was weighted by analytical scale (accuracy 0.001 g). A Principal Component Analysis (PCA) was carried out using the morphometric parameters shell length (L), shell height (H) and weight (W) (correlation matrix) for specimens belonging to the shell length class range 10-20 mm, common to all studied populations (N=626). The value of the morphometric variables did not reveal a normal distribution according to the Shapiro-Wilk test and this justified the use of nonparametric statistics. The significance of the differences between the various morphometric parameters of the individuals was evaluated using a rank-based nonparametric ANOVA Kruskal-Wallis and post hoc comparison (Z test). All statistical analysis were performed with the Statistica 13.1 software (Dell Inc., Tulsa, OK, USA), setting the significant level of $P \leq 0.05$.

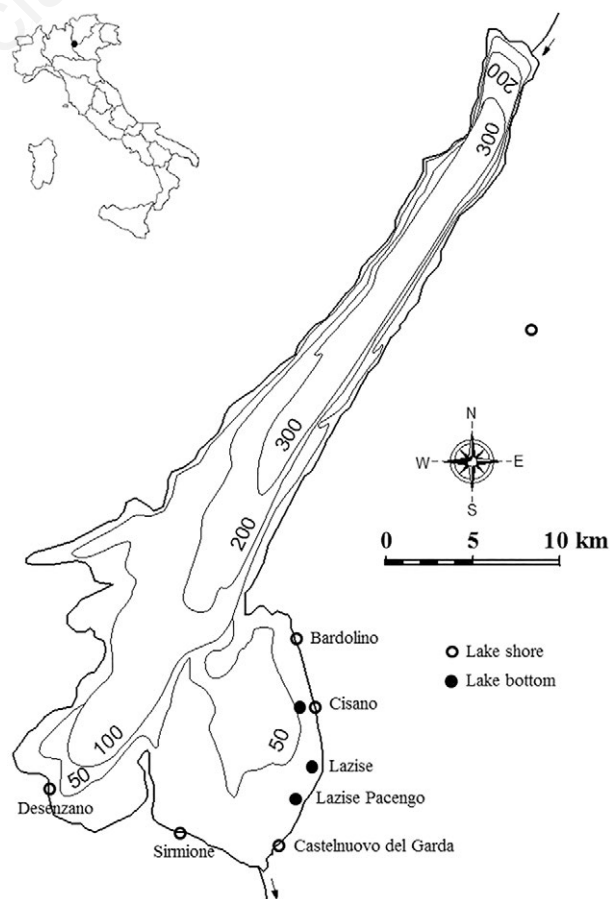


Fig. 1. Sampling stations in Lake Garda.

RESULTS

Based on morphological features, two new morphs of *Corbicula* clams found in Lake Garda, living syntopically with *C. fluminea* and *C. fluminalis*, were determined as *C. largillierti* and *C. leana*, showing similarities with Spanish populations (Quiñonero Salgado and López Soriano, 2016a, 2016b). *Corbicula* specimens collected in Lake Garda are shown in Fig. 2. Morphometric data of *C. fluminea*, *C. fluminalis*, *C. largillierti* and *C. leana*

from Spain and Lake Garda showed a high variability in shell dimensions (Tab. 1). In particular, *C. fluminea* and *C. leana* specimens from Spain were longer and heavier, with maximum shell length (L) up to 40 mm and maximum weight of 13.7 g and 12.8 g respectively.

An evident separation of *C. fluminalis* and *C. leana* for both Lake Garda and Spanish populations was observed with PCA, with the two principal components explaining most variation (99.5%); *C. fluminea* and *C. largillierti* were less clearly separated (Fig. 3). Significant differences



Fig. 2. Specimens of *Corbicula* from Lake Garda. *C. fluminea* (scale: A, 0.5 cm; B-D, 1 cm); *C. fluminalis* (scale: 1 cm); *C. leana* (scale: A, 0.5 cm; B-D, 1 cm). *C. largillierti* (scale: A, 0.5 cm; B-D, 1 cm).

for all considered variables (L, H, W, H/L and L/W) were observed for two length classes: 10-20 mm and 21-30 mm (Kruskal-Wallis test) (Tab. 2). In the 10-20 mm class (common to all populations from Lake Garda and Spain) no significant differences were observed between Lake Garda and Spain after post hoc comparison for *C. leana* and *C. largillierti* for all variables. Also within the 21-30 mm class, *C. leana* from Lake Garda was not different from the Spanish population. Significant differences were observed for *C. fluminea* and *C. fluminalis* in the size range 10-20 mm and for *C. fluminea* in the size range 21-30 mm. A comparison within the 31-40 mm class was not performed because it included just two populations (*C. leana* and *C. fluminea* from Spain). The height/length ratio (H/L) and the length/weight (L/W) ratio can be descriptive of the differences in the shape and weight of the species

