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Bad taxonomy can kill: Molecular re-evaluation of *Unio mancus* Lamarck, 1819 (Bivalvia: Unionidae) and its accepted subspecies

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Abstract

The conservation status of European unionid species rests on the scientific knowledge of the 1980s, before the current revival of taxonomic reappraisals based on molecular characters. The taxonomic status of *Unio mancus* Lamarck, 1819, superficially similar to *Unio pictorum* (Linnaeus, 1758) and often synonymized with it, is re-evaluated based on a random sample of major French drainages and a systematic sample of historical type localities. We confirm the validity of *U. mancus* as a distinct species occurring in France and Spain, where it is structured into three geographical units here ranked as subspecies: *U. m. mancus* [Atlantic drainages, eastern Pyrenees, Spanish Mediterranean drainages], *U. m. turtonii* Payraudeau, 1826 [coastal drainages East of the Rhône and Corsica] and *U. m. requienii* Dupuy, 1843 [Seine, Saône-Rhône, and coastal drainages West of the Rhône]. Many populations of *Unio mancus* have been extirpated during the 20th century and the remaining populations continue to be under pressure; *U. mancus* satisfies the criteria to be listed as "Endangered" in the IUCN Red List.

Les risques d’une mauvaise taxonomie : ré-évaluation moléculaire d’*Unio mancus* Lamarck, 1819 (Bivalvia : Unionidae) et de ses sous-espèces.

Résumé

Le statut de conservation des espèces d’unionidés européennes repose sur les connaissances scientifiques des années 1980, avant le renouveau des ré-évaluations taxonomiques basées sur des caractères moléculaires. Le statut taxonomique de la Mulette méridionale *Unio mancus* Lamarck, 1819, superficiellement semblable à la Mulette des peintres *Unio pictorum* (Linnaeus, 1758) et souvent mise en synonymie avec elle, est ici ré-évalué sur la base d’un échantillonnage à l’aveugle des grands bassins versants français et d’un échantillonnage ciblé des localités-types historiques. Nous confirmons la validité d’*Unio mancus* comme espèce distincte, présente en France et en Espagne, où elle est structurée en trois unités géographiques ici traitées comme sous-espèces : *U. m. mancus* [bassins versants atlantiques, est des Pyrénées, bassins versants méditerranéens espagnols] *U. m. turtonii* Payraudeau, 1826 [bassins versants des fleuves côtiers à l’est du Rhône et de Corse] et *U. m. requienii* Dupuy, 1843 [Seine, Saône-Rhône, et fleuves côtiers à l’est du Rhône]. De nombreuses populations d'*Unio mancus* ont été éradiquées dans le courant du 20ème siècle et beaucoup de populations restantes restent menacées ; l'espèce répond aux critères "En Danger" pour la Liste Rouge de l'UICN.

Introduction

Freshwater mussels combine several life history traits (longevity, filter feeding, larvae parasitic on the gills of fishes) that make them particularly vulnerable to loss of habitat quality. Unionidae have the dubious privilege of being among the most threatened freshwater invertebrates (Lydeard *et al*. 2004), with 20% of North American species already listed as Extinct or Possibly Extinct (Master *et al*. 2000), and a number of European species protected under national or European regulations. Because legislative texts protect species through names, it is important that names reflect appropriate evolutionary and/or conservation units. However, the taxonomy of European unionids is still unstabilized, especially in southern Europe, and the current listings on legislative texts - which reflect the scientific knowledge of the 1980s - have not kept pace with advances in taxonomical knowledge.

The taxonomy of European unionids is plagued with a plethora of names. Based on a typological approach to within- and between-population phenotypic diversity, hundreds of nominal species have been described, especially as a result of the excesses of the 19th century French “Nouvelle École” (see Bouchet 2002). There is an important body of classical and modern literature that documents shell plasticity in unionoids (e.g., Ortmann 1920; Agrell 1948; Nagel 1992; McMurray *et al.* 1999; Watters 1994; Zieritz and Aldridge 2009; Zieritz *et al*. 2010) and, based on phenotypic resemblances, these 19th century names have ended up in synonymy (see, among others, Haas 1969). Molecular techniques are now allowing a fresh evaluation of these nominal species, confirming in many cases

morphology-based synonymies (Araujo *et al*. 2005; Araujo *et al*. 2009b), but sometimes leading to the revalidation of some abusively synonymized species (Araujo *et al.* 2009a, Araujo *et al*. 2009b; Reis and Araujo 2009; Khalloufi *et al*. 2011). Conservation programs now largely advocate the use of genetic approaches to evaluate the validity of the evolutionary and/or conservation units being managed (Schonewald-Cox *et al*. 1983; Machordom *et al.* 2003; Geist and Kuehn 2005).

"Bad taxonomy can kill" (May 1990, Mace 2004). Given the subjective basis of the taxonomic validity of many nominal species, it is not impossible that important conservation units remain unrecognized or buried in the "graveyard of synonymy". Clarifying the taxonomic status of potentially endangered taxa is thus a major concern. Among the unionoids of French hydrographic basins, the taxonomic status of *Unio mancus* Lamarck, 1819 has been controversial in the recent literature. Based on shell characters, Haas (1969) considered *U. mancus* as one of the subspecies of *U. elongatulus* C. Pfeiffer, 1825 (despite the nomenclatural priority of *mancus* over *elongatulus*). Nagel and Badino (2001), based on enzymatic studies, treated *U. mancus* as a subspecies of *U. pictorum* (Linnaeus 1758). In the taxonomic authority list of French non-marine molluscs, Falkner *et al*. (2002) listed *U. mancus* as a valid species with seven subspecies, largely inspired from Haas' (1969) subspecies of *U. elongatulus*: *U. m. mancus* [Seine, Loire, Charente basins, coastal rivers of Normandy], *U. m. aleronii* Companyo and Massot, 1845 [eastern Pyrenees and Garonne basin], *U. m. bourgeticus* Bourguignat *in* Locard, 1882 [Saône and Ain drainages, Lake

Bourget], *U. m. brindosianus* de Folin and Berillon, 1874 [coastal lakes of the Landes], *U. m. moquinianus* Dupuy, 1843 [piedmont of central and western Pyrenees], *U. m. requienii* Michaud, 1831 [Rhone basin downstream of Lyon], and *U. m. turtonii* Payraudeau, 1826 [Corsica], suggesting that their geographical segregation implied underlying genetic isolation. The status of *U. mancus* as a valid species was confirmed by Araujo *et al.* (2005) based on molecular and ecological data on Spanish populations. Except for *U. aleronii* (synonymized with *U. mancus* by Araujo *et al.* 2009c), the status of the subspecies recognized by Falkner *et al*. (2002) has not been further evaluated.

Because of the considerable phenotypic variation of the shell in unionids, the application of names beyond their type locality or, at least, beyond the hydrographic basin of their type locality, is problematic. In consequence, and through COI sequences obtained from topotypical populations, the purpose of the present study is (1) to re-evaluate the taxonomic status of *Unio mancus* and compare it specifically with *U. pictorum*, and (2) to evaluate the taxonomic status of the different nominal subspecies of *U. mancus*.

