Challenging exploration of troubled waters: a decade of surveys of the giant freshwater pearl mussel Margaritifera auricularia in Europe

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Acknowledgments:

This work was conducted within the scope of the LIFE project “Life13BIOFR001162 Conservation of the Giant Pearl Mussel in Europe”. We thank Dominique Tesseyre from the Adour-Garonne Water Agency; Julie Marcinkowsky and Gérard Tardivo from the DREAL Centre as well as the DREAL Picardie for providing financial support for large-scale surveys of *M. auricularia* in France; Elodie Hugues, Guillaume Métayer (Conseil Général de Charente Maritime), David Bécart (Voies Navigables de France) and Amandine Szurpicki (COSEA), Frédérique Moinot and Olivier Guerri (EPIDOR) for financing focus surveys in the Charente, Seine, Vienne and Garonne Rivers; and for Spain FMC Foret S.A., Enagas, Gas Natural, INYPSA, Hidroeléctrica La Zaida, Edison Mission Energy and EID Consultores.

Abstract

The critically endangered Giant Freshwater Pearl Mussel *Margaritifera auricularia* was presumed extinct before its re-discovery in Spain in 1985 and France in 2000. Since then, numerous surveys have been set up to search for living populations in France and Spain. This article presents an up-to-date distribution of the species based on available data, i.e. literature, Museum collections and recent field surveys; and provides unpublished molecular data for France. The Giant Freshwater Pearl Mussel is still living as three populations in the Ebro River in Spain, and eight populations in France (two in the Loire drainage, one in the Charente drainage, two in the Garonne drainage and three in the Adour drainage). The biggest population lives in the Charente River with an estimated 100.000 individuals. Recruitment is very scarce in all populations but living specimens estimated to be less than 10 years old have been found in the Ebro in Spain and in the Vienne, Charente, Dronne and Adour rivers in France. Recent populations rediscovery in France were mainly a result of intensive dedicated surveys including scuba-diving. Subsequent advances in knowledge show how large rivers and downstream ecosystems remain a *terra incognita* for the hydrobiologist.

Distribution; museum collections; historical data; scuba diving surveys; large rivers; conservation

Introduction

Freshwater ecosystems are the most threatened ecosystems worldwide (Dudgeon et al. 2006) and freshwater bivalves rank amongst the most threatened animals in the world (Lydear et al. 2004; Lopes-Lima et al. 2016). One of them, the Giant Freshwater Pearl Mussel *Margaritifera auricularia* (Spengler, 1793), ranges amongst the most imperilled bivalve species. Although it was considered widespread in most of the western Europe rivers at the beginning of the 20th century, it is now considered as critically endangered by the IUCN (Araujo & Ramos, 2001; Prié 2010). The Giant Freshwater Pearl Mussel had become so rare during the 20th century that it was not even considered when the European Habitat Directive species lists have been established. Indeed, the Giant Freshwater Pearl Mussel is difficult to observe: it lives downstream in large rivers, a habitat that is difficult to survey due to deepness, turbidity, current and often navigation. Hence, not surprisingly, it has been overlooked by malacologists of the 20th century. However, it nowadays still survives as a few populations in south-west France and eastern Spain.

The species was first rediscovered in Spain in 1985 (Altaba, 1990) and in France in 2000 (Cochet, 2001). Since 1998, the biology, distribution and lifecycle of the Giant Freshwater Pearl Mussel in Spain were described (Araujo & Ramos, 1998a, b; Araujo & Ramos, 2000 a, b; Araujo & Ramos, 2000; Grande *et al.*, 2001; Araujo *et al.*, 2001; 2002; 2003; Gómez & Araujo, 2008). Since then, some few news of the species in Spain have been released in national and international congresses (i. e. Nakamura et al., 2015; Online Resource 1), but, apart from Araujo & Álvarez-Cobelas (2016) there are no new scientific results published since 2008. In France, focused surveys have led to the rediscovery of many populations since 2007, but most of these results are unpublished (but see Prié et al., 2007; Prié et al., 2008; Prié et al., 2010) or available only as grey literature (Online Resource 1).

An extensive review of all available data on *Margaritifera auricularia*'s distribution is provided here for the first time, together with new data from museum collections and recent field surveys. This article clarifies the past and present distribution of the species, presents the results of the last ten years' surveys in France and Spain and discusses conservation perspectives.

Material and Methods

Bibliography review

The bibliography since 1793 (species description date) has been extensively reviewed. Local publication and grey literature were also consulted when available. Bibliographic data was generally imprecise, but allowed figuring a broad image of the original distribution and ecology of *M. auricularia* (Fig. 1). Bibliography review thus provided the first indications for where to look for this species.

Museum collections

A first review of museum collections had been performed by Araujo & Ramos (2000a) at a global scale. This review mostly aimed at large national museum collections and included also *Margaritifera marocana* (Pallary, 1918), a species living only in Morocco (Araujo et al., 2009a). We then inventoried all the regional museum and Universities collections in France. Fifty-eight local natural history collections were identified. Each of them was contacted and questioned about the presence of malacological collections, freshwater bivalves and eventually *M. auricularia* specimens. When *M. auricularia* specimens were recorded in the inventories or discovered in the collection by the curator, pictures were sent to us to confirm identification. Eventually, some of the most important collections (Musée des Confluences in Lyon, Museum d'Histoire Naturelle in Bordeaux, Museum d'Histoire Naturelle in Toulouse, Museum national d'Histoire naturelle in Paris, Museum d'Histoire Naturelle in Lille, Museum d'Histoire Naturelle in Nantes, Museum d'Histoire naturelle in Orleans, University of Rennes, University of Montpellier) were visited by one of us.

Specimens collected since 2000, year of the re-discovery of the species in France, were not included in the results presented here.

Field surveys and population sizes

Numerous field surveys aiming at freshwater mussels have been performed in France and Spain (Fig. 1, Table 1).

These dedicated surveys aimed at places most likely to host the species, i.e. places identified by literature data, museum collection data or, for France, species habitats modelling (Prié et al., 2014). Moreover, some surveys took place into the frame of impact studies. These impact studies were triggered when *M. auricularia* was living

- or when available data suggested that it could still be living - in an area impacted by a development project. The results of these impact studies are generally not published, consisting only in various cryptic reports (but see Prié et al., 2007; Prié et al., 2008; Araujo & Alvarez-Cobelas, 2016). We here summarize for the first time all the grey literature related to *M. auricularia* in France and Spain (Online Resource 1).

M. auricularia mainly lives in downstream ecosystems. Surveying this habitat is challenging because it is often deep, turbid, strongly flowing and navigable. In the Ebro historic channels, sampling depends on the hydraulic works made by the Confederación Hidrográfica del Ebro; it is necessary to decrease the water level in order to wade the channel bottom to find the specimens (Gómez & Araujo, 2008). In France, some populations are readily accessible, living in the banks (Vienne River) or in shallow waters (Creuse, Luy or Arros River). For those populations, snorkelling or wading with viewing glasses allowed efficient surveys. However, cumbersome methods based on a team of scuba-divers were needed in most cases. For some surveys, a boat was used to shuttle the divers from a place to another. For others, divers dove from the river banks and sampling plans were then constrained by river accessibility.

Population sizes given here were estimated based on exhaustive counts of observed living individuals (Luy, Creuse and Vienne Rivers); statistical analyses (Ebro, Arros and Charente Rivers), or in the worst case, by a subjective appreciation based on the density of specimens observed (Dronne, Adour and Save Rivers).

