

Conservation status and a novel restoration of the endangered freshwater mussel *Unio crassus* Philipsson, 1788: Poland case

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Abstract – Bivalves play an important part in freshwater ecosystems and improve water quality; the thick-shelled river mussel *Unio crassus* is classified as a bioindicator. Unfortunately, the species is regarded as endangered and is under species protection in the whole of Europe. The reasons for the drastic decline of its populations are: considerable eutrophication of waters, anthropogenic influence in its broad sense and the presence of invasive species. The life cycle of *U. crassus* includes the stage of larva which is an obligatory parasite of fish. This makes it possible for the species to disperse and populate new territories but it limits the development to places where appropriate host species are available. Intensive measures have been taken in Poland to protect *U. crassus*, while in France numerous new localities of the species have been bar-coded. In 2010–2014 active protection measures were taken in southern Poland, including inventorying, studies of genetic diversity and reintroductions. The project contributed to the increase in population abundance and in the number of localities of the thick-shelled river mussel, which resulted in a twofold increase in the range of occurrence of the species in the river. The procedures presented here can and should be used in further restitution of *U. crassus* not only in Poland but also elsewhere in Europe.

Keywords: Conservation genetics / freshwater mussels / metabarcoding / DNA markers / Unionidae

Résumé – Etat de conservation et restauration inédite de la moule d'eau douce menacée *Unio crassus* Philipsson, 1788: cas en Pologne. Les bivalves jouent un rôle important dans les écosystèmes d'eau douce et améliorent la qualité de l'eau; la moule de rivière à coquille épaisse *Unio crassus* est classée comme bioindicateur. Malheureusement, l'espèce est considérée comme menacée et fait l'objet d'une protection dans toute l'Europe. Les raisons du déclin drastique de ses populations sont: l'eutrophisation considérable des eaux, l'influence anthropique au sens large et la présence d'espèces envahissantes. Le cycle de vie de *U. crassus* comprend un stade larvaire qui est un parasite obligatoire de poissons. Cela permet à l'espèce de se disperser et de peupler de nouveaux territoires mais limite le développement à des endroits où des espèces hôtes appropriées sont disponibles. Des mesures intensives ont été prises en Pologne pour protéger *U. crassus*, tandis qu'en France, de nombreuses nouvelles localités de l'espèce ont été dotées de codes-barres. En 2010–2014, des mesures de protection active ont été prises dans le sud de la Pologne, notamment des inventaires, des études sur la diversité génétique et des réintroductions. Le projet a contribué à l'augmentation de l'abondance de la population et du nombre de localités de la moule de rivière à coquille épaisse, ce qui a entraîné un doublement de l'aire de répartition de l'espèce en rivière. Les procédures présentées ici peuvent et doivent être utilisées pour la poursuite de la restauration de *U. crassus* non seulement en Pologne mais aussi ailleurs en Europe.

Mots clés : Génétique de la conservation / moules d'eau douce / métabarcodage / marqueurs ADN / Unionidae

1 Introduction

Bivalves of the order Unionida are among the most endangered freshwater invertebrate taxa. According to the

International Union for Conservation of Nature (IUCN), which is currently the world's most comprehensive source of information on the global conservation status of animal, fungi and plant species and is compiling the International Red List of IUCN (www.iucnredlist.org), as many as 30% of freshwater mollusc species are threatened with extinction. Depending on the degree of threat they are assigned categories of critically

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endangered (CR), endangered (EN) and vulnerable (VU). Among the 164 endangered mollusc species, the Unionida constitute ca. 16%.

Among the 16 European species of the order Unionida nine have status of near threatened, endangered, or critically endangered according to the IUCN Red List (IUCN, 2017). For this reason environmental, ecological, population and genetic studies aimed, among others, at conservation, are thoroughly justified.

These bivalves have an unusual life cycle which includes incubation of eggs and larvae in female's ctenidia, and a larval stage (glochidium), which is an obligatory parasite of fish (Labecka and Domagała, 2018, 2019; Labecka and Czarnoleski, 2019). Besides, they exhibit a unique mode of inheritance of mitochondrial genome (mtDNA), which differs from the strictly maternal inheritance (SMI) found in plants, most animals and humans. The phenomenon is called doubly uniparental inheritance (DUI) and was first described in 1990 in marine bivalves (Fisher and Skibinski, 1990). At present the number of bivalves known to practice DUI exceeds 100 marine and freshwater species of 12 families (Liu *et al.*, 1996; Zouros, 2000; Walker *et al.*, 2006; Boyle and Etter, 2013; Gusman *et al.*, 2016; Zouros, 2000, 2020; Guerra *et al.*, 2019; Śmietanka *et al.*, 2018; Plazzi and Passamonti, 2019; Soroka, 2020). Most of them are freshwater members of the order Unionida (Guerra *et al.*, 2019). In Europe DUI inheritance was described in nine freshwater species of Margaritiferidae and Unionidae and in four marine species of Mytilidae and Donacidae (Lubośny *et al.*, 2020; Soroka, 2020).

Freshwater bivalves play an important part in ecosystem services. They improve water quality through biofiltration, bioaccumulation and biosedimentation (Vaughn and Hakenkamp, 2001; Howard and Cuffey, 2006; Chowdhury *et al.*, 2016; Douda and Čadková, 2018). A single individual can filter ca. 40 l of water per day, and a population of bivalves can retain 50% sestone in a river during summer (Lopes-Lima *et al.*, 2017a). Bivalves have an influence on matter circulation in the system (the so called nutrients focusing) and the composition of plankton, using up the excess of phytoplankton and selectively removing microorganisms, mainly algae, from the water (Nalepa *et al.*, 1991; Welker and Walz, 1998; Thorp and Casper, 2002; Vaughn and Spooner, 2006). Due to their sensitivity to all kinds of water pollution some bivalve species, for example the thick-shelled river mussel *Unio crassus*, are used as environmental bioindicators (Oertel and Salánki, 2003).

The unionid bivalves occupy an important position in the web of biotic interactions between organisms since they are hosts to many parasite species of various taxa (Reichard *et al.*, 2010, 2011; Cichy *et al.*, 2016; Pavluchenko and Yermoshyna, 2017). Life cycles of many species are closely linked with bivalves (Brian and Aldridge, 2019). Moreover, in order to metamorphose, glochidia have to temporarily parasitise fishes, inducing immunological reactions and pathological conditions in the hosts (O'Connell and Neves, 1999; Douda *et al.*, 2017; Gopko *et al.*, 2018; Modesto *et al.*, 2018). As larvae, the bivalves migrate with their hosts, sometimes covering great distances, and spreading to new areas (Kat, 1984; Rogers-Lowery and Dimock, 2006; Domagała *et al.*, 2007; Douda *et al.*, 2012b). This mode of reproduction makes it possible for the bivalves to function in the metapopulation system, that is, system in which bivalve colonies which disappear as a result of

hydrological changes are replaced by new colonies which arise as a result of fish-mediated dispersal (Zajac *et al.*, 2019).

