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CHARACTERISTICS OF MITOCHONDRIAL DNA OF UNIONID BIVALVES (MOLLUSCA: BIVALVIA: UNIONIDAE). I. DETECTION AND CHARACTERISTICS OF DOUBLY UNIPARENTAL INHERITANCE (DUI) OF UNIONID MITOCHONDRIAL DNA

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ABSTRACT: Doubly Uniparental Inheritance (DUI), a peculiar way of inheritance of mitochondrial DNA in animals, has been detected in seven families of marine and freshwater bivalves, including Unionidae. DUI involves two independently inherited mitochondrial genomes: maternal (F genome) and paternal (M genome), which show different tissue localisation and wide genetic variation. F genomes occur in somatic tissues of both sexes and are inherited maternally (Strict Maternal Inheritance, SMI). M genomes are located in male germ cells and transmitted to next generations along the male lineage, i.e. from fathers to male offspring. The objective of this study was detection of M genomes and characteristics of DUI in unionid bivalves from Poland, based on sequential analyses of seven mitochondrial genes. This is the study to analyse F and M haplotypes at intra- and interspecific level in seven species of freshwater mussels. DUI was first observed in species of the genus *Unio* (*U. crassus*, *U. pictorum* and *U. tumidus*), and the best M haplotype marker was gene *cox1*. In the studied bivalves F and M sequences showed a similar intraspecific variation, with differences among the genes. Three *tRNA* genes showed the smallest (ca. 20%) nucleotide variation, followed by the gene coding for RNA for the small ribosomal subunit, *srRNA* (24%); a significantly greater variation (exceeding 30%) was recorded for protein-coding genes (*cox1*, *cytb*) and the gene coding for RNA for the large ribosomal subunit, *lrRNA*. Interspecific variation of F sequences of the studied unionids ranged from 5% for *tRNAs* to 18% for *cytb*. Higher values were observed for M sequences: from 7% for *tRNAs* to 19% for *cox1*. The Chinese mussel occurring in Poland, despite the morphology-based identification as *Anodonta / Sinanodonta woodiana*, proved to be genetically more similar to *A. arcaeformis* than to Asian specimens of *A. woodiana*. Phylogenetic analyses showed that in the genus *Unio* the youngest species were *U. pictorum* and *U. mancus*, and the earliest species was *U. tumidus* showing the greatest genetic distinctness.

KEY WORDS: Doubly Uniparental Inheritance (DUI), mtDNA, rRNA, tRNA, freshwater mussels, Unionidae

INTRODUCTION

Besides large molecules of nuclear DNA, eukaryotic cells contain closed circular molecules of cytoplasmic DNA, located in mitochondrial matrix and, in plants, also in chloroplasts (cpDNA). The content of mitochondrial DNA (mtDNA) in animal cells is constant, of ca. 1–2% of total DNA. The size of animal mitochondrial genomes varies and is not associated with the degree of complexity of the organism; it ranges from 11,153 bp in the rotifer *Brachionus plicatilis* to 32,115 bp in the bivalve *Placopecten magellanicus* (GISSI et al. 2008). Most often, its size in animals is ca. 16,000 bp: for example in the marine blue mussel *Mytilus edulis* it is 16,740 bp (BOORE et al. 2004), in the freshwater bivalve *Lampsilis ornata* – 16,060 bp (SERB & LYDEARD 2003), and in man – 16,569 bp (ANDERSON et al. 1981).

In animals typical mitochondrial DNA (mtDNA) contains 37 genes encoding 13 protein subunits of

the enzymes of the respiratory chain complexes, the two rRNAs of the mitochondrial ribosome, and the 22 tRNAs necessary for the translation of the proteins encoded by mtDNA (AVISE 1986, MORITZ et al. 1987). This typical number of 37 genes is usually constant in animals, and their homologues have been identified in plant, fungal and protist mtDNA (BOORE 1999). In mitochondrial genomes all genes can be located on one DNA strand, e.g. in nematodes (OKIMOTO et al. 1992), annelids (BLEIDORN et al. 2006) or marine bivalves (MIZI et al. 2005, MILBURY & GAFFNEY 2005, ZBAWICKA et al. 2007). Most often however the genes are located on both strands of mtDNA, with most of them on the heavy strand (H strand), e.g. in man (ANDERSON et al. 1981), or on the light strand (L strand), in unionid bivalves (SERB & LYDEARD 2003, BRETON et al. 2009, SOROKA & BURZYŃSKI 2010). Animal mtDNA includes a single large non-coding region, whose components control replication and transcription, though it has not been identified in all the studied genomes (SHADEL & CLAYTON 1997, BOORE 1999, BRETON et al. 2009). This control region has ca. 800 bp; in vertebrates, echinoderms and molluscs it is known as "D-loop", in insects as "A+T-rich" (AVISE et al. 1987, WENNE 1993, PODSIADLOWSKI 2006, PODSIADLOWSKI et al. 2006, COMANDI et al. 2009).