The Seine (downstream) and Eure Rivers could only be surveyed by dredging. The dredger used had an aperture of 50 cm, a 25-mm mesh, weighted 11 kg and was propelled by a 30-horsepower engine Zodiac by means of a 30 m long rope. In the Eure River, different biotopes and flow facies were aimed at (mud, sand, stones, riffles, vegetation). In the Seine River, water was up to 6 meters deep and too troubled for operators to see the river bed. Catches were then randomly positioned. Catches were 8 to 10 m long in the Eure River, and up to 40-50 m long in the Seine River. Sediment collected by the dredger was pulled up and sorted out on the boat. Wading surveys were adopted upstream the Seine River.

In the Somme River, a boat was used to shuttle divers and 82 bank to bank transects were sampled on a 26 km long river stretch. In the Oise River, the divers were also transported by boat from a spot to another, but diving plans were constrained by river condition (from very strong current to muddy bottoms). Areas with very strong current were sampled combining scuba-diving and climbing technics, with a 100 m long static rope secured on a tree on the bank. The diver used a climbing harness and caving equipment in addition to scuba diving gear to progress on the rope. Fins were used to go from side to side in the current, allowing to cover a ca.90 m long cone-shaped surface on the river bottom. Altogether, 115 dives have been carried out on a 35 km long stretch of the river, from the confluence with the Aisne River downstream to a few kilometres upstream the town of Sempigny. Upstream this stretch of river, surveys were carried out wadding randomly in suitable habitats.

In the Charente River, the population was estimated based on scuba-diving transects surveys. A boat was used to shuttle the divers from a transect to another. A 20 m long line was settled down on the bottom of the river and scuba divers counted every living specimen left and right of the line at a distance of 2 m. Each sample then covered 80 m^2. Transects were repeated every kilometer in the river stretch where mussels were present, and then every three kilometers downstream and upstream the population's distribution limits. A total of 43 transects were repeated on a stretch of 60 km. Detection probability has been estimated at 75% using iterated observations analysed with the software MARK (White & Burnham, 1999). Geographical statistics (Anselin, 1996) were performed using GeoDa software (Anselin et al., 2006). Suitable habitat length in the whole river was delimited downstream by the limit of the mud cover due to the influence of the Saint-Savinien's impoundment, upstream by the limit of the living population. Between these limits, the substrate and general ecological quality of the river was very homogenous. In this stretch of favourable habitat, live specimens have been observed wherever we have dived between 2010 and 2016, thus confirming that the population is uniformly distributed.

Fourteen sampling surveys were undertaken between September 2000 and June 2006 in the Ebro River, totalising 25 km, wading in shallow waters and with a team of divers in the deeper parts of the river. Divers used submerged ropes to perform bank to bank or longitudinal transects (survey methods were detailed and reported in Gómez & Araujo, 2008; Araujo & Álvarez-Cobelas, 2016).

In the Dronne and Isle Rivers, about 100 km stretch of each river upstream their confluence was surveyed, both by wading and scuba-diving from the banks. The estimation of the population size was based on author's appreciation only, and is likely underestimated: over 50 specimens have been observed during the surveys, with a subpopulation of 30 specimens in the lower location (exhaustive count). We estimate that about half of the living individuals have been observed during surveys, which is unlikely given the detection probability in this large river.

About 60 km of the Save River was surveyed by wading and scuba-diving, aiming at an exhaustive count of the few remaining specimens which were found only in the lower section of the river. Most of the sampling in the Adour River was undertaken by wading and snorkelling, with scuba divers requested only for a few deeper places. As for the Dronne River, few specimens were found in isolated places, with biggest subpopulation numbering about ten specimens. Population size is estimated based on experts' appreciation only. The Arros River is highly impacted by agriculture practices. The remaining favourable habitats were found isolated between the numerous impoundments' influences. A first survey was conducted by scuba divers, but the deepest places did not have suitable habitats. A more intensive survey was then organized by a team wading with viewing glasses. The total length of river stretches having suitable habitats was 54 km. Within these 54 km, sixteen sites were sampled. On each sampled site, stretches of 100 m to 1 km were exhaustively surveyed. Population size was estimated based on average densities observed during surveys, multiplied by favourable habitat's surface. In the Luy River, divers explored the deepest pools while most of the river can be explored by wading. The main population is found in a very shallow place, and exhaustive counts were performed three times (years 2010, 2011, 2012) by five persons wading in a line, about one meter apart, ensuring efficient scanning of every single place of the river bed. However, detection probability is never 100%. Some specimens may spend some time completely buried in the sediment and are overlooked (see below the results for the Luy River). The results of these assumed exhaustive counts are therefore likely underestimated.

The most intensive surveys took place in the Vienne and Creuse Rivers. The surveys aimed at providing exhaustive counts of all living specimens. Observers with viewing glasses and divers (depending on the depth) were lined one meter apart and moved forward upstream, ensuring efficient scanning of every single place of the river bed. Sampling was reiterated several time between 2009 and 2016 using the same methods.

In this study, when shells only have been collected, we considered "ancient shells" those that were worn and uncomplete, without periostracum nor ligament remains. "Recent shells" include shells with at least periostracum and ligament remains. We consider as "juveniles" specimens with shell length lower that 11 cm, "subadults" specimens from 11 to 14 cm. Occasionally, some adult specimens had very short shells, especially in the Charente River, but these were obviously very old given the growth lines density and shell wear.

Genetic analyses

Tissue samples have been collected from ten specimens from the Ebro River in Spain, and ten specimens from the Vienne River (Loire coastal drainage), two specimens from the Luy River (Adour River coastal drainage), two specimens from the Charente River and one specimen from the Save River (Garonne River coastal drainage) in France. Foot tissue samples were snipped in the field and preserved in 90° ethanol for molecular analysis.

For Spanish specimens, DNA was extracted using CTAB protocol: tissue samples, preserved in ethanol or frozen, were ground to a powder in liquid nitrogen before adding 600 m L of CTAB lysis buffer (2% CTAB, 1.4 M NaCl, 0.2% b-mercaptoethanol, 20 mM EDTA, 0.1 M TRIS [pH = 8]) and subsequently digested with proteinase K (100 $mg.ml^{-1}$) for 2–5 h at 60° C. Total DNA was extracted according to standard phenol/chloroform procedures (Sambrook & Maniatis, 1989). For French specimen DNA was extracted using the Nucleospin Tissue Kit (marketed by Macherey–Nagel), following the manufacturer's protocol. Extractions, amplifications and sequencing were performed by Genoscreen (France).

To test genetic variability between populations, we examined fragments of two mitochondrial genes, COI and 16S, used previously by Huff et al. (2004); these showed the greatest phylogenetic resolution power for relationships among margaritiferids. 28S nuclear gene fragments were also amplified, but different fragments were targeted for French and Spanish specimens. The COI, 16S and 28S gene were amplified by polymerase chain reaction (PCR) using the protocol described by Prié & Puillandre, 2014 for French specimens, and described by Machordom et al. (2003) and Araujo et al. (2016) for Spanish specimens. The amplified fragments were purified by ethanol precipitation prior to sequencing both strands using BigDye Terminator kits (Applied Biosystems, ABI). Products were electrophoresed on an ABI 3730 genetic Analyser (Applied Biosystems). The forward and reverse DNA sequences obtained for each specimen were aligned and checked using the Sequencer program (Gene Code Corporation) after removing primer regions. Sequences were automatically aligned using ClustalW multiple alignments implemented in BioEdit 7.0.5.3 (Hall, 1999). The accuracy of automatic alignments was confirmed by eye. Genebank accession numbers are provided in Table 2.