Material and methods

Material studied

The main drainage systems of France (Rhine River excepted) were sampled, with special consideration for the type localities of the accepted subspecies (Figure 1, Table I). Sampling was successful for type localities of *Unio m. mancus* Lamarck, 1819, *Unio m. aleronii* Companyo and Massot, 1845, *U. m. bourgeticus* Bourguignat, 1882, *U. m. brindosianus* de Folin and Bérillon, 1874, *U. m. requienii* Michaud, 1831 and *U. m. turtonii* Payraudeau, 1826. *U. m. moquinianus* Dupuy, 1843, seems to have been extirpated from its type locality and we sampled nearby localities belonging to the same drainage. In addition, specimens from Lake Maggiore in Italy were included in the dataset. A total of 151 specimens were sampled in 38 localities (Figure 1, Table I).

DNA analysis

A piece of foot was clipped from living specimens and preserved in 90% ethanol. Soft tissues and shells are kept in the Muséum national d'Histoire naturelle in Paris (see Table I for voucher numbers). DNA was extracted from these samples using the Nucleospin Tissue Kit (marketed by Macherey Nagel), following the manufacturer's protocol. A fragment of the cytochrome oxidase subunit I (COI) gene was amplified by PCR using the universal primers LCO1490 and HCO2198 (Folmer *et al*. 1994) or our own designated primers (CO1UnioF: TGTTAGCTTTGTGATCTGGGTTAATTGG; CO1UnioR: AAATTGGATCACCTCCCCCAGTAGGG). The PCR mix contained 20 ng of DNA, 1U of Fast Start Taq (commercialized by Roche), 6 pmol of dNTP mix,

37.5 pmol of $MgCl_2$, 1 pmol of each primer and H_2O was added for a final volume of 50 ml. PCR started with an initial denaturation cycle at 95 °C for 10 min, followed by 50 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were purified using Nucleofast Plates (commercialized by Macherey Nagel) and sequenced in a 3730XL genetic analyser (Applied Biosystems). Extractions, amplification and sequencing were performed by Genoscreen (France) and Eurofins (Germany).

Fifteen sequences from Araujo *et al.* (2005) (for *U. mancus*) and from Soroka (2010) (for *U. pictorum*) were downloaded from GenBank and included in the analyses (see Table I for GenBank accession numbers). These were selected because the sequences are linked to voucher specimens.

The 154 sequences obtained in this study and the 15 sequences downloaded from GenBank were aligned using ClustalW multiple alignment implemented in Bio-Edit (Hall, 1999). MEGA 5 (Tamura *et al*. 2011) was used to calculate the p distances between each pair of specimen. The GTR + I + G model was identified as the best-fitting substitution model by JModeltest 0.1.1 (Posada, 2008) following the Akaike criterion. This model has been used for the Bayesian analysis, performed running two parallel analyses in MrBayes (Huelsenbeck *et al.* 2001), consisting each of two Markov chains of 15,000,000 generations each, sampled every 5,000 generations. A consensus tree was then calculated after omitting the first 25% trees as burn-in.

The Bayesian analyses were run on the 26 different haplotypes obtained from the 169 sequences involved in this study. *Unio tumidus* Philipsson, 1788 and *Unio*

crassus Philipsson, 1788 were chosen as outgroups, as they are considered to be closely related to *U. mancus* and *U. pictorum* (Nagel and Badino 2001). *Margaritifera auricularia* (Spengler, 1793) was chosen as distant outgroup to assess the monophyly of *U. mancus* and *U. pictorum*.

Results

On the phylogenetic tree (Figure 2), three main clades were defined. Sequences from GenBank identified as *Unio mancus* by Araujo *et al.* (2005) clustered in clade 1 together with our own material of *U. mancus* and its subspecies. Specimens from the Italian lake cluster in clade 2. Sequences of specimens identified as *U. pictorum* by Soroka (2010) all clustered in clade 3 together with our own material of *U. pictorum*. Average divergence within the *U. pictorum* clade was 0.3 % (max: 1 %), average divergence within the *U. mancus* clade was 0.5 % (max 1.3 %), minimum distance between *U. mancus* and *U. pictorum* was 2.9 %. *U. mancus* and *U. pictorum* were found monophyletic.

U. pictorum has been found mainly in northern France (Seine and Meuse basins), with isolated populations in Lake Bourget, Lake Brindos and the Garonne basin, suggesting a broader distribution but scattered occurrences (Figure 3, Table I). *U. mancus* is found in the Mediterranean drainages of France and eastern Spain to the Seine basin in the north. Beyond this presumably natural distribution, *Unio mancus* occurs in the Rhine, and Glöer and Zettler (2005) have suggested that this is the result of an expansion through the Rhine-Rhône canal (established in 1833), where it was not reported before 1911 (as *Unio voltzii* Kobelt, 1911; see Haas 1969). *U. mancus* has not been found in the Meuse River.

Specimens from Lake Maggiore in Italy have been referred to "*U. pictorum mancus*" by Ravera *et al*. (2007), while Gavetti *et al*. (2008) identify them as *U. mancus*. The specimens from Lake Maggiore form a distinct lineage from *U.*

mancus (Figure 2, clade 2), which is identifiable as *Unio elongatulus*, after Bodon *et al*. (1995) and the Italian fauna Checklist (www.faunaitalia.it).

Within the *U. mancus* clade, populations were divided in three geographic groups (Figure 3): Group 1a included the populations from Atlantic drainages (Loire, Garonne), plus the Mediterranean populations from Spain and from the Basse River near Perpignan (posterior probabilities = 1); group 1b included the populations from Corsica and South-East France, i.e. drainage systems East of the Rhône (posterior probabilities = 0.86); group 1c the populations from the Rhône, Hérault and Seine drainages (posterior probabilities = 0.45). These three evolutionary units are characterized by several diagnostic characters: clade 1a differs from all other by three fixed apomorphies (bp 448: T instead of C; bp 478: A instead of G; bp 518: G instead of A), clade 1b by one apomorphic character (bp 169: A instead of G) and clade 1c by one apomorphy (bp 346: G instead of A). On the three specimens from Lake Bourget attributable to *U. mancus*, one constitutes a distinct haplotype with only one different character (bp 455: G instead of T). *U. m. turtonii* is not distinct from continental populations of South-East France (Argens). *U. m. moquinianus* and *U. m. aleronii* cluster in the same group as the nominate *U. m. mancus* (Loire), and their haplotypes are shared by most of the specimens sampled (none of them constitute a distinct clade). Specimens from the type locality of *U. m. brindosianus* were found to belong to the *U. pictorum* clade, as well as two specimens from Lake Bourget that did not differ in shell shape from *U. mancus* from the same locality (Figure 4I-J).

Discussion

Validity of *Unio mancus*

Haas (1969) suspected the possible existence of hybrids between *U. mancus* and *U. pictorum* in the Seine and Marne Rivers. Nagel and Badino (2001) considered them conspecific. Our results support the distinction between *U. mancus* and *U. pictorum*, the former currently present in the Atlantic drainages from the Pyrenees to the small drainages of Brittany, in the Seine basin in upstream Aisne and Marne Rivers, and in Mediterranean drainages, Corsica included. Furthermore, these populations are conspecific with *U. mancus* as identified by Araujo *et al.* (2005) in Spain. The species level status of *mancus* is further supported by the syntopic co-occurrence of *U. mancus* and *U. pictorum* in Lake Bourget and in the Cher, Ourcq, Seine and Aisne Rivers (Figure 3, Table I).