Mitochondria and, consequently, mitochondrial DNA have endosymbiotic origin from photosynthetic purple bacteria which entered into symbiosis with ancestors of Eukaryota, and then lost their autonomy through the reduction of their genome and transfer of their genes to the host's nucleus (MARGULIS 1981, KURLAND & ANDERSSON 2000). The endosymbiotic concept has been corroborated with molecular studies. Sequential analyses of ribosomal RNA (rRNA) in mitochondria have shown a similarity with rRNA sequences in prokaryotes of the α-proteobacteria group, suggesting monophyly (KRZANOWSKA 2002, GOLIK 2009).

As a consequence of its endosymbiotic origin, mitochondrial DNA bears many characters of bacterial genome, it does not conform to an array of rules of transfer of genetic material which are characteristic of nuclear genes, and shares no common past with these genes. Compared to nuclear DNA, mtDNA contains significantly less genetic information (only about 0.00055% of the total human genome), has no post-replication mechanisms and its replication, transcription and translation depend on the nuclear DNA (BALLARD & WHITLOCK 2004). Mitochondrial DNA does not conform to the rule of universality of genetic code (except plants), since some codons have a different meaning than in the nuclear DNA (KRZANOWSKA 2002, GOLIK 2009). Textbook examples are different stop codons and methionine codon. Genetic code of mitochondrial genes in *Drosophila* is the same as that in mammals, with very few exceptions (WENNE 1993). The observed differences in the mitochondrial code may be remains of the code that was present in organisms which through endosymbiosis gave rise to mitochondria of eukaryotic cells. It can not be excluded however that these are secondary changes, since departures from the mitochondrial code in insects (e.g. *Drosophila*) and mammals have been observed also in Protista (e.g. *Paramecium*) and Echinodermata (HIMENO et al. 1987, KRZANOWSKA 2002).

The evolution of eukaryotic cells includes cases of unilateral transfer of genetic information from mitochondrial to nuclear DNA, hence the presence of mitochondrial pseudogenes in the nucleus (nuclear pseudogenes of mitochondrial origin, numts) (LOPEZ et al. 1994, ZOUROS 2000, BENSASSON et al. 2001). Numts occur more often in plants than in animals and have been found in nuclear genomes of more than 64 species, including man (for review see BENSASSON et al. 2001), but they are absent in the marine bivalve *Mytilus trossulus* (ZBAWICKA et al. 2007). Till 2000 it was believed that, contrary to nuclear DNA, mtDNA did not undergo recombination. However recently, the body of evidence supporting this phenomenon in animal mtDNA has been increasing (LADOUKAKIS & ZOUROS 2001, PIGANEAU et al. 2004, SHAO et al. 2005, TSAOUSIS et al. 2005, CIBOROWSKI et al. 2007), including marine bivalves (LADOUKAKIS & ZOUROS 2001, BURZYŃSKI et al. 2003).

Nuclear and mitochondrial genomes differ also in the mutation rate which in mtDNA is 10 times greater than in the single-copy fraction of nuclear genome, and in higher primales it is ca. 2% per 1 million years (BROWN et al. 1979). However these data can not be applied to all species and their mitochondrial genomes, since the mutation rate varies with the site in the mtDNA, and even with the technique applied to evaluate it. In mitochondrial protein-coding genes synonymous (silent) substitutions are 4–6 times more frequent than substitutions causing a change in the encoded amino acid, and tRNA genes evolve at least 100 times faster than their nuclear counterparts. The fast evolution rate is also characteristic of non-coding sequences of mtDNA (for review see AVISE 1986). Other data suggest than in *Drosophila* the rate of synonymous substitutions in mtDNA is 1.7–3.4 times greater than in the fastest-evolving nuclear genes and 4.5–9.0 greater than in the average nuclear genes (BALLARD & WHITLOCK 2004). Animal mtDNA contains no introns, repetitive DNA, pseudogenes and mobile elements (AVISE 1986, AVISE et al. 1987, MORITZ et al. 1987). Also, intergenic sequences are generally small or absent (AVISE 1986, MORITZ et al. 1987) and biases toward A/T-ending codons have been observed (BALLARD & WHITLOCK 2004). Mitochondrial DNA is haploid and uniparentally inherited, contrary to the biparental inheritance of nuclear DNA.