Being rather large-sized and having indeterminate growth, the bivalves constitute a significant and often dominant part of benthos biomass (even up to ca. 90%) (McMahon and Bogan, 2001; Labecka and Czarnoleski, 2019) forming a food base for predators: leeches (Bolotov *et al.*, 2019), crabs and crayfish (Klocker and Strayer, 2004), fishes (Bradshaw-Wilson *et al.*, 2019), birds (Urbańska *et al.*, 2013) and mammals (Zajac, 2014). Their shells are important structural components of the bottom of water bodies and provide settling substratum for other organisms (Bołtrusko, 2010; Sousa *et al.*, 2011; Dzierżyńska-Białończyk *et al.*, 2018). They are very durable and preserve well as fossils, hence they are used in archaeomalacology. Mollusc remains form the largest invertebrate group in archaeological materials from the Palaeolithic, through the Iron Age to modern times. The species composition of shell remains provides information on climate and habitat conditions of the studied periods. C14 dating of mollusc remains facilitates precise determination of chronology of archaeological sites (Alexandrowicz and Alexandrowicz, 2011). Unionid shells play an important role in such studies (Ożgo *et al.*, 2012; Kovalchuk *et al.*, 2018; Märgärit *et al.*, 2018; Apolinarska and Kurzawska, 2020).

Bivalves are of great economic importance as source of food as well as providers of valuable shells and pearls. For thousands of years people have used bivalves as food, and their shells as ornaments and tools. In many regions of south-eastern Asia their overexploitation for food has led to depletion of populations of some species (Zieritz *et al.*, 2016, 2017). Since the 1850s freshwater bivalves have been exploited for pearls and mother-of-pearl for button production (Humphries and Winemiller, 2009). During the peak of this exploitation in 1912, 50 000 tonnes of bivalves were harvested from North American rivers (Haag, 2012).

Threats to bivalves worldwide have been observed for a long time. Molluscs constitute 42% of species extinct since 1500: 269 snail species and 31 bivalve species (Lydeard *et al.*, 2004). In 2015, 44% of freshwater bivalve species were classified as threatened or nearly threatened with extinction. More than 70% of bivalve species in North America are regarded as threatened with extinction, and 37 species have become extinct. Fifty three species of freshwater bivalves occur in Europe (Piechocki and Wawrzyniak-Wydrowska, 2016), three of them are critically endangered (CR: *Margaritifera margaritifera*, *Pseudunio auricularius* and *Unio gibbus*), two are endangered (EN: *Potomida littoralis* and *U. crassus*), and four are vulnerable (VU: *Anodonta cygnea*, *Pseudanodonta complanata*, *U. delphinus* and *U. mancus*) (based on IUCN Red List).

Till as late as the 19th c. *U. crassus* was still common in rivers and streams of Central Europe. In the second half of the 20th century its distribution range was reduced by ca. 50%; this pertains to both the abundance of individual populations and the number of populations. The reasons for the decline were industrial development, canalisation of rivers and increasing level of water pollution. The changes in species composition of the river ichthyofauna contributed to the decline, making it difficult or sometimes impossible for the glochidia to metamorphose because of limited availability or lack of hosts. Besides, the introduction of muskrat (*Ondatra zibethicus*) into

Table 1. Current conservation status of *U. crassus* in Europe according to Lopes-Lima *et al.* (2017a) and Bolotov *et al.* (2020).

Threat level	Country
Extinct in the wild, EW	Lithuania, Netherlands
Critically Endangered, CR	Austria, Germany, Switzerland
Endangered, EN	Czech Republik, Poland, Romania, Sweden,
Strictly protected	Belgium, Croatia, Germany, Poland,
Protected	Bulgaria, Czech Republik, France, Hungary, Latvia, Slovakia, Sweden
Rare	Hungary
Vulnerable, VU	Albania, Belarus, Finland, Latvia, Slovakia
Least Concern, LC	Kazakhstan, Russia

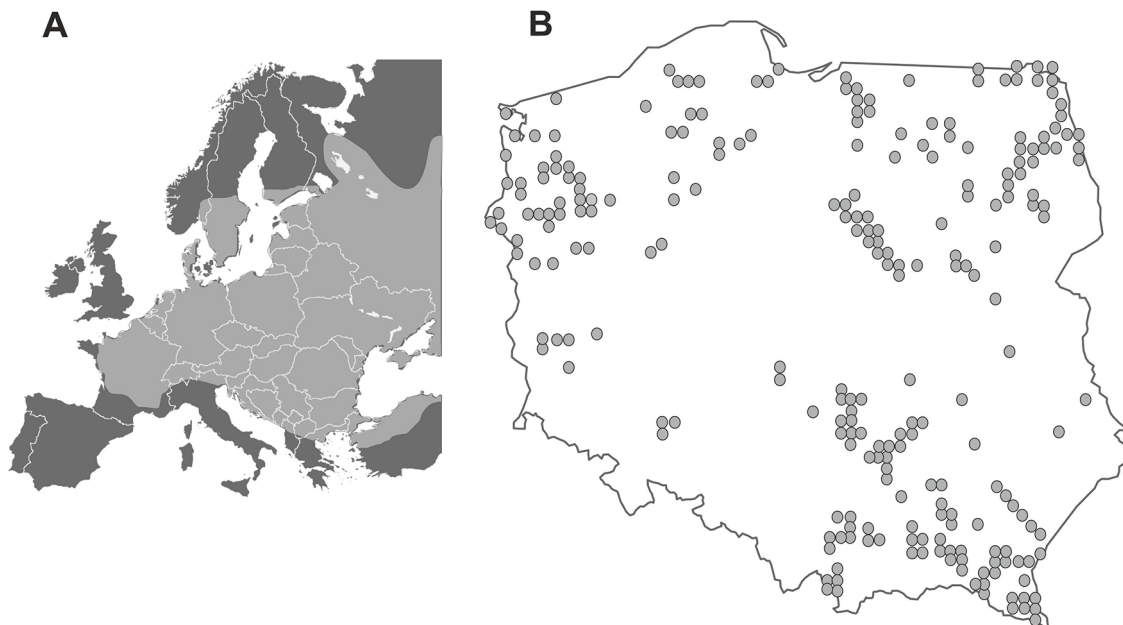


Fig. 1. Maps of the range and distribution of the thick-shelled river mussel *Unio crassus*: A – European part of *U. crassus* range (light grey polygon) according to Welter-Schultes (2012) and Lopes-Lima *et al.* (2017a); B – *U. crassus* occurrence in Poland according to Zajac (2018, modified): grey dot – squares 10 × 10 km with *U. crassus* sites.