Within *Unio mancus*, the French populations cluster in three COI sequences groups (1a, 1b and 1c) that do not correspond to the subspecies delimitations proposed by Haas (1969) and retained by Falkner *et al.* (2002). However, although sampling topotypes is in principle the most reliable approach to evaluate the status of a nominal (sub)species, it is not certain that material collected in 2010-2011 belongs to the same gene pool as the specimens occupying the type localities in the mid-19th century, when the nominal species were described. Aquatic ecosystems have been strongly impacted by human activities over the last century. Water quality has been globally declining, sediment quality has been modified by dams and channelization (Sabater 2008, Sabater and Tockner 2009), and these changes may have locally led to extirpation of populations. Previously

isolated drainage systems are now connected by canals and through fish translocation, which induce translocation of associated bivalves through the larval parasitic stage (Nagel 2000; Gherardi *et al.* 2008; Cappelletti *et al.* 2009), resulting in new gene pools being introduced to old localities. Because of the potential colonization and introgression, the geographical genetic structuring observed in the 21st century may thus be different from that in the 19th century when names were established. Sampling of specimens at the type locality is thus not sufficient to guarantee that the specimens are genuinely representative of nominal (sub)species originally described from that locality. To avoid a wrong application of the name to supposedly topotypical specimens, it is thus essential to compare them morphologically to the historical type specimens (when available).

With this caveat in mind, it is not entirely certain that present-day populations in Lake Bourget (type locality of *U. m. bourgeticus*) represent the same gene pool as in the 1880s. The specimens sampled (Figure 4G-J) have uniformly small, brownish and rounded shells that do seem similar to the type specimens (MNHN 22139, Figure 4K), but the latter are more kidney-shaped. Furthermore, whereas based on COI sequences evidence both *U. pictorum* and *U. mancus* are now present in the lake, we found no morphological differences between them. If this situation existed already in the 19th century, then the allocation of the type material of *Unio bourgeticus* to either *U. mancus* or *U. pictorum* is problematical.

Likewise, the specimens sampled in Lake Brindos (type locality of *U. m. brindosianus*) were all attributable to *U. pictorum* according to COI sequences, but it is not clear whether they correspond genuinely to what was historically

described as *U. brindosianus* (which would then be a synonym of *U. pictorum*), or whether *U. mancus* has been extirpated from that locality and replaced by the ecologically more tolerant *U. pictorum*.

Evaluation of the morphological variability

Important morphological variations exist in shell size, shape and colour of *U. mancus* (Figure 4, more material is illustrated in Prié 2012).

Specimens from the Loire (Figure 4B) and Seine drainages (Figure 4C), including the nomenclatural lectotype designed by Falkner (1994) (Figure 4A), have small-sized, elongated, and rather yellowish shells; by contrast, specimens from Brittany have larger and much darker shells (Figure 4D). The population from the Basse River (type locality of *U. m. aleronii*, Figure 4E) is characterized by small, ovate, rather greenish shells. Their COI sequences are similar to those from Atlantic drainages and to the morphologically very different specimens from Spain (Araujo *et al*. 2005; Araujo *et al*. 2009c). The sampled population from the Adour drainage (Pyrenees) has small and rounded shells (Figure 4F), with a very dark color, that corresponds to the original description of *U. m. moquinianus* and its taxonomical extension in Haas (1969). Their COI sequences belong to the same clade as populations from the Loire and Charente Rivers, which have very different shell shape and color.

Specimens from Lake Bourget (Figure 4G-J) are morphologically homogeneous, with shells resembling those from the Aisne River, but their COI sequences correspond to two species, *U. pictorum* and *U. mancus*. However, none of the

specimens sampled matches perfectly the kidney-shape and dark shells of 19th century historical type specimens (Figure 4K).

Populations from Corsican and Mediterranean drainages have large, often kidney-shaped shells with a brownish colour (Figure 4L-O). They belong to two distinct clades, one of them, despite different shell shapes, including the Lake Bourget and Seine basin populations. Material from Corsica matches the original description of *U. m. turtonii*, but this description is vague enough to correspond to any *U. mancus* population from southern France. Characters such as the thickness of the shells are most probably linked to water quality: Corsican populations of *U. mancus* live on an acid substrate whereas the mainland populations sampled live mainly on limestone.

Incongruence of morphology with DNA sequences data has been noted in previous studies (Nagel 1992; McMurray *et al.* 1999; Zieritz *et al*. 2010). Because the historically restricted-range *U. m. aleronii*, *U. m. bourgeticus* and *U. m. moquinianus* cannot be separated by COI sequences, there is no support to regard them as separate taxa despite their morphological singularities.

<u>Structuring within *U. mancus*</u>

Despite the weak posterior probabilities, the genetic structure within *U. mancus* is biogeographically cohesive and coherent, supporting the recognition of three different evolutionary/conservation units corresponding to the three groups 1a, 1b and 1c (Figure 3).

Group 1a includes the populations from Atlantic drainages, from the Pyrenees to Brittany, including the Loire basin, as well as the population from the Basse River

in Perpignan. Outside France, it includes Spanish Mediterranean drainages as far South as the Jucar River basin. This clade corresponds to the subspecies *Unio m. mancus*, *Unio m. aleronii*, *U. m. moquinianus* as recognized by Falkner *et al.* (2002), and should thus be known as the nominotypical subspecies *Unio m. mancus*, with the other names as synonyms. The genetic similarity between populations from the Loire and the Adour-Garonne systems was not expected as the Adour and Garonne drainages have to this day remained unconnected by canals (Persat and Keith 2011). This distribution matches that of *Leusiscus burdigalensis* Valenciennes, 1844 (with the taxonomic extension of Keith *et al*., 2011). Interestingly, the genus *Leusiscus* Cuvier, 1816 is closely related to *Squalius* Bonaparte, 1837, which includes two species known as efficient host fishes for *U. mancus* (Araujo 2005), suggesting that *L. burdigalensis* should be tested as a larval host for *Unio mancus*. The co-clustering of populations from Spanish Mediterranean drainages and French Atlantic drainages evidences that, counter-intuitively, the Pyrenees are not a significant biogeographic barrier. Interestingly, fishes like the Iberian gudgeon *Gobio lozanoi* Doadrio and Madeira, 2004 or the Adour minnow *Phoxinus bigerri* Kottelat, 2007 are also distributed in both the Ebro basin in Spain and the Adour basin in the French Atlantic drainage (Kottelat and Freyhof 2007, Keith *et al.* 2001). The distribution of *Squalius laietanus* Doadrio, Kottelat and de Sostoa, 2007, including the Ebro basin and the southernmost French Mediterranean Rivers (Kottelat and Freyhof 2007, Keith *et al.* 2001), parallels that of clade 1a in the Basse River. Such distributions may

have their origin in the connections between French and Spanish hydrographic systems during periods of low sea levels (Persat and Keith, 2011).

The Rhône, Vidourle and Hérault rivers have been connected in the past when the sea level was lower, which can explain the similarity of fauna in the rivers draining to the Golfe du Lion (Persat and Keith, 2011). This is congruent with the COI sequences results in our analysis. By contrast, an unexpected result of our study was the allocation of specimens from the Seine drainage system to clade 1c. This was unexpected as the fauna of the Seine is in general more similar to that of the Loire due to (i) connections during geological time until the early Pleistocene, and (ii) historically (year 1642), the opening of the canal de Briare connecting the Loire and the Loing (a tributary of the Seine). The artificial connection between the Rhône and the Seine systems was established later in 1832 (through Canal de Bourgogne between Ouch and Armacon Rivers) and 1907 (canal between the Saone and Marne Rivers). We hypothesize that the occurrence of *U. mancus* in the Seine is the result of a modern colonization event, either *via* canals or fish translocation.