In animals, including man, maternal inheritance of mitochondrial DNA is commonly known (SMI –

Strict Maternal Inheritance); in SMI species all copies of mitochondrial genome in the organism's cells are identical in length and sequence of DNA, and the phenomenon is called homoplasmy. Mutations of mitochondrial DNA, being more frequent than in nuclear DNA, may result in appearance of its different forms in the organism, which was termed heteroplasmy. These forms arise independent of sex and may affect the functioning of the organism, but only the forms that arose in the female germ cells will be transmitted to the next generation. This maternal inheritance of mtDNA occurs also in plants, except sequoia in which both mitochondrial and chloroplast DNA is paternally inherited through pollen (NEALE et al. 1989). Sex-dependent heteroplasmy with two different forms of mtDNA transmitted to the next generations both maternally and paternally was first described in marine bivalves of the genus *Mytilus* in 1990 and called DUI, doubly uniparental inheritance (FISHER & SKIBINSKI 1990). Besides three species of *Mytilus*, DUI was detected in a few species representing six bivalve families, and is the most frequent in Unionidae, which have recently been intensely studied in this respect (HOEH et al. 1996a, b, LIU et al. 1996, PASSAMONTI & SCALI 2001, CUROLE & KOCHER 2002, 2005, KREBS 2004, MOCK et al. 2004, WALKER et al. 2006a, CHAKRABARTI et al. 2006, THEOLOGIDIS et al. 2008, SOROKA 2008a, b, BRETON et al. 2009, DOUCET-BEAUPRÉ et al. 2010).

In the case of DUI there are two types of mitochondrial DNA – 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 type of mtDNA – F type, which they have inherited from the mother and which they transmit to the next generations through female offspring. Males are heteroplasmic and have both forms of mtDNA. Mitochondrial genome of M type is located in male gonads; males receive it from the father and transmit it to their male offspring. In their somatic tissues males have F type DNA inherited from their mother and not transmitted to the next generations. The two mitochondrial genomes in bivalves are of similar size, close to that in birds and mammals, but evolve faster, and M type evolves faster than F type (STEWART et al. 1995, HOEH et al. 1996b, ZOUROS 2000). The presence of DUI in seven phylogenetically remote families (Mytilidae, Unionidae, Margaritiferidae, Hyriidae, Veneridae, Donacidae and Solenidae) suggests that the phenomenon may be widespread among bivalves (SKIBINSKI et al. 1994, ZOUROS et al. 1994, RAWSON & HILBISH 1995, HOEH et al. 1996a, LIU et al. 1996, PASSAMONTI & SCALI 2001, HOEH et al. 2002b, CUROLE & KOCHER 2005, WALKER et al. 2006b, THEOLOGIDIS et al. 2008, SOROKA 2008a, b). Though this unusual way of mtDNA inheritance (DUI) occurs mainly in marine and freshwater bivalves, the two groups show marked differences in the characteristics and fidelity of transfer of the two mitochondrial genomes. For this reason the mechanism of DUI and its evolutionary significance remain unknown and there is need for detection and characteristics of this model of inheritance of mtDNA in possibly the greatest number of species which will no doubt provide new and significant information.