Europe had a negative effect on local populations of *U. crassus*. The muskrat, though essentially herbivorous, readily feeds on bivalves (Zahner-Meike and Hanson, 2001).

A considerable reduction in the number of thick-shelled river mussel populations was observed in many European countries in the 1980s. In 2014 the species was included in the IUCN Red List (Lopes-Lima *et al.*, 2014, 2017a), but the degree of its threat varies across Europe and depends on the country (Tab. 1) (Lopes-Lima *et al.*, 2017a; Beran, 2019).

For example, according to the current regulations (decree of the Minister of Environment of December 16th 2016) seven species among the 36 which occur in Poland are under legal protection (Bogdanowicz *et al.*, 2008; Piechocki and Wawrzyniak-Wydrowska, 2016). Four of them are under partial and three, including *U. crassus* (continuous protection since 2001), under strict protection. The thick-shelled river mussel is listed in Annexes II and IV of the Habitats Directive of the European Council of 1992 (92/43/EEC), which obliges the EU countries to formulate protection plans, designate areas

which protect a representative part of the populations and monitor the state of the species' conservation. In the EU 818 such areas were designated: in Poland 68 areas "Natura 2000", where habitats of *U. crassus* are subject to protection according to the plans formulated by experts. The last report on the results of protection measures taken to conserve *U. crassus* in Poland, prepared for the needs of the European Commission, estimates the state of conservation of the population as adequate (FV), but the state of habitats as inadequate (U1) and in need of improvement (Zajac, 2018; Fig. 1).

Effective protection of *U. crassus* requires an extensive knowledge of its biology, abundance and population structure within the distribution range, as well as formulation of new tasks within conservation biology. Practical application of scientific achievements and proposed solutions guarantees maintenance of the species in the existing localities and may contribute to restoring *U. crassus* in the rivers from which it disappeared and in which it had once been the most abundant

bivalve species. This is dependent on financing of such activities; to date, there have been 49 projects within the LIFE programme (the European Union's funding instrument for the environment) devoted to the restoration of freshwater mussel habitats (13 projects concern *U. crassus*), with the total funding of over 90 million Euros (based on a search for mussel species *Margaritifera auricularia*, *M. margaritifera*, *Unio elongatulus*, *U. crassus* in the LIFE database (European Commission, 1995–2020); some projects target more than one species). The problem of restoring bivalve populations in rivers presents an especially serious challenge to the science, since in the whole of Europe, except two cases, attempts at permanent restoration of freshwater bivalves in nature keep failing (Lopes-Lima *et al.*, 2017a). Attempts at reintroduction of the pearl mussel *Margaritifera margaritifera* in the Karkonosze National Park (Poland) in the 1960s failed. Ca. 110 individuals from the Czech Republic were released into the wild, but already after two weeks no mussel could be found. It is estimated that till the 1990s the abundance of the European populations decreased by more than 90% (Bauer, 1988). An example of a successful restoration of the species in the wild is the Lutter River in northern Germany. Here the factors contributing to the success were reduction of the inflow of marine deposits and reconstruction of the whole catchment area (Geist, 2010). Another case of successful restoration is *U. crassus* in the Biała Tarnowska River in Poland, where the possibilities of fish migrations were improved, thus permitting re-colonisation by the species. The case is discussed in detail in the last chapter of this article.

At present one of the very important aspects of protection of all endangered species is the knowledge of their genetic resources at the species and population levels, combined with active protection of these resources during restitution and reintroduction (Ferreira-Rodríguez *et al.*, 2019). The knowledge is used by conservation genetics – a discipline of science aimed at protection of wild endangered species of plants and animals. The first publications dealing with the significance of genetics for nature conservation appeared in the 1970s (Frankel, 1974). Conservation genetics deals with management of small, fragmented populations in order to increase their genetic diversity and reduce the inbred effect. Besides, it is involved in restitution process, including reintroduction, through selection of source populations or adequate habitats for the endangered species. Genetic studies make it possible to create gene banks which are important for protection of endangered species and for solving taxonomic problems.

It follows from the 2019 report of Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES, 2020) published by the United Nations that 25% of animal species are threatened with extinction, and nearly one million will become extinct during the next decades. The process of extinction of taxa has been known for a long time, and in palaeobiology referred to as Five Great Extinctions, but the current extinction rate is alarming (Sepkoski *et al.*, 1981; Racki, 2009). The main reason for this situation is the damaging effect of man on the environment during the last five decades. It is estimated that human activities have led to transformation of 75% of the globe's area (www.ipbes.net) which may lead to the so called Sixth Great Extinction (Frankham *et al.*, 2010; Kmiec and Skorupski, 2010). Much hope is attached to conservation genetics which

may offer means of counteracting the process through slowing down the decrease in genetic and biocoenotic diversity.

2 Biology

Seven species of the family Unionidae occur in Poland (Soroka, 2020). Unfortunately, a decline in their abundance in rivers and lakes is observed, except the invasive Chinese pond mussel *Sinanodonta woodiana* (Kraszewski and Zdanowski, 2001; Domagała *et al.*, 2007; Spyra *et al.*, 2016; Labecka and Domagała, 2018; Beran, 2019). The high degree of water eutrophication, oxygen deficits, and toxic substances (compounds of sulphur and nitrogen, ammonia and hydrogen sulphide) as well as the effects of invasive species are the greatest contributors to the decline of protected species (Havlik and Marking, 1987; Piechocki and Dyduch-Falniowska, 1993; Naimo, 1995; Patzner and Müller, 2001; Popa *et al.*, 2015).

Thirty seven bivalve species have been recorded in freshwaters of Poland; they represent four families: Dreissenidae, Unionidae, Cyrenidae and Sphaeriidae (Skuzza *et al.*, 2009; Woźniczka *et al.*, 2015; Piechocki and Wawrzyniak-Wydrowska, 2016). The Unionidae are represented by seven species, and three of them are strictly (*U. crassus*) or partially (*Anodonta cygnea* and *Pseudanodonta complanata*) protected.

The thick-shelled river mussel *U. crassus* occurs in fresh, most often running, waters where its life span is 10–75 years depending on the water temperature (Piechocki and Wawrzyniak-Wydrowska, 2016; Lopes-Lima *et al.*, 2017a). It is essentially sedentary, often forming colonies, and most often inhabits beds of large streams and rivers of pure water. The bivalves bury themselves in bottom deposits, leaving exposed the posterior end of the shell with two siphons: inhalant and exhalant. The inhalant siphon serves sucking in water from the surroundings, to provide oxygen and food (algae, various microorganisms and macromolecules of organic matter). The exhalant siphon expels water with pseudo-faeces. When environmental conditions change, *U. crassus* actively moves, most often at a distance of a few metres, but distances of up to 200 m have also been recorded (Zajac *et al.*, 2019).