Results

Bibliography

Available literature provided valuable data, although generally without precise location nor date. Nevertheless, a first historical distribution map could be drawn from ancient literature data. *Margaritifera auricularia* is known from the Netherland, England and Germany from fossil records only. However, some shells collected in the Unstrut River in Germany are very well preserved and probably date back to historical times, at least until the early Middle Ages (Bössneck et al., 2006). Fossil data in Spain includes a Mediterranean Quaternary river in Yecla (Murcia) with 129.000-140.000 years old specimens (Andrés & Ortuño, 2014) and many other Atlantic rivers with 5.000 years old specimens (Araujo & Moreno, 1999). In France, fossil data near Marseille (coming from archaeological excavation) and in Massif Central (found amongst fossils collected in a cave) were presumably a result of human transportation.

According to historical data collected, *Margaritifera auricularia* was only found in large rivers, in a calcareous substrate, in France, Spain and Italy. In France, historical data mainly comes from the Atlantic and Channel sea coastal drainages, with only one occurrence in the Mediterranean coastal drainages, in the Saône River (Rhône tributary). In Italy and Spain, the species is historically known from two Mediterranean coastal drainages, the Po and Ebro Rivers (Araujo & Ramos, 2000a). In Spain *M. auricularia* lived in two historic channels from the Ebro River, the Canal Imperial and the Canal de Tauste, where there were about 5000 live specimens. The more recent data published about these Spanish populations were recorded in Araujo & Ramos (2000b), Gómez & Araujo (2008) and Araujo & Álvarez-Cobelas (2016).

Museum collections

The Museum collections have been examined first by Araujo & Ramos (2001) at a wide scale, focusing mainly on national museums worldwide. Prié et al. (unpublished data, Online Resource 1) have focused on French regional collections only. Out of the 58 collections identified, 25 had at least one specimen of *M. auricularia* (Fig. 2A): Musée du Château in Annecy, Musée des Confluences in Lyon, Museum of Perpignan, Musée zoologique of Strasbourg, Muséum - Aquarium of Nancy, Museum of Auxerre, Muséum d'histoire naturelle in Bordeaux, Muséum d'histoire naturelle in Bourges, Muséum d'histoire naturelle in Grenoble, Museum d'Histoire Naturelle in Nantes, Muséum d'Histoire Naturelle in Toulouse, Museum d'Histoire Naturelle Victor Brun in Montauban, Museum d'Histoires Naturelles in Colmar, Muséum of Orléans, Muséum of Dijon, Muséum Lecoq in Clermont-Ferrand, Muséum national d'Histoire naturelle in Paris, Muséum national d'histoire naturelle in Lille, Paraclet center of ONEMA in Boves, Pôle muséal of Troyes, Université of Bourgogne in Dijon, Université of Montpellier I, Université of Rennes I, Museum d'histoire naturelle in la Rochelle, Museum of Cherbourg-Octeville. Part of the data from Museum collections were fossil specimens. A total of 400 non-fossil specimens were found in Museum collections, including the 37 specimens already found by Araujo & Ramos (2001). Among them, 332 were localized at a river drainage scale. A third of the specimens came from the Garonne drainage, 19 % from the Saône River (half of them coming from a single batch collected by Coutagne in 1879) and 17% from the Ebro River (Fig. 2B). Other drainages represent less than 30% of the Museum collections specimens. About 80% of the specimens dated were collected before the beginning of the 20th century.

Field surveys and populations sizes

A total of 2.500 km of rivers has been surveyed for *M. auricularia* in France and Spain during the last ten years (see bibliography and Online Resource 1 for details). These surveys covered most of the river stretches for which literature or museum collections data was available. Eleven populations could be identified, eight in France and three in Spain, plus a single individual found recently in the Ebro River (pers. comm. from R. Álvarez-Halcón to

R. Araujo) upstream Zaragoza (Fig. 3, Table 3). In Spain, the main population, with 5.000 live specimens, live at the Canal Imperial in Aragón. Although there have been some recent mortalities, some young specimens probably under ten years old have been observed during the last years (pers. comm. from J. Guerrero to R. Araujo). The other two Spanish populations, on the Canal de Tauste and the lower Ebro River, are today practically testimonials (pers. com. of the Generalitat of Catalonia to R. Araujo). See Gómez & Araujo (2008), Araujo (2012) and Araujo & Álvarez-Cobelas (2016) for more information.

In France, field surveys allowed finding ancient shells in the Seine, in the Vesle and in the Aisne Rivers; in the Saône River (Rhône drainage) near Pontailler-sur-Saône and in the Garonne River near Agen, findings which corroborate historical data. We believe the species was extirpated long time ago in those rivers. In the Oise River (Seine drainage), very recent shells have been found in 2007 and 2008, some of them still embedded in their natural position, suggesting that the species became extirpated very little time before the surveys took place.

The populations of the Creuse and Vienne Rivers (Loire drainage) are the most studied in France. They live in shallow and clear water, allowing regular surveys using viewing glasses or snorkelling. Although these populations are rather small (about 250 specimens altogether), over 40 juveniles were found in the Vienne and Creuse Rivers, which represent about 15% of the population.

Three sites with a few tens of live specimens were discovered in the Dronne River, including one juvenile of about ten cm. Additionally, some isolated individuals were also observed, suggesting the population is scarce but relatively widespread. In the Save River, only 5 live specimens were observed. Sampling conditions are difficult, with variable depth and current strength, and very low visibility. We can therefore suppose that our detection probability is low. But based on survey results, we estimate that the population should not exceed a few tens of living individuals. It is likely rapidly declining given the bad condition of the river and the large number of recent shells collected compared to the very few living specimens observed. The Adour drainage rivers were known to host *M. auricularia* from both literature and Museum collections data. In the Adour mainstream, the population is now highly fragmented, with only three sites where live specimens could be found. One of them, the most upstream, is now extirpated (Prié et al., 2010). The total population is estimated to be about 300 specimens in the total length of the Adour mainstream, but we still need a better estimation based on an appropriate sampling protocol. On the Luy tributary, a population of about 150 specimens is found in a very small stretch of river. Interestingly, although this River is very shallow (from 30 cm to 1,5 m), clear and easy to survey (hence detection probability is optimal), successive counts of 2010, 2011 and 2012 lead to respectively 110, 96 and 145 specimens. We suppose that a significant part of the population lives buried in the sediment, which biases the results of the counts. The Arros River had been overlooked by literature review and field surveys up to 2016. Following the findings in Museum collections, dedicated field surveys were conducted in 2016, allowing the rediscovery of a living population. This population's size was estimated to about 200 individuals on the 54 km of favourable habitat. The Charente River was known from the ancient literature to host an important population of *M. auricularia* (Bonnemère, 1901). Shell fragments and very few live specimens had been found by naturalists since 2003 (Nienhuis, 2003; P. Jourde pers. com.). Intensive field surveys performed in 2007, 2010, 2016 led to the discovery of the largest population worldwide. Geographical statistics based on scuba-diving transects showed that the population was not aggregated. Hence the total population size could be estimated by multiplying the average density by the total surface of suitable habitat in the stretch of river inhabited by *M. auricularia*. The population size in the Charente River was estimated to be about 100.000 (80.000 – 120.000) individuals, between the towns of Cognac upstream to Port-d'Envaux downstream.