and the L/W ratio in particular seemed to be relatively constant for each taxon (Fig. 4). In the shell length 10-20 mm class, *C. fluminalis* from Lake Garda and Spain had significantly a higher height/length ratio (H/L), with mean values of 1.15 and 1.07 respectively, corresponding to a less elongated form; on the contrary, *C. leana* had the lowest values (0.88 and 0.80 respectively). The H/L distinction among other species was less clear. The post hoc comparison also indicated significant differences between *C. fluminea* from Lake Garda and Spain; differences were also observed between *C. fluminalis* (Italy and Spain). No differences between Lake Garda and Spanish populations were found for *C. largillierti* and *C. leana*. Within the shell length 21-30 mm class the H/L ratio showed no significant differences between Lake Garda and Spain for *C. fluminea*.

Tab. 1. Morphometric data of *Corbicula* shells from Lake Garda (Italy) and Spain.

		n	H (mm)			L (mm)			W (g)		
			mean	sd	range	mean	sd	range	mean	sd	range
<i>C. fluminea</i>	G	225	17.0	4.0	6.4-28.6	17.9	4.1	7.7-30.1	1.705	0.948	0.068-5.737
<i>C. fluminalis</i>	G	104	13.5	2.6	8.0-21.8	11.8	2.6	7.6-20.0	1.225	0.802	0.213-4.385
<i>C. largillierti</i>	G	60	14.0	2.1	8.0-18.2	14.9	2.2	8.8-19.2	0.704	0.291	0.112-1.361
<i>C. leana</i>	G	49	12.4	2.9	7.4-19.8	14.1	3.0	9.1-22.3	0.674	0.474	0.110-2.303
<i>C. fluminea</i>	S	130	22.5	9.6	7.0-37.8	24.5	9.7	8.2-40.4	3.921	3.294	0.124-13.700
<i>C. fluminalis</i>	S	168	18.2	3.8	6.0-25.3	17.2	3.7	5.9-24.3	2.756	1.312	0.094-6.357
<i>C. largillierti</i>	S	20	15.0	1.9	11.4-18.2	15.9	1.7	13.0-18.9	0.798	0.307	0.320-1.360
<i>C. leana</i>	S	123	20.0	8.4	7.5-37.0	23.3	8.8	9.8-40.8	3.034	2.822	0.145-12.841

G, Lake Garda, Italy; S, Spain.

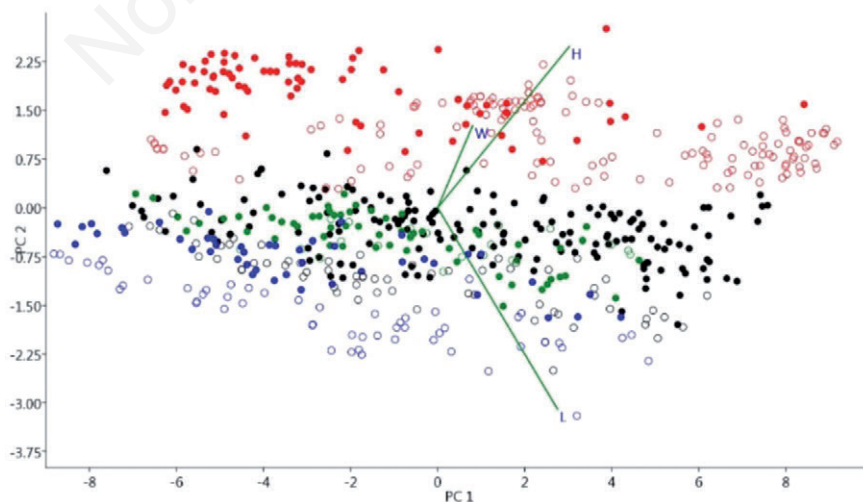


Fig. 3. The Principal Component Analysis showing the relationship of the PC1 and PC2 components of *Corbicula* populations from Lake Garda (Italy) and Spain (10-20 mm). Red, *C. fluminalis*; black, *C. fluminea*; green, *C. largillierti*; blue, *C. leana*. Filled circle, Lake Garda; empty circle, Spain.

Considering the shell length range common to all populations (10-20 mm), the L/W ratio was more informative to distinguish species. *C. fluminalis* from Lake Garda and Spain showed significantly lower values (9.96 and 7.98 respectively) in comparison to other taxa, corresponding to heavier shells. *C. leana* and *C. largillierti* had significantly higher values respect to *C. fluminea*. For the L/W ratio, no significant differences for all species from Lake Garda and Spain were observed.

DISCUSSION

With the new observation of *C. leana* and *C. largillierti* reported in this paper, Lake Garda *Corbicula* populations include at least four species, instead of the two previously cited (*C. fluminea* and *C. fluminalis*).