Among the many unionids, *U. crassus* is distinguished by its thick-walled, massive shell which is reflected by its specific name. The shell size and shape may vary depending on environmental factors of the habitat, for example latitude, flow in the river, or calcium content and trophic conditions. The shell length most often ranges from 3 to 7 cm, though some individuals may reach 9–11 cm (Nagel *et al.*, 2015; Piechocki and Wawrzyniak-Wydrowska, 2016; Lopes-Lima *et al.*, 2017a; Zajac *et al.*, 2018a,b).

U. crassus is dioecious, with a complex life cycle depending on availability of specific fish species. Reproduction takes place from March to July. In spring males release spermatozoa into the water; the spermatozoa, with their flagella directed outward and organised into spherical structures called spermatozeugmata, can move actively over considerable distances (Zajac and Zajac, 2020). Females of *U. crassus* lay eggs several times during the season – most often there are three broods, but up to five (Hochwald, 2001; Piechocki and Wawrzyniak-Wydrowska, 2016) or even up to seven broods have been observed (Zajac and Zajac, 2020). Eggs (9–16 thousand) are deposited in modified external

demibranchs (called marsupia), where they are fertilised and give rise to parasitic larvae – glochidia – 190–232 µm in size (Piechocki and Wawrzyniak-Wydrowska, 2016; Lopes-Lima *et al.*, 2017a). Depending on the temperature their incubation takes a few weeks, from late spring to early summer. In southern Poland eggs laid in March take 4–5 weeks to develop, and those laid in June and July take only a week or two (Zajac and Zajac, 2020). The female releases mature glochidia into the water where they have to encounter an appropriate fish species to metamorphose. In *U. crassus* the whole cycle – from gametes to juvenile mussels – is completed within one season, as in the other representatives of *Unio* (tachytictic species), as opposed to bradytictic species which retain their glochidia through winter till the next spring, for example, the genus *Anodonta* (Dillon, 2004).

An unusual strategy of acquiring fish hosts has been observed in *U. crassus*. The females move to the river bank and eject water from the exhalant siphon above the water surface, which disturbs the water table and attracts fish. The ejected portions contain mature glochidia (Vicentini, 2005; Aldridge *et al.*, 2018). The released larvae attach themselves to the fish body and there they metamorphose after 3–4 weeks (Piechocki and Wawrzyniak-Wydrowska, 2016; Lopes-Lima *et al.*, 2017a; Zajac and Zajac, 2020). Hosts of glochidia include ca. 16 fish species, the most frequent being: bullhead (*Cottus gobio*), minnow (*Phoxinus phoxinus*), chub (*Leuciscus cephalus/Squalius cephalus*), rudd (*Scardinius erythrophthalmus*), bleak (*Alburnus alburnus*), nase (*Chondrostoma nasus*), stickleback (*Gasterosteus aculeatus*), ide (*Leuciscus idus*) and perch (*Perca fluviatilis*) (Douda *et al.*, 2012a; Taeubert *et al.*, 2012b; Lamand *et al.*, 2016; Lopes-Lima *et al.*, 2017a). The density of *U. crassus* was shown to depend on the density of the hosts (Stoeckl *et al.*, 2015). Not every fish species can be host to *U. crassus*, since glochidia can be killed and removed by the fish's immune system (Schneider *et al.*, 2017, 2019). Larvae of *U. crassus* can not metamorphose on roach (*Rutilus rutilus*) (Taeubert *et al.*, 2012b). Invasive fish species which are alien to the fauna of Northern, Western and Central Europe, for example round goby (*Neogobius melanostomus*) and rainbow trout (*Oncorhynchus mykiss*), lost more than 98% of glochidia within 16 days, indicating that they are unsuitable hosts; another unsuitable host is sterlet (*Acipenser ruthenus*) (Taeubert *et al.*, 2012a,b). Following metamorphosis the juveniles leave the host and bury themselves in the bottom deposits where they are difficult to observe during the first months because of their microscopic size. In autumn their shells are ca. 0.5 cm long. It's already known that host compatibility can differ even within one species. Douda *et al.* (2014) proved differences in the ability of *U. crassus* to use particular fish species as hosts between both nearby and recently isolated populations. That variation was also associated with genetic and morphometric differences. An insufficient number of studies on differences at the interpopulation – level in terms of the host settlement mechanism results in limited restitution possibilities (Douda *et al.*, 2014).

U. crassus become sexually mature in 2–5 years. The sex ratio in the Polish populations of *U. crassus* is most often ca. 1:1 with a slight predominance of females (Piechocki and Wawrzyniak-Wydrowska, 2016). Because of its long, complex and fish-dependent life cycle the species has high ecological requirements which results in its great sensitivity to any

changes in the conditions or the ichthyofauna (Taeubert *et al.*, 2014).

The geographical range of *U. crassus* includes nearly the whole of Europe, from France in the west (except the British Isles, Apennine Peninsula and Iberian Peninsula) to Asia Minor and European Russia in the east, with the rivers of the Baltic, Black, Azov, and Caspian Seas drainage basins, all the way to the Ural (Yaik) River basin in Russia and Kazakhstan (Fig. 1) (Bank *et al.*, 2006; Graf, 2007; Lopes-Lima *et al.*, 2014; Piechocki and Wawrzyniak-Wydrowska, 2016; Lopes-Lima *et al.*, 2017a; Bolotov *et al.*, 2020). In Poland it occurs countrywide, though with varied frequency (Fig. 1B; Piechocki and Dyduch-Falniowska, 1993; Piechocki and Wawrzyniak-Wydrowska, 2016; Zajac, 2018). Unfortunately, there are documented cases of disappearance of some localities of *U. crassus* in the Carpathians, Upper and Lower Silesia, Małopolska Upland, Wielkopolska and Mazovia (Zajac, 2004; Lewin, 2014; Sulikowska-Drozd *et al.*, 2016; Zawal *et al.*, 2016; Tatoj *et al.*, 2017; Zajac *et al.*, 2018a,b).

3 Genetics

Compared to other unionid species, genetic studies on the protected *U. crassus* in Europe are few. This is mainly a result of the fact that obtaining specimens in the field requires a permit, that they are difficult to collect and that often it was necessary to kill the animals. Great hopes for solution of these problems are attached to newly-devised non-invasive methods of haemolymph sampling and brush-swabbing of the mantle, viscera and foot, to obtain material for bivalve DNA isolation (Geist and Kuehn, 2005; Henley *et al.*, 2006; Feind *et al.*, 2017). Since recently it has also been possible to determine sex *in vivo*, based on molecular methods, with 97.5% probability (Mioduchowska *et al.*, 2016).