Genetic diversity

Margaritifera auricularia is genetically remarkably homogenous. The specimens from France and Spain all shared the same 16S and COI haplotype, but two specimens from Spain: specimens vouchered FW1238-14 and FW1238-12, with for COI T-> A in position 37, T->A in position 50 and G->C in position 73; and for 16S T-> C in position 176. The French and Spanish specimens could not be compared for 28S as different gene fragments were amplified. But within France, all specimens shared the same haplotype and within Spain, all specimens shared the same haplotype.

Discussion

Historical and actual data

The number of specimens found in the various regional museum collections was unexpected. *Margaritifera auricularia* is a large species that retained collector's attention. Most data from museum collections corresponded to the literature data, excepted those from the Arros and Vezere Rivers in France. Surprisingly, most French specimens came from the Garonne and Saône Rivers, were the species is now believed to be extirpated or very rare. In contrast, very few specimens came from the Charente River, where the largest population is found nowadays, and where industrial fisheries were established to make nacre shirt buttons (Bonnemère, 1901). Similarly, museum collections host no specimen from the Vienne or Creuse Rivers, where healthy populations live in shallow and clear waters. In the Seine drainage, most shells came from upstream and the Aisne tributary, while the Oise tributary seems to have host the last population.

The historical review confirmed that *M. auricularia* was once present as far as the Thames in England and Netherlands and Germany where fossil specimens have been found and studied (Araujo & Ramos, 2001). On historical times, we found museum records (recent shells) from the Rhine in France or Germany (precise location being unknown), the Seine and the Rhône in France, the Pô in Italy and the Tajo in Spain, where the species is now believed to be extirpated (Araujo & Ramos, 2001). Today, *Margaritifera auricularia* is considered restricted to five coastal drainages: from north to south the Loire drainage (two close populations in the Vienne and Creuse Rivers), the Charente drainage, the Garonne drainage (two very isolated populations, in the Dronne and Save Rivers), the Adour drainage (at least three isolated populations, one in the Adour itself, one in the Luy and one in the Arros) and the Ebro River (three populations, two in channels and a small one remaining in the Ebro itself). As has been previously estimated (Prié et al., 2014), *Margaritifera auricularia*'s range contraction has probably reached about 90% in the last two centuries.

Surveying downstream ecosystems

Large rivers are amongst the most difficult ecosystems to sample. Deepness, turbidity and water current are challenging conditions. In addition, large rivers are subject to navigation, which makes scuba-diving potentially hazardous. Nevertheless, scuba diving appears to be the most efficient way to produce data for species such as *M. auricularia*: despite malacological surveys undertaken with canoes and dredging, only a few shell fragments had been collected in the Charente River before scuba diving sampling had been set up. Scuba divers met hundreds of shells and living specimens there. Similarly, scuba-divers collected the few living specimens, that today are probably dead by now, in the main Ebro River in Spain (Araujo & Álvarez-Cobelas, 2016). In the Oise River, a few ancient shell fragments had been collected on the banks by amateur malacologists, but scuba-diving allowed finding numerous shells in most of the river stretches investigated. In the Garonne River mainstream, a malacologist spent about 20 days wading and searching for shells on the gravelled banks. In two days, a team of three divers found four shell fragments.

While bivalve surveys have been conducted in the Saône River (ex. Mouthon & Daufresne, 2006), no shell fragments had ever been collected before 2016's scuba-diving prospections. The advances in the distribution knowledge of *M. auricularia* in France and Spain are directly linked to new investigation methods and scuba diving is so far the most efficient mean of survey for this species.

Conservation and further perspectives

Main threats

While overfishing may have contributed to the species decline in the past (Bonnemère, 1901; Prié et al., 2011; Araujo & Álvarez-Cobelas, 2016), it is obviously river management and agriculture impacts that nowadays cause the most important threats to the Giant Freshwater Pearl Mussel. Both causes are linked together, at least in the southern part of the species distribution area: river management aims at providing freshwater for corn culture, especially in summer. Hence, numerous dams are built, even in small rivers, to maintain pools for pumping water in the dry season. These dams produce lotic and silty conditions unsuitable for the Giant Freshwater Pearl Mussel. Altogether, these small dams can affect about than 70% of a given rivers stretch. In the Dronne, Arros and Save Rivers in France for example, the Giant Pearl Freshwater Mussel populations survive in the form of dashed lines, only in riffles (shallow parts of streams where water flows brokenly) with gravel or stony bottoms, between long portions of lotic conditions. Moreover, these dams constitute obstacles for potential fish hosts. The presumed natural host fish of the Giant Freshwater Pearl Mussel, the European Sturgeon *Acipenser sturio*, has been extirpated from almost all European rivers mainly because of dams (Lepage & Rochard, 1995; Gesner et al., 2010). River management has been an important threat in Spain too. Water regulation and the replacement of natural bottoms with concrete have been responsible for a massive death of Naïads. Recently, there has been an unusual high mortality of adults in the Imperial Channel (pers. com. from the Diputación General de Aragón to R. Araujo), but the causes are unknown.

Although a probable cause of recruitment failure, moderate levels of pollution and eutrophication have not demonstrated to be a significant threat to adult specimens. Some population survive in highly human-impacted waters. For example, one of the highest Giant Freshwater Pearl Mussel densities spot lies just downstream the Saintes sewage system in the Charente River. The same kind of conditions occurs at the Canal Imperial in Aragón with the water coming from the Ebro River, which is highly polluted. Overall, the species survives in rivers that are highly impacted by agriculture and domestic effluents. But we still don't know how these eutrophic and polluted waters may impact juvenile survival (Augspurger et al., 2007; Strayer & Malcom, 2012; Archambault et al., 2014).

Invasive species probably add to the threats *M. auricularia* is facing. Widespread invasive species such as *Corbicula fluminea* probably affect the freshwater mussels of Europe like it has been demonstrated for other species in North America (eg. Soussa et al., 2014). However, no clear impacts have been described for *M. auricularia*, and the healthiest populations survive in rivers largely colonized by *Corbicula*. The zebra mussel

Dreissena polymorpha attaches to the valves of *M. auricularia* in the Ebro, probably affecting filtration efficiency. This phenomenon has not been observed in France, where the zebra mussel remains at low densities in the rivers of the Atlantic coast.

Habitat management

Contrarily to the Freshwater Pearl Mussel *M. margaritifera*, for which experiments of habitat managements have proved to be successful (Altmüller & Dettmer, 2006), the Giant Freshwater Pearl Mussel lives in downstream ecosystems. Attempts to implement broad scale drainage system management are therefore unrealistic. However, some realistic management objectives can be achieved to improve the habitat quality locally, in a short or middle term. The deconstruction of the numerous impoundments (many of them being disused) seems the most efficient way to restore suitable riverbed conditions for the Giant Freshwater Pearl Mussel. Although the negative impact of pollution and eutrophication are not clearly known, they are for sure not needed for the species survival. Improving water quality through reasonable agricultural practices, with buffer strips or grass strips along waterways, should be a middle-term objective.

Farming projects

Breeding farms have been established for many endangered mussel species. In Europe, there is an abundant literature dealing with *M. margaritifera* breeding farms. Some trials are also ongoing for *U. crassus* and for various *Unio* species in Spain (Araujo et al., 2015). Regarding the Giant Freshwater Pearl Mussel, attempts of ex-situ breeding have been performed in Spain (Nakamura et al., 2015), and a LIFE project is ongoing in France to artificially breed the species in controlled conditions. Juveniles have been successfully produced (Nakamura et al., 2015), but we still face obstacles in the rearing of these juveniles (although some juveniles are still alive, Nakamura com. pers.).