Falkner *et al.* (2002) restricted the name *U. m. turtonii* to Corsican populations and questioned the identity of populations for continental rivers East of the Rhône. The clustering of COI sequences of specimens from South-East France and Corsica in group 1b is congruent with Germain's (1931) opinion, and the name *U. m. turtonii* is applicable to it.

Clade 1c corresponds to the subspecies *U. m. requienii* as recognized by Falkner *et al*. (2002), but with a distribution here extended to the Hérault River and to the

Seine system. (The identity of *U. m. bourgeticus*, from the Rhône drainage, remains in doubt as collected material seems different from type material, but it is probably a synonym of *U. m. requienii*).

Conservation issues

When the appendices to the Habitats and Species Directive (92/43/EEC) were put together back in the early 1990s (see Bouchet *et al.* 1999 for a history of how the list was compiled), the prevailing scientific opinion was that *Unio pictorum* was a polytypic species, with a broad range extending from Portugal to Estonia, and was not endangered. The then poorly known *U. mancus* was not explicitly considered, and was probably implicitly considered to be part of the variation of *U. pictorum* (e. g. in the Habitats Directive) or *U. elongatulus* (e. g. in the Bern Convention). The recognition of *U. mancus* as a distinct species, endemic to a few drainages in France and Spain, has of course radical consequences in a conservation perspective (Morrison *et al*. 2009).

Extensive sampling in the main drainages in the course of the present study resulted in the discovery of healthy populations of *Unio mancus*, particularly in Atlantic drainages, as well as healthy populations of *U. crassus*, a species considered endangered, protected in France and listed in the Habitats Directive. However, compared to *U. crassus*, *U. mancus* has a much more restricted range, with drainages in Mediterranean and southwestern France contributing most populations. These drainages are heavily impacted by human activities and systematic sampling of historical localities indicates an important decline of populations, giving cause of concern for their conservation status.

Intensive farming produces considerable pollutants effluents: 73,000 tons of phytosanitary products have been used in France in 2004, and pesticides have been detected in 96% of French rivers checkpoints (IFEN 2006). Dams and impoundments for irrigation are a primary cause of fragmentation and habitat loss due to siltation (Strayer 2006, Sabater 2008); water extraction and severe droughts heighten the concentration of pollutants and eutrophication effects (Sabater and Tockner 2009). It thus does not come as a surprise that the distributions and abundances of unionids in French rivers differ – sometimes considerably – from what can be inferred from 19th century publications. For instance, the type localities of *U. m. moquinianus* do not seem to host *Unio* species any more: the Echez and Arros Rivers are both highly impacted by intensive corn farming. Only a few live specimens were found in the Drée River (type locality of *U. m. mancus*), whereas numerous recently dead specimens covered the river bed, indicating a recent die-off. The Drée River is subject to recurrent pollution from malfunctioning sewage treatment plants, and an important pollution, involving fish mortality, occurred in summer 2010 during a low water event. Although we could find living *Unio* in Lake Brindos, these were very scarce and no juveniles were encountered, indicating a declining population. Indeed, the site is affected by heavy organic siltation, the substrate is dominated by anoxic sludge, and the survival of this population is obviously compromised. As noticed above, specimens collected in Lake Le Bourget differ morphologically from specimens historically collected in that lake, and a doubt remains concerning the survival of such historical *U. m. bourgeticus* morphotypes.

Populations in the Mediterranean drainages have become very restricted and demographically weak. Mediterranean rivers are affected by water restriction, which increases the effect of other stressors such as pollutants, UV-impact and thermal stress (Tockner *et al.* 2010), and should be even more impacted by human activities and global changes in the future (Alcamo *et al*. 2007; Sabater and Tockner 2009). French Mediterranean drainages are home to two subspecies, for which conservation efforts should be considered. In Corsica, *U. m. turtonii* was historically known from at least eight rivers (Payraudeau 1826), which have all been resurveyed (this paper and Araujo, pers. com.); *Unio m. turtonii* has likely been extirpated from three of these sites. It also seems to have recently been locally extirpated from the River Argens by a major flood that occurred in December 2010 after our sampling had taken place. The type locality of *U. m. aleronii* in the Basse River is now restricted to a stretch of a few hundreds of meters, between the suburbs of Perpignan downstream and the newly built railway line upstream.

Robust taxon delimitation of, and within, *U. mancus* thus suggests its conservation status should be revised to take into account the impact of habitat degradation on the size and number of populations of its constituent subspecies or evolutionary units.

<u>IUCN categorization</u>

U. mancus is currently considered “Near Threatened” on the IUCN European Red List of Non-Marine Molluscs (Cuttelod *et al*. 2011). At the time when the Red List was compiled (2009), criterion A (declining population) was not used as no

precise data were available to support it. This is the unfortunate fate of invertebrate taxa: despite evidence of an alarming global decline of non-marine molluscs (Lydeard *et al* 2004), we generally lack proper data to assess population decline for each taxon considered. In this paper, 18 historical localities have been re-sampled. Despite an intensive search involving several prospectors and appropriate approaches (aquascope, dredging, scuba diving), the result was that 10 of these 18 populations have been extirpated since the early 1900s and four out of the remaining eight are declining. Moreover, global decline can be inferred from the well documented decline of habitat and water quality. It is therefore likely that population reduction has reached over 50% during the 20th century. The causes are not clearly understood, they have not ceased and are in most cases not reversible; inferred decline is based on direct observation, decline in Area Of Occurrence (AOO), and effect of pollutants. Based on these considerations, *U. mancus* meets the criteria A2ace to categorize as "Endangered".

With regard to criteria B (restricted range and fragmentation, decline or fluctuations) and C (small population size and decline or fluctuations), *U. mancus* has a relatively large distribution (about 800,000 km², based on river basins) and healthy populations are known. Given this distribution and occurrence, criteria B and C are not globally applicable, but the present study stresses out the “severely fragmented” option in criterion B, which should be taken into account. Different COI lineages have been revealed in France but, although COI evolves at a fast rate, it is not enough to reveal population isolation nowadays. Therefore, fragmentation may be higher than suggested by COI sequences. Further studies on

population genetics may highlight distinct populations most likely isolated in independent hydrosystems.

Fragmentation and decline are an established fact, especially with regard to *U. m. turtonii* known only from six locations: one on the mainland (the population sampled by us has since been extirpated but specimens probably survive elsewhere in the River Argens), five in Corsica, with extirpation from three further localities within a century. However, total population probably exceeds 10,000 individuals, and criterion C is not applicable. Criterion D (very small population or very restricted distribution) and E. (Quantitative analysis of extinction risk) are not applicable either.

Each of the three subspecies of *Unio mancus* recognized in the present study should arguably be considered as "Endangered". Despite a more restricted range and higher anthropogenic pressure on *U. m. turtonii*, it does meet the IUCN criteria for "Critically Endangered".

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Table I: Samples localities, MNHN numbers and GenBank accession numbers.