Genetic studies on freshwater bivalves of the family Unionidae revealed an unusual mode of inheritance of mitochondrial DNA. This mode, unique among animals, is called doubly uniparental inheritance (DUI) (Hoeh *et al.*, 1996; Liu *et al.*, 1996). The phenomenon was described for the first time in 1990 in marine bivalves of the genus *Mytilus* (Fisher and Skibinski, 1990), and at present DUI is known to function in numerous species of 12 phylogenetically remote families, both marine (Mytilidae, Veneridae, Donacidae, Nuculanidae, Mactridae, Arctidae, Semelidae, Solenidae and Yoldiidae) and freshwater (Unionidae, Margaritiferidae and Hyriidae) (Soroka, 2020).

Two types of mitochondrial DNA are involved in DUI – F haplotype (F type or female genome), inherited from the mother and M haplotype (M type or male genome), inherited from the father. Females are homoplasmic and have only one mtDNA, of F type, inherited from the mother and passed on to the next generations through female offspring. Males are heteroplasmic and have both forms of mtDNA. Mitochondrial genome of M type, located mainly in the gonads, is inherited from the father and passed on to the male offspring. Male somatic tissues contain mtDNA of F type which they inherited from the mother; they do not pass it on to the next generations. The two mitochondrial genomes are of similar size (ca. 16 500 base pairs), and are genetically much differentiated; M type is usually longer and faster to evolve

Table 2. Average p-distances for *16S rRNA*, between and within populations of *Unio crassus* in Europe. Number in parentheses after country name refers to number of analysed specimens. The origin of the date is mentioned in and below the table.

	1	2	3	4	5	6	7	8
1. Germany (3) ^a	0.000							
2. Greece (8) ^a	0.019	0.017						
3. France (50) ^{a,b}	0.003	0.016	0.001					
4. Poland (3) ^c	0.007	0.019	0.004	0.000				
5. Russia (16) ^{d,e}	0.022	0.025	0.020	0.022	0.003			
6. Slovakia (21) ^a	0.004	0.018	0.003	0.006	0.021	0.005		
7. Sweden (1) ^f	0.023	0.026	0.021	0.023	0.003	0.023	n/c	
8. Ukraine (5) ^g	0.017	0.022	0.015	0.017	0.010	0.017	0.009	0.014

^aAraujo *et al.* (2018).^bPrié and Puillandre (2014).^cBurzyński *et al.* (2017).^dAnisimova *et al.* (2019).^eBolotov *et al.* (2020).^fKällersjö *et al.* (2005).^gYanovich *et al.* (2012) (unpublished, accession number in GenBank: JQ253855-8 and JQ253871).

compared to F type (Stewart *et al.*, 1995; Hoeh *et al.*, 1996; Zouros, 2000; Burzyński and Soroka, 2018; Soroka and Burzyński, 2018; Soroka, 2010b, 2020; Hoeh *et al.*, 1996).

Among the unionids, with their more than 670 species (Graf and Cummings, 2007; Bogan, 2008; Bogan and Roe, 2008), DUI was to date described in ca. 80 species (Gusman *et al.*, 2016; Soroka, 2020). In 2010 the phenomenon was described for the first time in *U. crassus* based on mitochondrial gene *cox1*, with 29–31% differentiation between F and M haplotypes (Soroka, 2010a; Mioduchowska *et al.*, 2016). In the Unionidae the differentiation between F and M haplotypes ranges from 28% to 35% for genes *cox1* and *16S rRNA*, respectively (Hoeh *et al.*, 1996; Krebs, 2004; Soroka, 2008a,b, 2010a; Froufe *et al.*, 2014; Soroka and Burzyński, 2015).

The whole mitochondrial female (F) and male (M) genomes of *U. crassus* were sequenced relatively recently (Burzyński *et al.*, 2017). Their size is 15 781 base pairs (bp) for F genomes and 16 633 and 16 646 bp for M genomes, which are by 5% longer. Each type of mitogenome contains a set of 37 genes which are typical of animals and an additional gene F or M ORF, characteristic of unionids and associated with a different mtDNA transmission and sex determination in dioecious species of the family (Breton *et al.*, 2009; Soroka, 2020; Zouros, 2020). The arrangement of the 38 genes in mitochondrial genomes of *U. crassus* is the same as in the other unionids; 27 of them are located on the light strand, and 11 on the heavy strand of DNA (Breton *et al.*, 2009; Soroka, 2010b; Burzyński *et al.*, 2017). Genetic differentiation of the entire female and male genomes of *U. crassus* is 54% and the value is slightly higher than in the other unionid species where it ranges from 50% to 53% (Breton *et al.*, 2009; Doucet-Beaupré *et al.*, 2010; Fonseca *et al.*, 2016; Soroka and Burzyński, 2016).

Much more genetic research was done on mitochondrial genes of F genomes of *U. crassus*, which can be obtained from any somatic tissue of either sex (most often gills, foot, and recently haemolymph). In contrast, studies on M genomes require DNA isolation from mature male gonads which

necessitates collecting material during the reproductive season and killing the animal. Analysis of 13 specimens of *U. crassus* from northern Poland (Brda River) showed a 3% differentiation within mitochondrial gene *cox1* (F haplotypes) (Soroka, 2010a; Mioduchowska *et al.*, 2016). Earlier sequencing of a single specimen from Sweden provided female sequences for mitochondrial genes *cox1* and *16S rRNA* and for nuclear non-coding regions *ITS1* and *ITS2* (Källersjö *et al.*, 2005). This year's studies hold in Poland and Lithuania concerned different molecular markers: nuclear ITS region and mitochondrial genes (*cox1* and *nd1*), and confirmed the existence of two genetically different lineages of different geographical distribution (Kilikowska *et al.*, 2020). Based on BLAST comparative analysis of sequences deposited in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) the similarity of European *U. crassus* from Poland, France, Sweden and Ukraine within mitochondrial gene *cox1* (F haplotype) is 100–97% (or differentiation up to 3%). The differentiation between *U. crassus* and two other species: *U. delphinus* and *U. pictorum*, is 90% and 89%, respectively. The Polish populations of *U. crassus* show the same level of differentiation as remote European populations (up to 3%). This indicates that they are genetically much varied, not under the effect of genetic drift and can be used as source populations in conservation practices.

GenBank data on another often studied mitochondrial gene *16S rRNA* are available for specimens from eight European populations (own analyses with software MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets, Kumar *et al.*, 2015). No variation was observed among the mussels from Germany and Poland, and the differentiation was the greatest in Greece and Sweden (Tab. 2). The mean genetic differentiation of *U. crassus* in Europe is 1% (0–3%), and among its populations (mean inter-population diversity) it is 2.2%, which is correlated with the geographical distance (e.g. Sweden and Greece). This is of great significance for species conservation, and restitution should take place in the nearest vicinity of the existing populations. Within this gene, *U. crassus* displays a 15% differentiation in relation

to *U. delphinus* and *U. pictorum* and 17% in relation to *U. tumidus* (Blast analysis).