Genetic diversity

The very low genetic diversity for the mitochondrial genes studied was unexpected as (i) the Giant Freshwater Pearl Mussel populations are geographically isolated for a long time; and (ii) strong morphological differences are found between populations (Fig. 4). (i) The populations from France belong to the Atlantic drainage and the population from Spain to the Mediterranean drainage, two geographically isolated bioregions. Strong genetic divergences are observed for other freshwater mussel species from the Iberian Peninsula: *U. delphinus* from the "*pictorum*" lineage and *U. tumidiformis* from the "*crassus*" lineage were recently considered as distinct species based on molecular divergences (Reis & Araujo, 2009; Araujo et al., 2009b). But on the other hand, some species do not show significant genetic divergences (ex. *U. mancus*, Prié et al., 2012; *Potomida littoralis*, Araujo et al., 2016; Froufe et al., 2016). (ii) The different populations known today have obvious morphological differences in shell size and shape (Fig. 4). The specimens from the Charente River population have a peculiarly small and conspicuously ear-like shell shape, contrasting to the Vienne and Dordogne Rivers populations, which are larger and more elongated; and to the Arros and Save Rivers populations, which are remarkable with their huge sizes. Some populations live in deep coastal rivers (ex. Ebro, Vienne and Charente populations) while others seem to be confined to shallow riffle sections of the upstream rivers (ex Save and Adour populations), but these ecological traits are not linked to shell morphological differences.

Margaritiferidae are known to have very low mitochondrial DNA evolution rates (Araujo et al., 2016; Bolotov et al., 2016). Population genetics based on microsatellites allowed to differentiate evolutionary units within the related species *Margaritifera margaritifera* (Geist et al., 2010; Stoekle et al., 2016) and *M. marocana* (Soussa et al., submitted). But first studies using microsatellites based on *M. margaritifera* primers have failed to reveal any population structure in France (Prié, unpublished data). If the ex-situ breeding projects are successful, the population genetics question will become unavoidable.

The fish host issue

The known host fish of *Margaritifera auricularia* are sturgeon species *Acipenser sturio*, *A. nacari* and *A. baeri*, the River Blenny *Salaria fluviatilis* and the Eastern Mosquitofish *Gambusia holbrooki* (Araujo & Ramos, 1998b; Araujo et al., 2000; Araujo et al., 2001; Altaba & Lopez, 2001; Lopez & Altaba, 2005; Lopez et al., 2007).

The only native *Acipenser* species in the area of occurrence of *Margaritifera auricularia* is the European sturgeon *A. sturio*. This species became extirpated from most European Rivers during the 20th century. Nowadays, it is almost extinct, with last documented natural reproduction dating back to 1994 in the Garonne River. The River Blenny is a Mediterranean species whose range does not overlap with the French populations of *M. auricularia*. The Eastern Mosquitofish, an introduced species, lives in shallow and standing to slow-flowing waters. It is not usually found in places favoured by *Margaritifera auricularia*. Reported success as host fish for *M. auricularia* glochidia was questionable. Experiments with other common fish species that occur within the distribution range of *M. auricularia* (*Anguilla Anguilla*, *Barbus graellsii*, *Barbus haasi*, *Parachondrostoma toixostoma, Cobitis paludicola, Liza aurata, Mugil cephalus, Alburnus alburnus, Carassius auratus, Cyprinus carpio, Gobio gobio, Scardinus erythrophthalmus* and *Tinca tinca*) failed to produce juveniles (Araujo et al., 2001; Lopez & Altaba, 2005).

The actual knowledge on *M. auricularia* host fish cannot explain the recruitment observed recently in the Atlantic drainage rivers. We therefore suspect an overlooked host fish species. For example, *Alosa* species, which are migratory fish and still breed in the drainages were *M. auricularia* produces juveniles, are good candidates (Llorente et al., 2015). But there must be another fish host to explain reproduction in the Dronne and Charente Rivers, which are isolated from the sea by impoundments; or in very upstream populations such as those of the Arros or Aisne Rivers, were migratory fishes do not breed. The other hypothesis could be that reproduction occurred in France periodically taking advantage of accidental releases of *A. baeri*, a common species in French fish farms (but not in Spain). We have recently succeeded in completing the full cycle on the three-spined stickleback *Gasterosteus aculeatus* in controlled conditions (Soler et al., in prep.). As this species is widespread within the range of *M. auricularia* and tolerant to brackish waters, it could also be a good candidate as a natural fish host. To find the natural host fish species of *M. auricularia* in France is now vital for the survival and conservation of this freshwater bivalve.

Conclusion

Margaritifera auricularia has become very rare in the last century, with an estimated range contraction of 90%. Only three populations were known worldwide before 2007. Intensive surveys in the last decade allowed re-discovering nine more. Given the magnitude of the efforts allocated to surveying the species in its historical range, we now believe that there are very little chances to rediscover unnoticed populations (excepted maybe in north-east France).

Although some juveniles were found recently, they remain very scarce and most extant populations seem to live on a borrowed time. Within the time lapse of this study, some populations already became extirpated in the Ebro and Adour Rivers. The status of the species therefore remains worrying. Priority populations for conservation are the Charente River’s population, because it is by far the largest worldwide; the Vienne and Creuse population, because it has the higher level of natural recruitment; the Adour drainage populations, because they form an important and unique metapopulation; and the Ebro population because it is now the only remaining one in the Mediterranean drainage. Conservation challenges for the next years are (i) an appropriate management of the rivers which host the priority populations; (ii) the development of farming projects, in order to reinforce existing populations; (iii) research on fish hosts, for a better comprehension of the species’ threats; ecological requirements, to understand which are the habitat factors driving the species’ recruitment success; population genetics to plan conservation efforts according to the genetic diversity of the remaining populations; (iv) a wide scale development of modern survey methods such as scuba diving and environmental DNA in order to discover the potentially remaining unnoticed populations.

Despite these efforts, we may fail to save the Giant Freshwater Pearl Mussel from extinction. However, current researches help to shed light on the obscure river downstream ecosystems' ecological functions and threats, as well as to develop exploring methods for this challenging environment.