Indeed, we confirm the presence of two species not previously cited in Italy, which expands to a third European country the distribution of both *C. leana* and *C. largillierti*.

Besides morphological features (ribs, colour of teeth and inner and outer shell), morphometric parameters (H/L and L/W ratios in particular) are informative to distinguish among the four analysed species (Quiñonero Salgado and López Soriano, 2016a, 2016b) (Tab. 3). *C. leana* is characterized by a very uniform orange umbo and violet lateral teeth, as described in Quiñonero Salgado and López Soriano (2016a; 2017); it has been described as having a shell that is consistently broader and lighter than *C. fluminea*, as also shown in the morphometric analysis. On the other hand, *C. largillierti* is not so clearly discernible by morphometric parameters but has peculiar

Tab. 2. Results of the ANOVA Kruskal-Wallis for the morphometry of *Corbicula* shells from Lake Garda and Spain grouped in three length classes: 6-9 mm, 10-20 mm, 21-30 mm

Lenght class	6-9 mm df=6; N=20		10-20 mm df=7; N=626		21-30 mm df=4; N=127	
	H	P	H	P	H	P
H (mm)	4,5102	ns	195,2877	***	31,6538	***
L (mm)	4,7255	ns	125,4989	***	66,7735	***
W (g)	7,3996	ns	279,6785	***	64,1655	***
H/L	16,8168	ns	548,8888	***	101,2741	***
L/W	11,8238	ns	386,3458	***	70,8472	***

Significance level P<0.05;(ns, not significant; ***P<0.001.

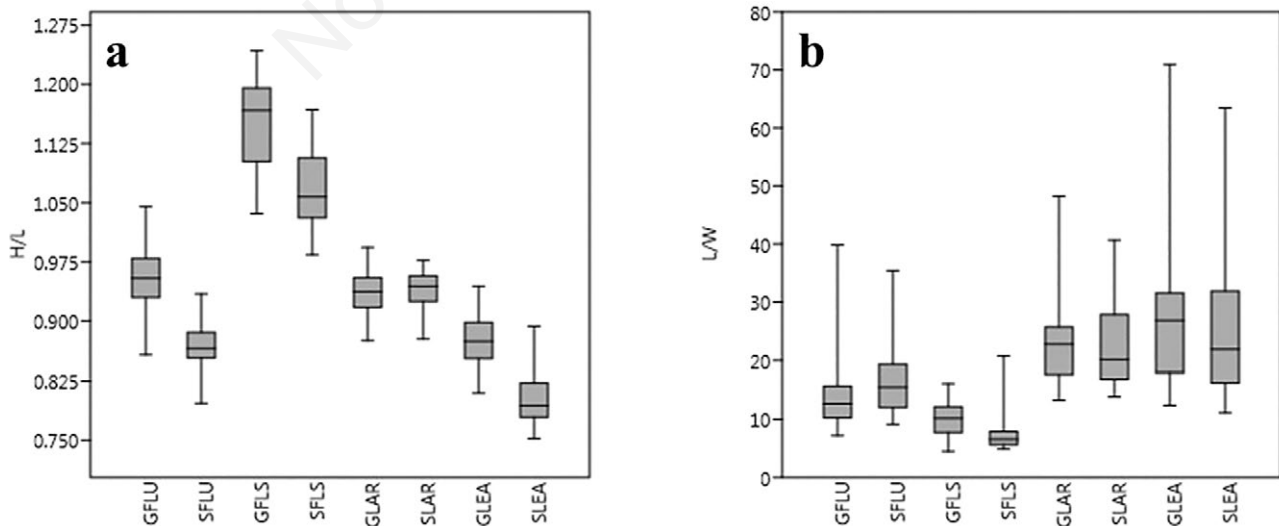


Fig. 4. Boxplot (median and 25-75% quartiles) of a) height/length (H/L) ratio and b) length/weight (L/W) ratio for specimens in the range 10-20 mm of *Corbicula* specimens. FLU, *C. fluminea*; FLS, *C. fluminalis*; LAR, *C. largillierti*; LEA, *C. leana*; G, Lake Garda; S, Spain.

morphological features: a dark violet coloration on the entire inner surface, and lacks the blue blotch in the umbo that is always present in *C. fluminea* (Quiñonero-Salgado and López Soriano, 2016a, 2016b). *C. largillierti* and *C. leana* do not have significant differences in their morphometric parameters and ratios between Lake Garda and Spanish populations. Not surprisingly, the most studied and widespread species, *C. fluminea* and *C. fluminalis*, show significant biometric differences between Lake Garda and Spain, as already observed in other countries (Araujo *et al.*, 1993; Sousa *et al.*, 2007; Skuza *et al.*, 2009; Gomes *et al.*, 2016). The syntopic presence of up to four species in a single environment is rather a new scenario, but it could indeed be a more widespread situation than expected, since no in-depth analyses have been performed in most of the *Corbicula* invasive populations.