GenBank sequences available for mitochondrial gene *cytb* pertain exclusively to Polish specimens of *U. crassus* which show an over 3% differentiation among themselves and ca. 15% differentiation compared with *U. delphinus* and *U. pictorum* (own analyses with Mega 7.0 software).

Studies of nuclear genome of *U. crassus* pertain only to gene *28S rDNA* for more than 200 specimens from France, to non-coding regions ITS1 and ITS2 (for one specimen from Sweden) and to microsatellite loci from populations in Poland, Germany and Sweden (Källersjö *et al.*, 2005; Sell *et al.*, 2013; Prié and Puillandre, 2014; Feind *et al.*, 2017).

An excellent instrument for ecological, population genetics and conservation management studies is provided by nuclear polymorphic microsatellite loci. However, they require analysis of numerous specimens and multiple loci. In 2013 they were for the first time devised for populations of *U. crassus* from the north (Drawa River, 27 specimens) and south (San River, 30 specimens) of Poland (Sell *et al.*, 2013). Subsequent analyses of microsatellite markers showed genetic and spatial differentiation of populations of *U. crassus* into northern ones (including Sweden, northern Germany and Poland) and southern ones (southern Germany and Poland), suggesting two post-glacial refugia of the species in Europe (Feind *et al.*, 2017).

Genetic research on the thick-shelled river mussel is essentially of basic character, but in recent years it has become intensified with focus on conservation genetics (Sell *et al.*, 2013; Douda *et al.*, 2014; Mioduchowska *et al.*, 2016; Feind *et al.*, 2017). Its main aims are recognising of the gene pool, structure and level of variation of the populations for the purposes of conservation and restitution of the species.

4 Genetic studies and species conservation

Genetic studies on *U. crassus* include, among other aspects, phylogenetic and taxonomic analyses of bivalves of the order Unionida (Källersjö *et al.*, 2005; Doucet-Beaupré *et al.*, 2010; Huang *et al.*, 2013; Prié and Puillandre, 2014; Burzyński *et al.*, 2017; Fonseca *et al.*, 2016; Klishko *et al.*, 2017; Lopes-Lima *et al.*, 2017b). The results are used for species identification and for resolving phylogenies at higher taxonomic levels within a new discipline of biology called integrative taxonomy (Dayrat, 2005). The discipline includes traditional taxonomic tools and museum collections, knowledge of phylogeography, phylogeny and evolution, and in particular techniques of sequencing and visualisation, for example computer tomography. Such a complex approach was used to update the knowledge of unionid occurrence and distribution in Russia and Kazakhstan, including ascertaining of the eastern distribution border of *U. crassus* (genetic studies based on genes *cox1*, *16S rRNA* i *28S rDNA*) (Bolotov *et al.*, 2020).

Adequate classification of taxa guarantees conservation of the largest possible gene pool and preservation of rare taxa. This is also true of the genus *Unio*, which is now undergoing systematic revision using, among other methods, genetic approach (Araujo *et al.*, 2005, 2009, 2018; Reis and Araujo, 2009; Prié *et al.*, 2012; Fonseca *et al.*, 2016; Froufe

et al., 2016). Prié and Puillandre (2014) proposed a division of *U. crassus* in two subspecies which in the future will probably turn out to be distinct species. The results of molecular analyses suggest the existence of two genetically different clades: *Unio crassus crassus* and *Unio crassus* cf. *courtillieri* Hattemann, 1859. Both forms occur in Poland (Mioduchowska *et al.*, 2016), and in some other European countries (Klishko *et al.*, 2017; Kilikowska *et al.*, 2020). Mioduchowska *et al.* (2016) described two haplogroups corresponding to clades: the first clade – haplogroup I – represented by individuals from northern Poland (*Unio crassus* cf. *courtillieri*), the second clade – haplogroup II – with individuals from southern Poland (*Unio crassus crassus*). The localities in central Poland hold individuals of both clades – haplotypes. Kilikowska *et al.* (2020) used different molecular markers (ITS region of nuclear ribosomal DNA and mitochondrial genes *cox1* and *nd1*) to study inter-population genetic differentiation of *U. crassus* at multiple spatial scales (within rivers, among rivers within catchment areas, and between catchment areas of the Neman and Vistula rivers in Lithuania and Poland). They found a high genetic differentiation between the two catchment areas, indicating two different genetic units, probably of species rank in the future.

Successful conservation of bivalves necessitates precise information on their occurrence and distribution. However, gaining such information is time-consuming and extremely laborious – the bivalves frequently occur in places which are difficult of access, and their identification often requires an expert (Lamand and Beisel, 2014; Leppänen, 2019). The method of assessment of bivalve species diversity used in recent years, especially for surveying and monitoring endangered species, and for early detection of introduced alien and invasive species, is environmental DNA (eDNA) metabarcoding (Stoeckle *et al.*, 2016; Blackman *et al.*, 2020; Egeter *et al.*, 2020; Gasparini *et al.*, 2020; Prié *et al.*, 2020). Surveys based on eDNA sampling have potential for applied conservation through detection of rare or cryptic species that may be overlooked with standard methods.

In the case of aquatic species genetic material for metabarcoding analyses is obtained through filtration of large quantities of water from the habitat which harbours the organisms in question. Their identification is based on polymerase chain reaction (PCR) which makes it possible to detect and quantify species-specific DNA sequences. Primers for mitochondrial gene *16S rRNA* were used in the studies on European freshwater bivalves, and the result was on average 137 and 128 bp for the Unionida and the Venerida, respectively (Prié *et al.*, 2020). Using eDNA metabarcoding Prié *et al.* (2020) described an array of new localities of *U. crassus* in France.

A molecular key was constructed for eight species of the Unionida of Northern and Central Europe (*Unio crassus*, *U. pictorum*, *U. tumidus*, *Margaritifera margaritifera*, *Anodonta anatina*, *A. cygnea*, *Pseudanodonta complanata* and *Sinanodonta woodiana*) (Zieritz *et al.*, 2012). It makes it possible to identify glochidia from infected fish, as well as juveniles and adults. The molecular key also contains protocols for fast isolation and amplification of DNA of a larval bivalve from the host's branchial arches. It uses the PCR-RFLP (Restriction Fragments Length Polymorphism) method, based on amplification of nuclear non-coding markers ITS (Internal

Transcribed Spacer) followed by their digestion with restriction enzyme. The method made it possible to identify 90 samples from adult bivalves with 100% certainty (Zieritz *et al.*, 2012). ITS regions are characterised by fast evolution rate and are thus used in phylogenetic studies at low taxonomic levels: genus, species and even population.