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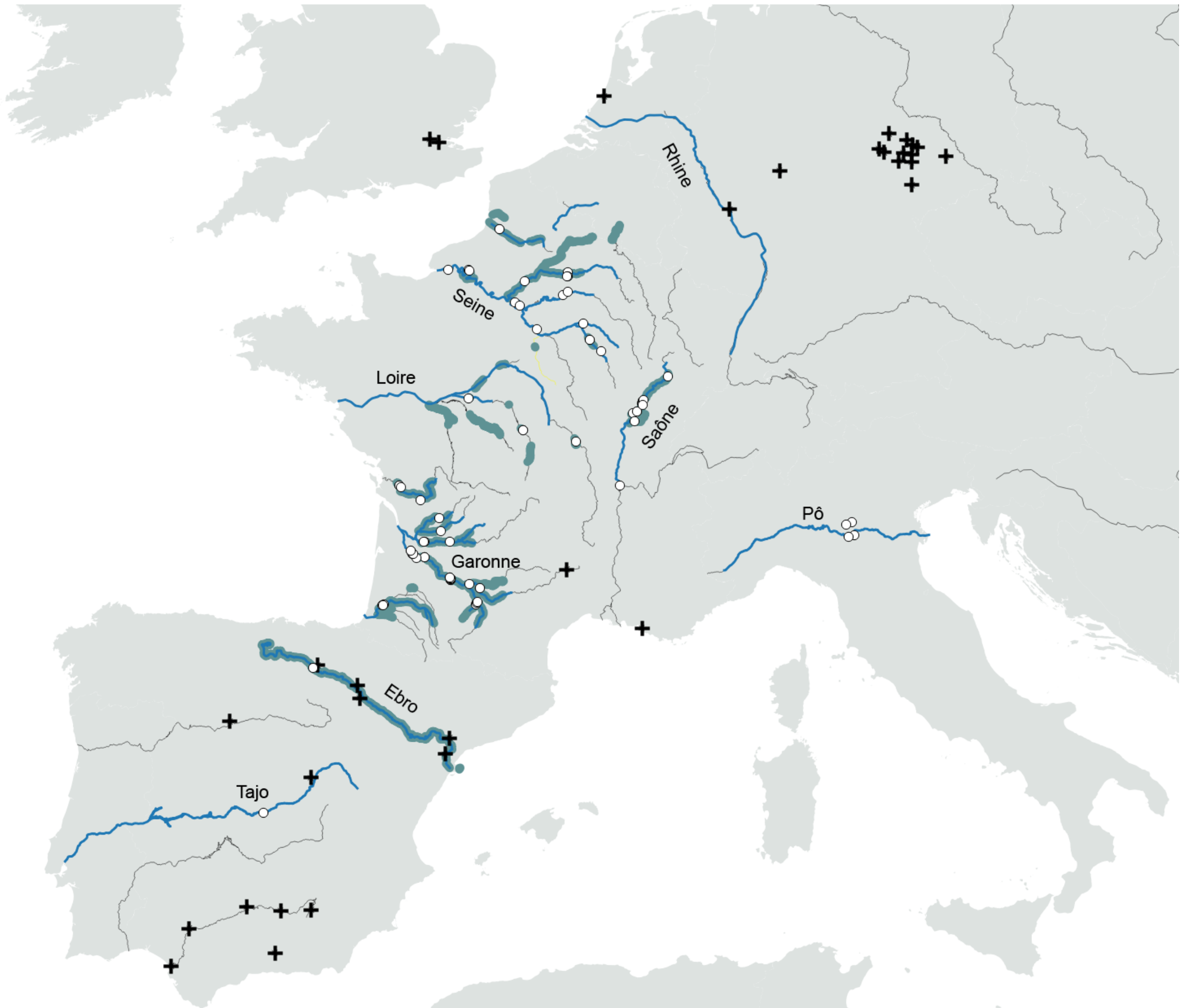

Figure 1: fossil (black crosses) and historical data (white dots for precise locations, blue lines for rivers names only) collected from the literature and Museum collections; and subsequent intensive field surveys locations (polygons).

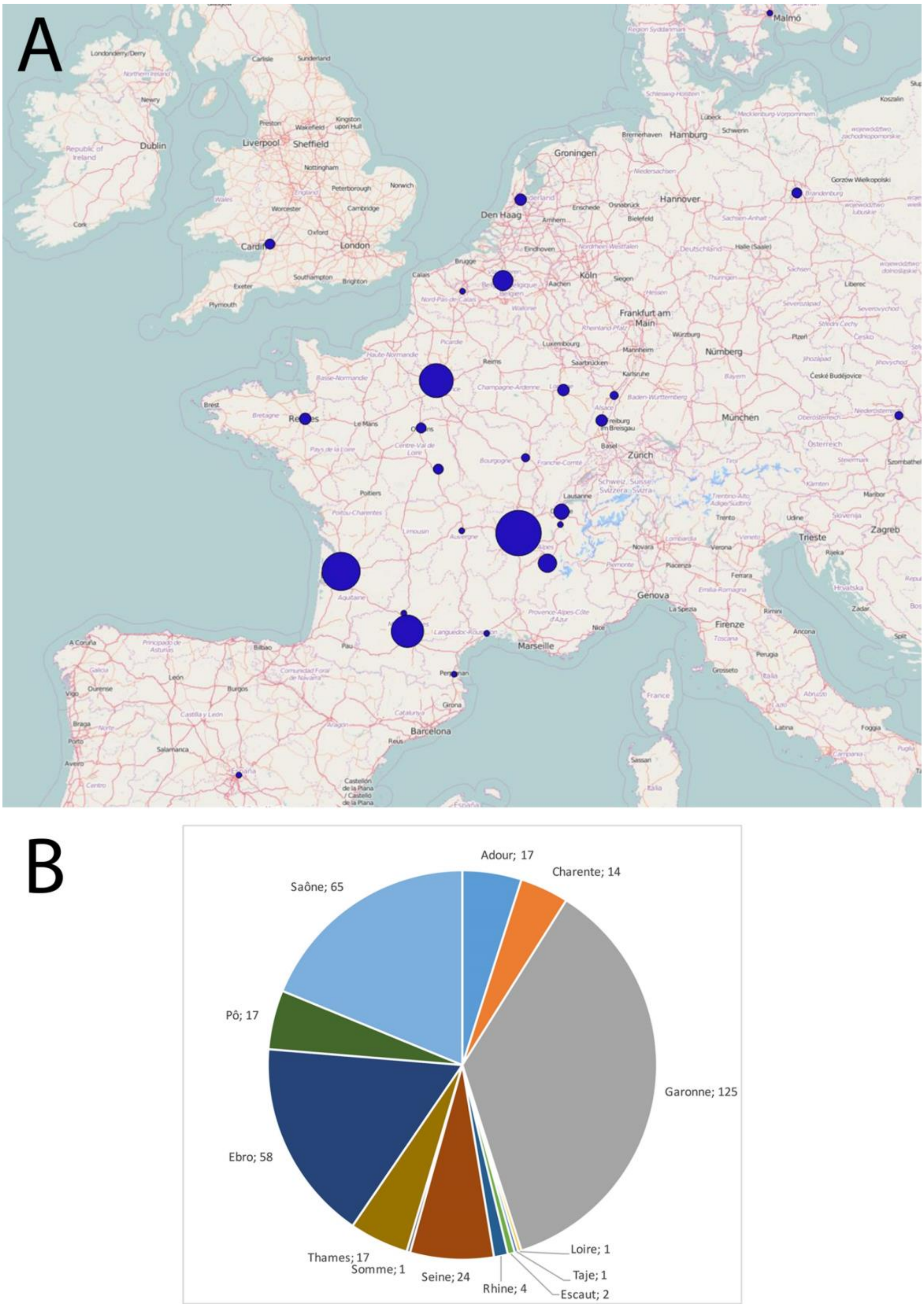

Figure 2: A: location of main museum collection investigated (dot size according to number of *M. auricularia* specimens). B: number of specimens held in Museum collections per main coastal drainages (the Saône River is actually a tributary of the Rhône, but all the specimens are located in the Saône and none elsewhere in the Rhône).