*: type localities.

	Maritime Facade	Major drainage system	River	Species	Clade	Haplotype	Voucher n°	Genebank accession n°
Sequences produced for this paper	Atlantic	Adour	Loutz	*Unio mancus mancus*	1c	1	IM-2009-12598	*En cours*
				Unio mancus mancus	1c	1	IM-2009-12599	*En cours*
			Lake Brindos *	*Unio pictorum*	2	18	IM-2009-17835	*En cours*
				Unio pictorum	2	18	IM-2009-17836	*En cours*
				Unio pictorum	2	18	IM-2009-17837	*En cours*
				Unio pictorum	2	18	IM-2009-17838	*En cours*
				Unio pictorum	2	18	IM-2009-17839	*En cours*
			Gave de Pau	*Unio pictorum*	2	17	IM-2009-21214	*En cours*
				Unio pictorum	2	19	IM-2009-21215	*En cours*
				Unio pictorum	2	17	IM-2009-21216	*En cours*
		Charente	Belle	*Unio mancus mancus*	1c	1	IM-2009-12710	*En cours*
				Unio mancus mancus	1c	1	IM-2009-12711	*En cours*
				Unio mancus mancus	1c	1	IM-2009-12713	*En cours*
				Unio mancus mancus	1c	1	IM-2009-12714	*En cours*
			Né	*Unio mancus mancus*	1c	1	IM-2009-12715	*En cours*
				Unio mancus mancus	1c	6	IM-2009-12716	*En cours*
				Unio mancus mancus	1c	6	IM-2009-12717	*En cours*
			Seugne	*Unio mancus mancus*	1c	3	IM-2009-12602	*En cours*
				Unio mancus mancus	1c	1	IM-2009-12604	*En cours*
		Dordogne	Lary	*Unio pictorum*	2	17	IM-2009-12588	*En cours*
				Unio pictorum	3	17	IM-2009-12589	*En cours*
		Loire	Authion	*Unio mancus mancus*	1a	6	IM-2009-21274	*En cours*
				Unio mancus mancus	1a	1	IM-2009-21275	*En cours*
				Unio mancus mancus	1a	1	IM-2009-21276	*En cours*
				Unio mancus mancus	1a	6	IM-2009-21277	*En cours*
				Unio mancus mancus	1a	1	IM-2009-21278	*En cours*
				Unio mancus mancus	1a	6	IM-2009-21279	*En cours*
				Unio mancus mancus	1a	6	IM-2009-21280	*En cours*
				Unio mancus mancus	1a	6	IM-2009-21281	*En cours*
				Unio mancus mancus	1a	6	IM-2009-21282	*En cours*
				Unio mancus mancus	1a	1	IM-2009-21283	*En cours*
			Boivre	*Unio mancus mancus*	1c	6	IM-2009-12591	*En cours*

			Drée*	*Unio mancus mancus*	1c	6	IM-2009-21267	*En cours*
				Unio mancus mancus	1c	6	IM-2009-21268	*En cours*
				Unio mancus mancus	1c	6	IM-2009-21269	*En cours*
			Cher	*Unio mancus mancus*	1c	2	IM-2009-21293	*En cours*
				Unio mancus mancus	1c	4	IM-2009-21294	*En cours*
				Unio mancus mancus	1c	6	IM-2009-21295	*En cours*
				Unio mancus mancus	1c	6	IM-2009-21296	*En cours*
				Unio pictorum	2	17	IM-2009-21297	*En cours*
				Unio mancus mancus	1c	6	IM-2009-21298	*En cours*
				Unio mancus mancus	1c	1	IM-2009-21299	*En cours*
				Unio mancus mancus	1c	2	IM-2009-21300	*En cours*
				Unio pictorum	2	17	IM-2009-21301	*En cours*
			Vonne	*Unio mancus mancus*	1c	1	IM-2009-12666	*En cours*
				Unio mancus mancus	1c	1	IM-2009-12667	*En cours*
				Unio mancus mancus	1c	6	IM-2009-12668	*En cours*
		Aër	Ellé	*Unio mancus mancus*	1c	6	IM-2009-17787	*En cours*
				Unio mancus mancus	1c	6	IM-2009-17788	*En cours*
	Atlantic *via* Manche	Blez Jean	Beaufort	*Unio mancus mancus*	1a	5	IM-2009-21302	*En cours*
				Unio mancus mancus	1a	5	IM-2009-21303	*En cours*
				Unio mancus mancus	1a	5	IM-2009-21304	*En cours*
				Unio mancus mancus	1a	5	IM-2009-21305	*En cours*
				Unio mancus mancus	1a	5	IM-2009-21306	*En cours*
				Unio mancus mancus	1a	5	IM-2009-21307	*En cours*
		Seine	Aisne	*Unio pictorum*	2	17	IM-2009-17764	*En cours*
				Unio pictorum	2	17	IM-2009-17798	*En cours*
				Unio pictorum	2	17	IM-2009-17799	*En cours*
				Unio pictorum	2	17	IM-2009-17801	*En cours*
				Unio pictorum	2	17	IM-2009-17802	*En cours*
			Armance	*Unio pictorum*	0	19	IM-2009-21250	*En cours*
			Eure	*Unio pictorum*	1	23	IM-2009-21285	*En cours*
				Unio pictorum	2	23	IM-2009-21286	*En cours*
				Unio pictorum	2	17	IM-2009-21287	*En cours*
			Oise	*Unio pictorum*	2	21	IM-2009-12688	*En cours*
			Ourcq	*Unio mancus requienii*	1b	13	IM-2009-21218	*En cours*
				Unio mancus requienii	1b	13	IM-2009-21222	*En cours*
				Unio mancus requienii	1b	13	IM-2009-21224	*En cours*
				Unio mancus requienii	1b	13	IM-2009-21228	*En cours*
				Unio mancus requienii	1b	13	IM-2009-21229	*En cours*