The life cycle of *U. crassus* includes metamorphosis of glochidium on the appropriate fish species; the tissue compatibility of the mussel population and their potential hosts guarantees successful metamorphosis (Douda *et al.*, 2014). This was the focus of studies on mtDNA (*cox1* and *nd3-tRNA-nd2* fragments) and 12 polymorphic microsatellite loci of *U. crassus*, with material obtained *in vivo* (Sell *et al.*, 2013). They showed a variation in the host–parasite interactions through individual experimental testing of host compatibility, which indicated the presence of different management units within a single river basin. The populations differed in their ability to infest various host species, which may have a major influence on their reproductive success under a common management regime. The inter-population differences in this critical ecological trait were accompanied by differences in neutral genetic markers and in morphometric characters. The genetic data indicated significant reproductive isolation of the two populations, which may have allowed adaptive or random changes responsible for the observed differences in their host compatibility. Hence the origin of both bivalves and their hosts should be taken into account when planning conservation of *U. crassus*.

A new approach to conservation of freshwater mussels includes genetic studies of their endosymbionts, parasites and pathogens which affect the condition of their hosts and whole populations. An example is the occurrence of bacteria of the genera *Cardinium* and *Wolbachia* inside the cells of *U. crassus* (Mioduchowska *et al.*, 2020b). These endosymbionts are known to have an effect on the host's reproductive success through cytoplasmic incompatibility, killing of developing males, induction of parthenogenesis and feminisation. Another example is provided by studies on the microbiome, *i.e.* profile of symbiotic organisms of the alimentary tract of *U. crassus* (Mioduchowska *et al.*, 2020a). Molecular identification of the bacteria was performed with the technique of high-throughput sequencing of the V3-V4 hypervariable regions in the bacterial *16S rRNA* gene fragment. The alimentary tract microbiome of *U. crassus* varies widely among the populations in the studied rivers, suggesting adaptation to local conditions. This is why the kind of co-occurring organisms, infestation and the character of bivalve microbiome should also be considered while planning effective conservation of the species.

5 Species protection and restitution in Poland

Unio crassus requires strict protection in many countries of the European Union, though in the eastern part of its range it is not threatened (Bolotov *et al.*, 2020). Accordingly, it was listed in Annexes II and IV of the Habitats Directive. It was also placed on the IUCN Red List as worldwide threatened, and has the same status in Poland in the Red Book of Animals (Zajac, 2004; Mioduchowska *et al.*, 2016; Lopes-Lima *et al.*, 2017a).

The Directive obliges the EU countries to protect a representative part of populations of the species from Annex II and to designate protected areas within the network “Natura 2000”. The objective is to maintain the population of the protected species and its habitats in adequate state of conservation. The Directive points to the necessity of monitoring based on assessment of the state of populations in designated localities and assessment of their habitats. The procedures should not be invasive and should not cause habitat changes. This kind of monitoring is practiced in 28 EU countries and in the UK.

The monitoring concept adopted in Poland is based on quantitative assessment done directly in the wild using the same, reproducible methods; it makes it possible to observe demographic changes in populations in individual localities and habitats. Indices which are treated as equivalent are used for the assessment, and they include for example abundance in the locality, age structure, or habitat indices such as settlement of a river section or anthropogenic changes (Zajac, 2010).

In the case of the thick-shelled river mussel the choice can not be random; the species never occurs along the whole length of the watercourse. It is suggested that monitoring should include 1/3 of the Polish rivers (ca. 30), and the selection should consider the species' distribution countrywide (Zajac, 2010).

Despite the occurrence of *U. crassus* in protected areas: national parks (*e.g.* Wigry and Drawa National Parks), landscape parks (*e.g.* Suwałki Landscape Park) and nature reserves, there is no action plan for the species as well as no conservation measures aimed especially at the thick-shelled river mussel protection. The area protection within the parks is not enough on counteracting the negative effects on the species (Zajac, 2004). For example, localities of *U. crassus* in the Nida River (tributary to upper Vistula), which flows through a landscape park, were found to disappear (Lewandowski, 2004). Likewise, designation of 68 areas of habitat and species protection within the EU programme “Natura 2000” is insufficient for improvement of the situation of *U. crassus* in Poland (Zajac, 2018). The matter is additionally complicated by the recent discoveries of genetic diversity of *U. crassus* in Europe and it is not excluded that it is a complex of species with two distinct taxa (Mioduchowska *et al.*, 2016; Kilikowska *et al.*, 2020). Little is known about the possible differences between these taxa, especially with respect to their host species.

Conservation procedures for the thick-shelled river mussel should not be limited to passive protection and monitoring, but should include actions aimed at improvement of water and habitat quality and preservation of their natural character. An important aspect is the effect on the ichthyofauna of the rivers which hold *U. crassus*, due to the life cycle including glochidium which requires appropriate fish hosts to metamorphose. The absence of adequate hosts may lead to disappearance of populations of the species (Douda *et al.*, 2012a,b; Taeubert *et al.*, 2012a,b; Ćmiel *et al.*, 2018; Schneider *et al.*, 2019). It was found that larvae of *U. crassus* failed to metamorphose on roach (*Rutilus rutilus*), which is a very common species (Taeubert *et al.*, 2012b). Likewise, glochidia fail to metamorphose on bitterling (*Rhodeus amarus*). The species is protected in the EU, and the number of its localities in Poland is increasing. Its presence in the habitat may even

have a negative effect on the bivalve's reproduction success. This is a result of the bitterling's mode of reproduction: laying eggs into the bivalve's mantle cavity where the embryos develop till hatching, deteriorating the bivalve's condition and the conditions of larval development (Reynolds *et al.*, 1997; Tatoj *et al.*, 2017).

Because of canalisation of many rivers in Poland, *U. crassus* does not encounter conditions which would favour population rebuilding. Despite the constant improvement of water quality in Poland, transport of glochidia by fish over long distances is very difficult and the possibilities of colonisation of new localities are limited. Despite the measures taken to reduce the harmful effect of anthropogenic changes, the reconstruction rate of populations of *U. crassus* is not fast enough not only in Poland but in the whole of Europe (Bogan, 2008; Lopes-Lima *et al.*, 2017a).

The anthropopressure dates back to the Neolithic and has been increasing since the industrial revolution; attempts at counteracting its negative effects are numerous. It has led to threat to or even extinction of many plant and animal species. One of the measures taken in attempt to counteract the process is protection of biocoenoses and restitution of endangered species. Restitution includes procedures which make it possible to restore the endangered species in its natural habitats. The procedures are, among others, artificial breeding in order to increase the species' abundance and, at least to a certain extent, reconstruction of the historic distribution range. For this purpose artificially bred individuals are reintroduced in the localities where the species lived prior to extirpation. Most often such procedures are supplementary to conservation of the still existing populations in their natural habitats.

Restitution-reintroduction programmes in nature conservation have been used for a long time. Numerous programmes of this kind helped prevent extirpation of some species or reinforce their natural populations. Unfortunately not all attempts at species restitution are successful. Restitution of the freshwater pearl mussel *Margaritifera margaritifera* (L.) failed and the species is now regarded as extinct in Poland (Każmierczak, 1966; Zajac, 2009; Piechocki and Wawrzyniak-Wydrowska, 2016). Reasons for such situations are always complex and may partly result from the absence of advanced research, including genetic studies.