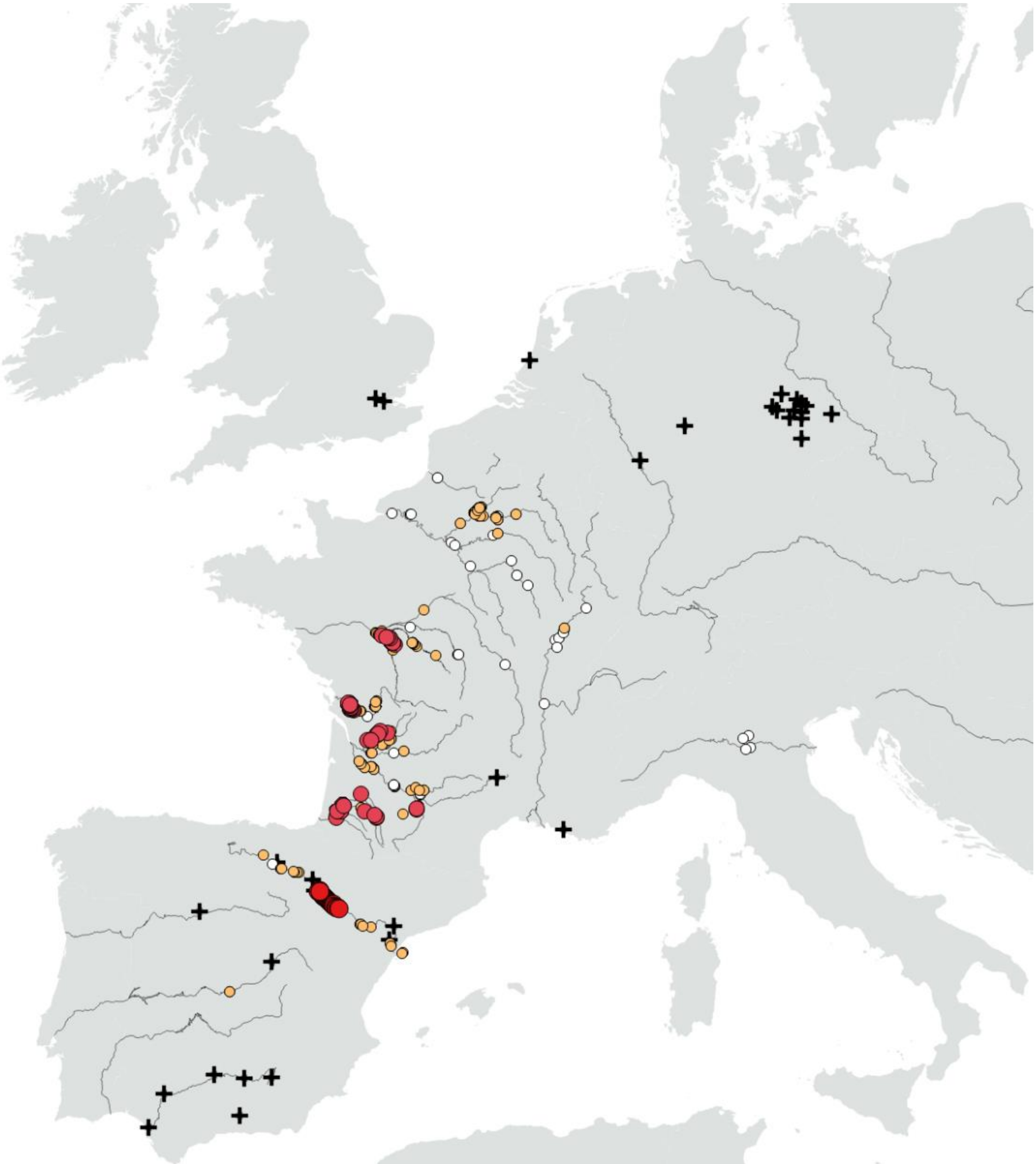

Figure 3: results of last ten years' field surveys and known past and actual distribution of *M. auricularia*. Fossil data (black crosses), historical data (white dots), shells collected in the last 10 years (orange dots) and still living populations (red dots).

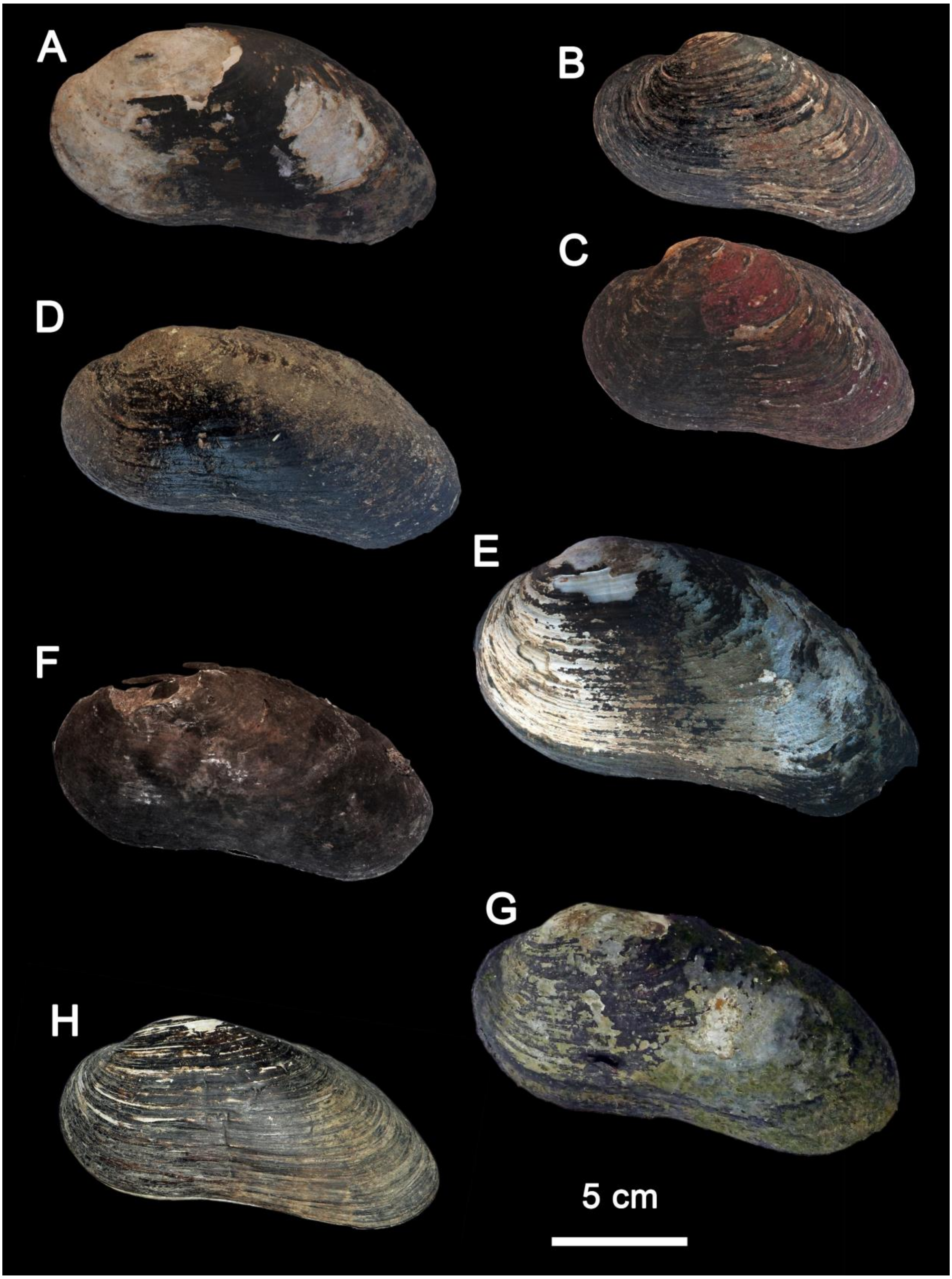

Figure 4: morphological variability of *Margaritifera auricularia*. A: Vienne River; B-C: Charente River; D: Dronne River; E: Save River; F: Luy River; G: Arros River, H: Ebro River.