				Unio mancus requienii	1b	13	IM-2009-21231	*En cours*
				Unio mancus requienii	1b	13	IM-2009-21233	*En cours*
				Unio pictorum		23	IM-2009-21234	*En cours*
				Unio mancus requienii	1b	13	IM-2009-21235	*En cours*
				Unio pictorum	2	23	IM-2009-21236	*En cours*
			Petit Morin	*Unio mancus requienii*		11	IM-2009-17803	*En cours*
				Unio mancus requienii	1b	13	IM-2009-17810	*En cours*
				Unio mancus requienii	1b	13	IM-2009-17812	*En cours*
				Unio mancus requienii		12	IM-2009-17813	*En cours*
				Unio mancus requienii	1b	13	IM-2009-17814	*En cours*
			Seine	*Unio mancus requienii*	1b	13	IM-2009-21242	*En cours*
				Unio mancus requienii	1b	13	IM-2009-21245	*En cours*
				Unio pictorum	2	21	IM-2009-12695	*En cours*
				Unio pictorum	2	17	IM-2009-12696	*En cours*
				Unio pictorum	2	17	IM-2009-12697	*En cours*
		Somme	Somme Canal	*Unio pictorum*	2	17	IM-2009-17771	*En cours*
				Unio pictorum	2	21	IM-2009-17772	*En cours*
	Mediterranean	Argens	Argens	*Unio mancus turtonii*	1a	9	IM-2009-12571	*En cours*
				Unio mancus turtonii	1a	10	IM-2009-12579	*En cours*
		Basse	Basse*	*Unio mancus mancus*	1c	6	IM-2009-17815	*En cours*
				Unio mancus mancus	1c	6	IM-2009-17816	*En cours*
				Unio mancus mancus	1c	6	IM-2009-17817	*En cours*
				Unio mancus mancus	1c	6	IM-2009-17818	*En cours*
				Unio mancus mancus	1c	6	IM-2009-17819	*En cours*
				Unio mancus mancus	1c	6	IM-2009-17820	*En cours*
				Unio mancus mancus	1c	6	IM-2009-17821	*En cours*
		Hérault	Hérault	*Unio mancus requienii*	1b	14	IM-2009-12569	*En cours*
				Unio mancus requienii	1b	13	IM-2009-12572	*En cours*
			Lamalou	*Unio mancus requienii*	1b	14	IM-2009-12570	*En cours*
		Rhône	Ardèche	*Unio mancus requienii*	1b	13	IM-2009-12582	*En cours*
			Lake Bourget*	*Unio mancus requienii*	1b	13	IM-2009-12701	*En cours*
				Unio pictorum	2	17	IM-2009-12703	*En cours*
				Unio mancus requienii	1b	13	IM-2009-12704	*En cours*
				Unio pictorum	2	17	IM-2009-12705	*En cours*
				Unio mancus requienii	1b	13	IM-2009-12706	*En cours*
				Unio mancus requienii	1b	15	IM-2009-12708	*En cours*
			Cèze	*Unio mancus requienii*	1b	13	IM-2009-12581	*En cours*
			Lake Gravière*	*Unio mancus requienii*	1b	13	IM-2009-17775	*En cours*

			Rhône	*Unio mancus requienii*	1b	13	IM-2009-12605	*En cours*
			Suran	*Unio mancus requienii*	1b	13	IM-2009-12699	*En cours*
				Unio mancus requienii	1b	14	IM-2009-17806	*En cours*
				Unio mancus requienii	1b	13	IM-2009-17807	*En cours*
				Unio mancus requienii	1b	14	IM-2009-17808	*En cours*
				Unio mancus requienii	1b	13	IM-2009-17809	*En cours*
		Corsica	Fium Orbu*	*Unio mancus turtonii*	1a	9	IM-2009-12735	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12736	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12737	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12738	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12739	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12740	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12747	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12748	*En cours*
				Unio mancus turtonii	1a	8	IM-2009-12749	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12751	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12754	*En cours*
			Golo*	*Unio mancus turtonii*	1a	9	IM-2009-17784	*En cours*
			Taravu*	*Unio mancus turtonii*	1a	9	IM-2009-12725	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12726	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12727	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12728	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12729	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12730	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12731	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12732	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12733	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12734	*En cours*
			Taviganu*	*Unio mancus turtonii*	1a	9	IM-2009-12758	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12759	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12760	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12761	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-12762	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-21815	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-17750	*En cours*
				Unio mancus turtonii	1a	9	IM-2009-17751	*En cours*
		Pô	Lake Maggiore	*Unio elongatulus*	3	16	IM-2009-12673	*En cours*
				Unio elongatulus	3	16	IM-2009-12674	*En cours*

				Unio elongatulus	3	16	IM-2009-12675	*En cours*
	Northern Sea	Meuse	Meuse	*Unio pictorum*	2	22	IM-2009-12573	*En cours*
				Unio pictorum	2	17	IM-2009-12574	*En cours*
				Unio pictorum	2	22	IM-2009-12576	*En cours*
				Unio pictorum	2	17	IM-2009-12577	*En cours*
Genebank sequences	North Sea (Poland)			*Unio pictorum*	2	13	?	AF468684.2
				Unio pictorum	2	14	233	EU548056.1
				Unio pictorum	2	19	253	EU548057.1
	Mediterranean (Spain)			*Unio mancus mancus*	1c	7	Fw1238-15	AY522847.1
				Unio mancus mancus	1c	7	Fw1238-18	AY522848.1
				Unio mancus mancus	1c	7	Fw1487-1	AY522849.1
				Unio mancus mancus	1c	7	Fw1487-2	AY522850.1
				Unio mancus mancus	1c	7	Fw1487-3	AY522851.1
				Unio mancus mancus	1c	7	Fw1487-4	AY522852.1
				Unio mancus mancus	1c	7	Fw1487-5	AY522853.1
				Unio mancus mancus	1c	7	Fw1535-N1	AY522854.1
				Unio mancus mancus	1c	7	Fw1536-N2	AY522855.1
				Unio mancus mancus	1c	6	Fw1544-N50	AY522856.1
				Unio mancus mancus	1c	6	Fw1545-N53	AY522857.1
				Unio mancus mancus	1c	7	Fw1585-N250	AY522858.1
Outgroups (own sequences)	North Sea	Meuse	Meuse	*Unio tumidus*	IM-2009-12575			*En cours*
	Atlantic	Loire	Vienne	*Unio crassus*	IM-2009-12564			*En cours*
				Margaritifera auricularia	IM-2009-12611			*En cours*

Figure captions

Figure 1: Distribution of *Unio mancus* subspecies in France according to Falkner *et al.* (2002) (red dotted lines). Type localities are indicated by grey squares. Localities sampled for this study are represented by green dots.

Figure 2: Bayesian phylogenetic tree of French and Spanish populations of *U. mancus*, *U. elongatulus* and *U. pictorum*. Posterior probabilities are indicated for each node (when > 0.70). H1 to H23: haplotype numbers. Rivers are given after haplotypes numbers. Black lines link up haplotypes from the same locality. Clades 1, 2 and 3 and groups 1a, 1b and 1c are highlighted.

Blue = clade 1a, red = clade 1b, orange = clade 1c, purple = *U. mancus*, green = *U. elongatulus*, yellow = *U. pictorum*.

Figure 3: Putative distribution of the subspecies of *U. mancus* inferred from main drainage systems. Blue, *U. m. mancus*; orange, *U. m. requienii*; red, *U. m. turtonii*. Yellow circles, *U. pictorum* sample sites; green circle, *U. elongatulus* sample site.

Figure 4: Morphological variability of *U. mancus* in France.

A: *U. m. mancus*, lectotype. River Drée, Bourgogne, MNHN 24632; B: *U. m. mancus*, River Vonne at Soudan (Loire-Atlantique), MNHN IM-2009-12668; C: *U. m. mancus*, River Ellé at Meslan (Morbihan), MNHN IM-2009-17788; D: *U. m. mancus*, River Basse at Toulouges (Pyrénées-Orientales), MNHN IM-2009-17815; E: *U. m. mancus*, River Loutz at Lacajunte (Landes), MNHN IM-2009-

12599; F: *U. m. requienii*, River Aisne at Bourg-et-Comin (Aisne), MNHN IM-2009-17803; G: *U. m. requienii*, Lake Bourget at Chindrieux (Savoie), MNHN IM-2009-12701; H: *U. m. requienii*, Lake Bourget at Chindrieux (Savoie), MNHN IM-2009-12707; I: *U. pictorum*, Lake Bourget at Chindrieux (Savoie), MNHN IM-2009-12703; J: *U. pictorum*, Lake Bourget at Chindrieux (Savoie), MNHN IM-2009-12705; K: *U. bourgeticus*, syntype, MNHN 22139; L: *U. mancus requienii*, possible topotype, Lake Gravières at Arles (Bouches-du-Rhône), MNHN IM-2009-17775; M: *U. mancus requienii*, River Rhône at Sablons (Isère), MNHN IM-2009-12605; N: *U. mancus requienii*, River Hérault at Bélarga (Hérault), MNHN IM-2009-12569; O: *U. mancus turtonii*, River Orbu at Ghisonaccia (Haute-Corse), MNHN IM-2009-12736; P: *U. mancus turtonii*, River Argens at Le Muy (Var), MNHN IM-2009-12579.