Active protection of *U. crassus* should consider results of extensive research as well as actions aimed at restitution of the species and increasing its abundance in the wild. In Poland some populations of *U. crassus* are sufficiently abundant to be able to play an important role as source populations in the process of restitution. Individuals selected from these populations are placed in special breeding stations to reproduce (Ćmiel *et al.*, 2018). The breeding station consists of modules. In one type of oxygenated water containers, mussels and fish are placed to attach glochidia to the fish. The second type of modules are containers in which pediveligers (young mussels) that fall off from fish after transformation are collected. Then the young mussels are bred in long containers with flowing water and sediment. The water used in the breeding station is supplied from the river. The resulting large numbers of juveniles are placed in habitats in which the conditions have improved and offer a chance of restitution. Restitution should necessarily be preceded by genetic studies of natural populations in order to preserve the existing genetic

diversity of the species in both the original and the newly introduced populations. Besides, important factors are close geographical location and similar habitat conditions of the source population and the site of restitution. The new population should be as similar as possible to the extinct one.

An example of such an extended strategy is the project "Restoring patency of ecological corridor of the Biała Tarnowska River valley", of 2010–2014, by the Regional Water Management Board in Krakow in cooperation with the Institute of Nature Conservation, Polish Academy of Sciences. The tasks realised by the Institute included re-colonisation (reintroduction) of the population of *U. crassus* in a tributary of the Dunajec River (Vistula catchment area). The thick-shelled river mussel populations in the Biała Tarnowska catchment area, as in the whole Carpathians, were sparse and unevenly distributed, and the lack of protection measures might have led to complete extirpation of the species in the area. The multi-directional tasks were aimed at maintaining and connecting these habitats through genetic studies of source populations, artificial breeding of the mussels and introduction of juveniles and gravid females with glochidia into natural or re-naturalised sections of the Biała Tarnowska. The project is now being continued by the Polish Waters through improvement of living conditions of fishes, including host species for glochidia of *U. crassus*. The river channel is being made patent – obstacles across the channel are being modified or removed in order to facilitate fish migrations. This is of special significance for nase *C. nasus*, which migrates upstream to spawn and is an important host to the thick-shelled river mussel glochidia.

The project was preceded by inventory of the Biała Tarnowska with its tributaries, which made it possible to ascertain in which sites the species had become extinct or the population continuity disrupted, and to assess the abundance of the existing populations. The greatest concentration of the mussel was observed in the Biała River, in its natural, non-canalised section with slow flow (Zajac *et al.*, 2018a,b). Single individuals were recorded in mouth sections of the remaining tributaries. The only fairly dense population was found in the tributary Bieśninka, but the observed considerable discontinuities in the age structure (absence of some age classes) indicated some seasons without recruitment. In the remaining populations from the region of Tuchów the distribution of age classes was continuous (Zajac *et al.*, 2018a,b).

Genetic analysis of these two populations from the catchment area of the Biała Tarnowska showed that they formed distinct units, as indicated by the presence of private alleles. The large population from the Biała contains the total of 4 alleles in each of 5 loci, and the population in the Bieśninka only 1 allele per locus. At the level of genetic analysis it does not suggest any significant phenomena, but combined with the unbalanced age structure in the Bieśninka it indicates frequent periods of low population abundance. In such periods genetic drift eliminates private alleles, which leads to a reduction of genetic variation in the population. This causes deterioration of regeneration potential of individuals and populations and starts the so called extinction vortex. Further isolation of the Bieśninka population from the abundant population in the Biała might accelerate the process.

In the light of the available results the population of *U. crassus* from the Biała Tarnowska is part of a homogeneous genetic group which occurs in the whole Carpathians

(Sell *et al.*, – unpublished data). Hence the adults for breeding were selected from the most abundant populations in the rivers of the Carpathians and their foothills. The source populations did not differ significantly in terms of their gene pool. The mussels were taken from colonies which were threatened, for example by necessary hydrotechnical works or from very abundant populations which would not be affected by removal of some individuals.

A special breeding station was created for the purpose of thick-shelled river mussel breeding, and a device was constructed which made it possible to breed the mussels in the river channel. At the same time hydromorphological survey made it possible to select appropriate localities for the reintroduction of *U. crassus*. The presence of host fish species was also taken into consideration. It was also found that the fish species with the highest survivorship of thick-shelled river mussel glochidia was nase *C. nasus*. Four procedures were used in order to increase the chances of successful reintroduction:

- introduction of adult females with glochidia-filled marsupia,
- release of glochidia-infested fish,
- introduction of juvenile mussels from *ex situ* breeding (adult females with glochidia-filled marsupia were obtained in the field, the hosts were infested with glochidia which then metamorphosed in the breeding station)
- at the same time juveniles were also obtained from the experimental *in vitro* culture – glochidia were obtained in the wild, and placed in a special medium which makes it possible to metamorphose without host (Gąsienica-Staszczek *et al.*, 2017).

Subsequent monitoring of the localities in 2013–2014 confirmed the presence of juvenile individuals in the places of introduction of adult females (Zajac *et al.*, 2018a), indicated an unaided maintenance of the population, and thus successful re-colonisation by the thick-shelled river mussel in the Biała Tarnowska and its tributaries. Both breeding methods are effective, but it would be better if the mussels reproduction and juveniles growing take place in the river in the wild. A promising method in this case would be the introduction of *ex situ* infected hosts, but this requires further research and standardization.

All the procedures mentioned above (introduction gravid females as well as juvenile mussels from *ex situ* breeding and release of glochidia-infested fish to the river) used were successful as indicated by the increased number of the thick-shelled river mussel localities, increase in abundance of the populations and the twofold expansion of the species' distribution range in the valley of the Biała Tarnowska as it has been showed by results of the monitoring and reported in the publications (Ćmiel *et al.*, 2018; Zajac *et al.*, 2018a, 2019). Based on the results and experience, other projects aimed at conservation of freshwater bivalves are currently under way. One of them is project LIFE17 NAT/PL/000018 “Renaturalisation of the inland delta of the Nida River”, which is mostly financed from the EU fund LIFE and the National Fund for Environment Protection and Water Management in Warsaw. Its main objective is improvement of hydrological conditions of the inland delta of the Nida

River which should contribute to restoration of unique natural values, including restitution of the thick-shelled river mussel populations.

The results of conservation procedures applied in the restitution of *U. crassus* in Poland are very promising and can be used in further restitution of the species elsewhere in Europe. Though they pertain to only one bivalve species, they provide a very good example of planned actions with the use of conservation genetics and may aid conservation of other endangered species of animals.

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