Table 1: summary of the methods used in France for surveying M. auricularia. For Spain, survey methods were detailed and reported in Gómez & Araujo, 2008 and Araujo & Álvarez-Cobelas, 2016.

Coastal drainage	River	Year	Use of a boat	Scuba-diving	Other methods	Authors involved
Somme	Somme River	2011	X	4 divers, 5 days		XC, VP
	Seine River (downstream)	2011	X		Dredging (2 persons for 4 days)	XC
	Seine River (upstream)	2015			Snorkeling (2 persons for one days)	XC
	Oise River	2007-2008	X	3 divers, 20 days		VP, LP, XC
Seine	Aisne River	2011		2-4 divers, 5 days		XC, VP
Rhône	Saône and Doubs Rivers	2016		4 divers, 5 days	Wading with viewing glasses (2 persons, 5 days)	VP, LP, NL, NP, BA
	Loire, Indre and Cher Rivers	2010-2011		2-3 divers, about 5 days	Wading with viewing glasses (2-4 persons, estimated to about 10 days altogether)	LP, VP
Loire	Vienne and Creuse Rivers	2009-2016		2 to 6 divers, over 20 days altogether	Wading with viewing glasses and snorkeling (2 to 6 persons, estimated to over 30 days altogether)	VP, XC, LP
Charente	Charente River	2007-2016	X	2 to 3 divers, about 20 days altogether		VP
	Dronne and Isle Rivers	2012-2014		2 divers, about 10 days altogether	Wading with viewing glasses (1-2 persons, about 5-10 days altogether)	VP
	Dordogne River	2016	X	2 divers, 3 days altogether	Wading with viewing glasses (1 person, about 5 days)	VP
	Vézère River	2016		3 divers, 3 days	Wading with viewing glasses and snorkeling (2 persons 3 days)	VP, LP, NP, BA, NL
	Garonne River	2016		3 divers, 2 days	Wading with viewing glass (1 person, 30 days)	VP, NL
Garonne	Save River	2009		2 divers, 1 day	Wading with viewing glass and snorkeling (1 person, 5 days)	VP, BA
	Adour River	2012-2014		2 divers approx. 9 days	Wading with viewing glasses and snorkeling (2 to 6 persons, 10 days)	VP, BA, NL
Adour	Arros River	2016		3 divers, 3 days	Wading with viewing glasses (6 persons, 4 days)	VP, BA, NL

Table 2: genes and Genbank accession numbers of French specimens used for DNA analyses.

Coastal drainage	**River**	**Specimen voucher number**	**Genbank accession number**		
			COI	**16S**	**28S**
Charente	Charente	MNHN-IM-2009-12596	MF494673	MF494681	MF494677
		MNHN-IM-2009-12597	MF494674	MF494682	MF494678
Garonne	Save	MNHN-IM-2009-12601	MF494675	MF494683	MF494679
Adour	Luy	MNHN-IM-2009-12662	MF494671	MF494696	MF494676
		MNHN-IM-2009-12663	MF494672	MF494697	
Loire	Vienne	Maur91	MF494670	MF494695	MF494680
		MNHN-IM-2009-12611	MF494661	MF494684	
		MNHN-IM-2009-12615	MF494662	MF494685	
		Maur70	MF494663	MF494686	
		Maur72	MF494664	MF494687	
		Maur74	MF494665	MF494688	
		Maur76	MF494666	MF494689	
		Maur77	MF494667	MF494690	
		Maur78	MF494668	MF494691	
		Maur85	MF494669	MF494693	
		Maur79		MF494692	
		Maur88		MF494694	

Table 3: summary of literature data, museum collections (fossil data are not considered) and field surveys.

Country	Coastal drainage		Literature	Museum collections (number of specimens)	Dedicated field surveys (references with an * refer to grey literature, summarized in Online Ressource 1)	Recent surveys results	Estimated population size
France	Charente	Charente	X	9	Prié et al., 2007*; Prié, 2010; Prié & Mouton, 2016*	Live specimens and juveniles	100.000
	Garonne	Garonne (mainstream)	X	125	Prié et al., 2016*	Ancien shells	
		Isle	X	0	Prié, 2012*	Recent shells	
		Dronne	X	0	Prié, 2012*; Prié, 2013*	Live specimens and juveniles	> 100
		Save		0	Prié, 2012*	Few live specimens, declining population	< 30
	Adour	Adour (mainstream)	X	9	Prié, 2012*	Live specimens and juveniles	> 300
		Arros		6	Prié & Néri, 2016*	Live specimens and subadult	200
		Luy		0	Prié, 2012*	Live specimens and subadults	150
	Loire	Loire (mainstream)	X	1		Recent shells	
		Vienne		0	Cochet, 2006*; Philippe et al., 2009*; Philippe et al., 2010*, 2011*, 2012*	Live specimens and juveniles	>100
		Indre		0	Dohogne, 2008*; Philippe et al., 2009*	Recent shells	
		Creuse		0	Philippe et al., 2012*; Philippe et al., 2013*, 2014*, 2015*, 2016*	Live specimens and juveniles	>150
		Cher		1	Prié et al., 2011*; Prié et al., 2016*	Nothing	
	Seine	Seine (mainstream)	X	12	Cucherat et al., 2011*	Ancien shells	
		Oise		0	Prié et al., 2007*	Recent shells	
		Aube	X	1	Cucherat et al., 2011*	Ancien shells	

		Aisne	X	6	Philippe et al., 2009*; Cucherat et al., 2011*	Ancien shells	
	Escaut	Escaut	X	2		No survey	
	Somme	Somme	X	1	Cucherat & Prié, 2011*	Nothing	
	Rhône	Saône	X	65	Prié et al., 2016*	Ancien shells	
Italy	Po	Po	X	15		No survey	
Spain	Ebro	Upper Ebro	X		Araujo et al., 2009b*; Araujo & Álvarez-Cobelas, 2016 ; pers. comm. from R. Álvarez-Halcón to R. Araujo	Live specimens and juveniles (at Gallur)	1
		Ribera alta			Nakamura & Guerrero, 2008 ; Araujo & Álvarez-Cobelas, 2016	Used to be 38-40 live specimens, today likely extirpated	
		Canal Imperial de Aragon	X	55	Gomez & Araujo, 2008; Araujo et al., 2009; Araujo & Alvarez-Corbela, 2016 ; pers. comm. from J. Guerrero to R. Araujo	Live specimens and juveniles	4.000
		Canal de Tauste	X		Araujo et al., 2009b*; pers. comm. from J. Guerrero to R. Araujo	Live specimens and juveniles	200
		Quinto ditch			Gomez & Araujo, 2008; Nakamura et al., 2017	Recent shells	
		Lower Ebro	X		Araujo et al., 2009b*; Araujo & Alvarez-Corbela, 2016	Used to be 70 live specimens, today extirpated	
	Tajo	Tajo	X	1	Villasante et al., 2016	Nothing	
Fossil data							
U.K.	Thames	Thames		17		No survey	
Germany	Rhine	Rhine	X	3		No survey	
Netherland	Rhine	Rhine	X			No survey	

Online resource 1: grey literature of the last decade. References of the various reports and impact studies aiming specifically at *M. auricularia*.

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