All specimens with an IM catalog number prefix are sequenced vouchers.

Figure 1

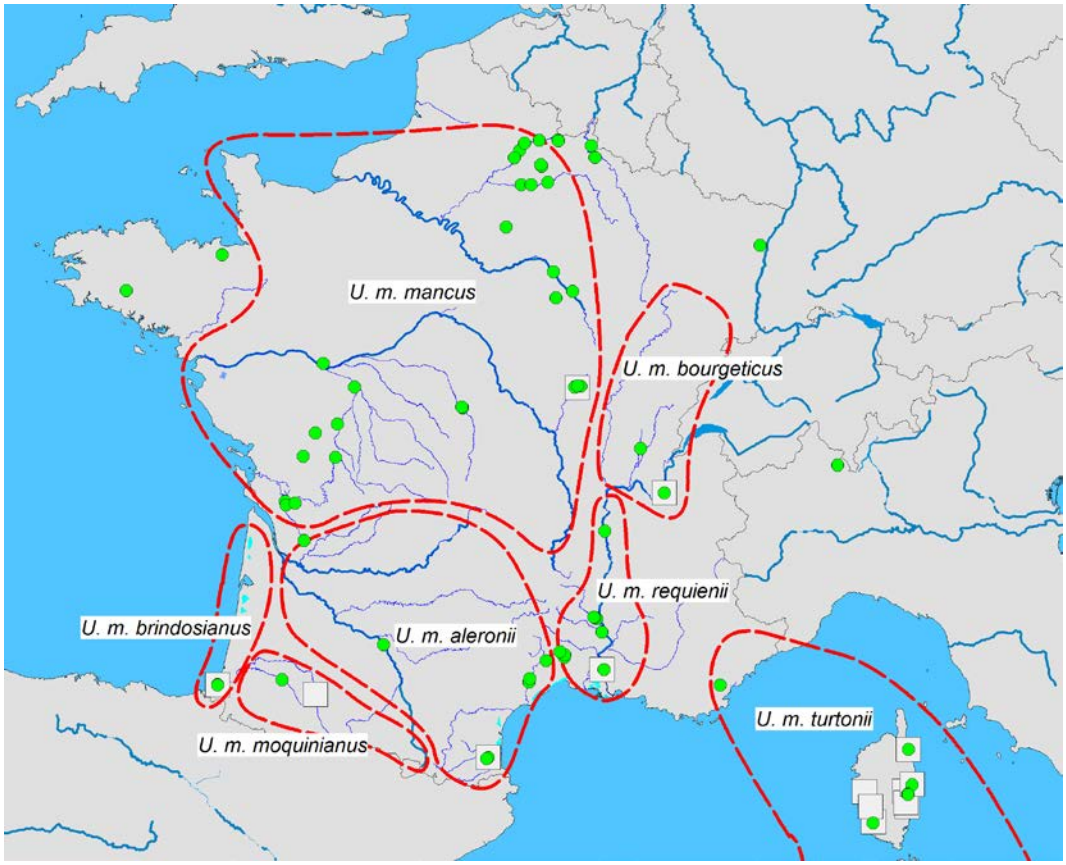
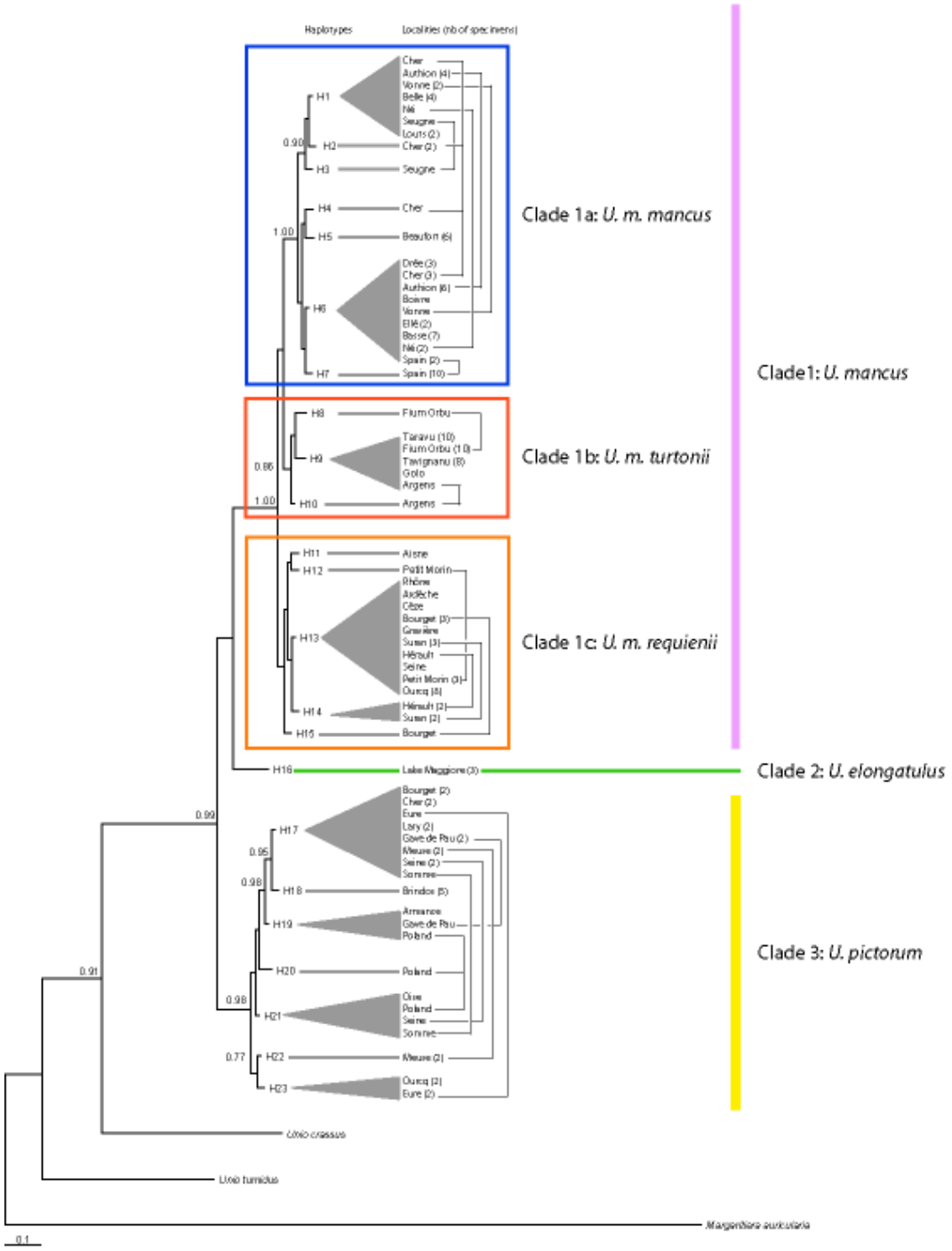