It has to be considered that the number of alien aquatic species in Lake Garda, -up to 42 species of fish, invertebrates, macrophytes and macroalgae- is quite high and constantly growing, probably due to tourism and recreational activities such as fishing and sailing (Ciutti *et al.*, 2011; Ciutti and Cappelletti, 2017). Indeed, the abundance of these invasive species has shown dramatic increases, as seen in accumulation of shells in the shore of the lake. Only a few years after their introduction, *Corbicula* clams are now widespread in the southern part of the lake and large deposits along the shore can be observed, resulting in an impressive scenario (Cappelletti and Ciutti, 2017) (Fig. 5).

We cannot speculate on the likely origin and time of arrival for these two newcomer species. However, no large specimens of *C. leana* were found in Lake Garda, compared to those found in the Ter River in Spain, which may reach up to 40 mm when adult. Thus, a likely recent origin could be postulated for this population in the lake. In this sense, this species was not detected in this lake

before 2017. Instead some specimens of *Corbicula* sp., now assigned to *C. largillierti*, were already found in March 2015 (shell length up to 16.7 mm).



Fig. 5. Large deposits of *Corbicula* shells along the Lake Garda shore.

Tab. 3. Main morphological features of *C. fluminea*, *C. fluminalis*, *C. largillierti* and *C. leana* (according to Quiñonero Salgado and López Soriano, 2016a, 2016b). Morphometric data from this study (mean and standard deviation).

	<i>C. fluminea</i>	<i>C. fluminalis</i>	<i>C. largillierti</i>	<i>C. leana</i>
Shell outer color	Olive green/olive/brown	Brown	Brown	Yellow/brown
Shell inner color	Ivory/light violet	Purple/dark violet	Purple/dark violet	Ivory/orange
Shell outline	Oval to triangular	Triangular	Oval to triangular	Oval, wide/lengthened
Lateral teeth	Violet blotch on base	No blotches	No blotches	Stained violet
Umbo	Olive green/brown; vertical violet line barely visible	Brown	Violet, without ribs	Orange
Ribs	Coarse, sparse	Regular, thick	Regular, thick	Coarse, sparse
Juveniles	Green, violet line in umbo	Purple/violet, no line in umbo	Purple/violet, wide stripe in umbo	Orange, no line in umbo
H/L Lake Garda (I)	0.95 (0.04)	1.15 (0.06)	0.94 (0.03)	0.88 (0.03)
H/L Ebro/Ter (S)	0.87 (0.03)	1.07 (0.04)	0.94 (0.03)	0.80 (0.03)
L/W Lake Garda (I)	14.00 (5.33)	9.96 (2.78)	23.39 (6.62)	28.65 (14.38)
H/L Ebro/Ter (S)	16.65 (5.65)	7.98 (3.94)	22.62 (7.84)	27.32 (14.73)

The new analysis of *Corbicula* populations in Lake Garda confirms that some populations may hide other *Corbicula* species apart from *C. fluminea* and *C. fluminalis*, as already suggested for different sites in North and South America, France, Germany and Spain (Torre and Reyna, 2013; Quiñonero Salgado and López-Soriano, 2016a; Tiemann *et al.*, 2017; Neesemann, 2018). Indeed, one of the species present in the Ebro River has been found in public and private collections, sampled around the year 2000 (Quiñonero Salgado and López Soriano, 2016a), thus confirming a cryptic presence for many years, misidentified as *C. fluminea*. In fact, the identification of *C. fluminea* has not followed a true “gold standard” criterion in the last decades, which is also evident because most of the published studies do not even include pictures of the collected specimens. Furthermore, in many cases citations were only based on few, bad preserved and dead specimens, which may not maintain some important diagnostic characteristics.

In the light of our results, and those by other authors, a detailed revision of the invasive populations of *Corbicula* is advisable, as some populations may have a richer species composition than reported. Indeed, our results confirm the predictions made by Reyna *et al.* (2018), based on bioclimatic models and known distribution ranges of *Corbicula* species, who postulated that *C. largillerti* could potentially expand its invasive range to different countries in Europe, with likely sympatric occurrence in some places with *C. fluminea* and *C. fluminalis*. Increase in global trade and tourism may favor in a near future these episodes of colonization.

CONCLUSIONS

With these results, we increase the number of *Corbicula* species present in Italy to four, and describe the first known populations of *C. leana* and *C. largillerti* in this country. For Europe, *C. leana* is present in Spain and France, while *C. largillerti* has only been reported in Spain and Germany. Other Italian populations should be studied, in order to detect the expansion of the four species already found in Lake Garda.

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