U. m. mancus
U. m. bourgeticus
U. m. requienii
U. m. brindosianus
U. m. aleronii
U. m. turtonii
U. m. moquinianus

Figure 2



681

682

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Figure 3

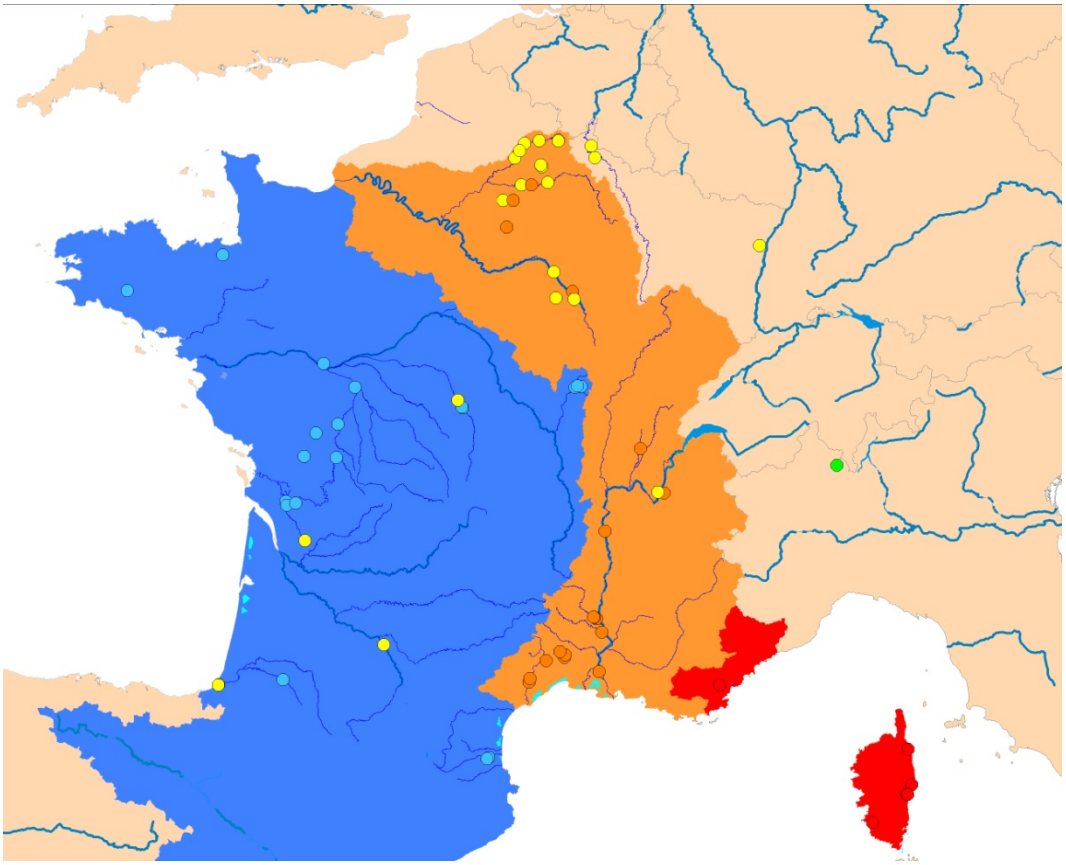
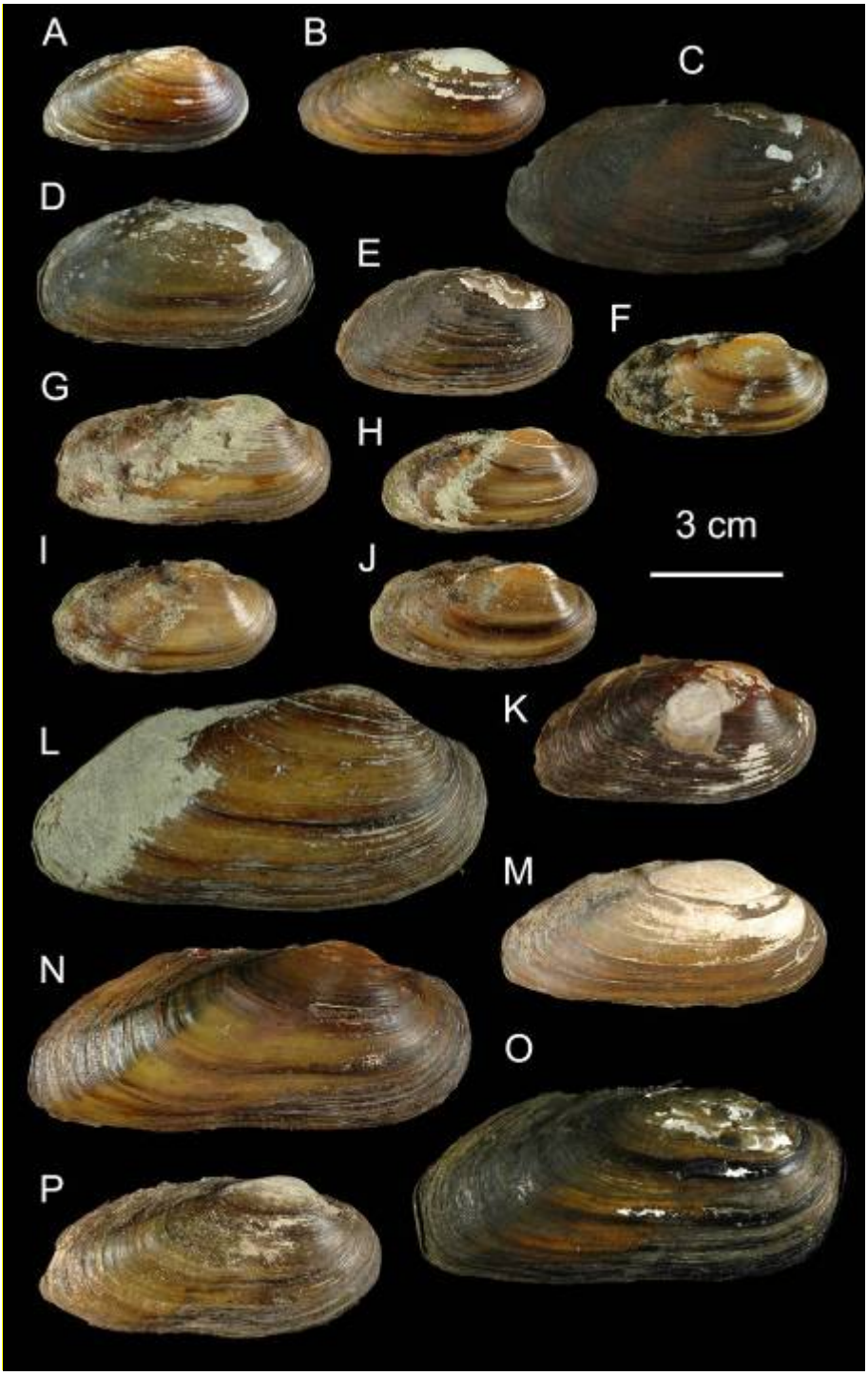
Figure 4