The genetic divergence between F and M types observed in the marine genus *Mytilus* is 2–20% for a fragment of gene *cox1* (STEWART et al. 1995, HOEH et al. 1996b, 2002b) and 21–26% for entire genomes (MIZI et al. 2005, ZBAWICKA et al. 2007, 2010). Besides the male gonad, M haplotype has been observed in small quantities also in the male somatic tissues and in few female tissues in such species as *M. edulis* and *M. trossulus* (GARRIDO-RAMOS 1998, DALZIEL & STEWART 2002). In the venerid clam *Tapes philippinarum* M type of mtDNA has been observed as dominant in somatic tissues (PASSAMONTI & SCALI 2001). Heteroplasmy of mtDNA observed in marine bivalves pertains to both the sequence (in *M. edulis)*, and molecule length (in *M. trossulus*) (WENNE 1993). Heteroplasmy of the length of mtDNA is evolutionarily younger than the sequence heteroplasmy, and variants of mtDNA size may arise within one generation through addition or deletion of repeatable sequences. Maintaining of heteroplasmy is associated with inheritance of male forms of mtDNA and replication supremacy of some variants of mtDNA (WENNE 1993). The frequency of heteroplasmy in *M. trossulus* from the Gulf of Gdańsk, Poland is 32.7% of males and 3.5% of females, and the phenomenon is not a result of hybridisation (WENNE 1993). In the case of marine bivalves there is also evidence for recombination, and reversal or masculinisation events between these molecules of mtDNA, hence in phylogenetic analyses the two mtDNA molecules may not form separate clades (HOEH et al. 1996b, 1997, 2002a, QUESADA et al. 1999, BURZYŃSKI et al. 2003, BRETON et al. 2007).

Masculinisation (role-reversal events) consists in the loss of the male genome by males and its replacement with female genome which is then transmitted to male offpsring (ZOUROS et al. 1994, SAAVEDRA et al. 1997, QUESADA et al. 1999). Following masculinisation, the difference between male and female genomes decreases to zero and their divergence starts de novo (HOEH et al. 1996a, 1997). Numerous such cases have been described in *Mytilus*, where some male haplotypes are more closely related to female haplotypes than to other conspecific male haplotypes (HOEH et al. 1996a, 1997). Masculinised genomes have become fixed at least six times in the evolutionary history of DUI: four within the Mytilidae, one before the origin of *Geukensia*, and one before the origin of Unionidae (HOEH et al. 1997). Breakdown of DUI is often observed in hybridization zones of *Mytilus*, e.g. in *M. trossulus* (WENNE & SKIBINSKI 1996, QUESADA et al. 1999, ZBAWICKA et al. 2010). However, break-

down of DUI is not limited to hybridization events and appears to be male-dependent (ZOUROS et al. 1994, SAAVEDRA et al. 1997). These masculinisation events are common in marine bivalves but have not been reported in the freshwater bivalves despite their history of over 450 MYA, suggesting that the fidelity of DUI varies among the taxa (CUROLE & KOCHER 2002). CUROLE & KOCHER (2002, 2005) have found the presence of ca. 185-codon extension of *cox2* gene in male genomes of Unionidae in 12 species, which may be responsible for the absence of masculinisation in this taxon. In marine species *M. edulis*, *M. trossulus* and *M. galloprovincialis* no large insertions or deletions in protein-coding polymorphisms have been detected in F and M genomes (BOORE et al. 2004, MIZI et al. 2005, ZBAWICKA et al. 2007).

In freshwater bivalves (Bivalvia: Unionidae) the observed differences between F and M haplotypes are greater and range from 28% to 35% for genes *cox1* and *16S rRNA* (synonym of *lrRNA*), respectively (HOEH et al. 1996a, KREBS 2004, SOROKA 2008a, b). When the whole genomes are compared, the values reach 50–52% (DOUCET-BEAUPRÉ et al. 2010). As a result of the high diversity and the absence of masculinisation of mtDNA in freshwater bivalves, separate F and M clades are formed in phylogenetic analyses and can be independently used for phylogeny reconstruction (CUROLE & KOCHER 2002, 2005, HOEH et al. 2002a, MOCK et al. 2004, SOROKA 2005, 2008a, b).

The origin of DUI is probably fairly ancient, since differences between sex-dependent types precede the speciation processes leading to *M. edulis, M. galloprovincialis* and *M. trossulus*. Based on the first estimates of divergence of F and M genomes in the bivalves of the genus *Mytilus*, the age of DUI in the taxon has been estimated as ca. 5 mln years (RAWSON & HILBISH 1995, STEWART et al. 1995). With increasing knowledge of DUI it has become obvious that the M-F divergence is not proportional to the age of DUI, since masculinisation in marine bivalves leads to effective resetting of the M-F divergence (HOEH et al. 1996a). Such periodic disappearance of M-F divergence in marine bivalves makes it difficult to reliably estimate the age of DUI. Analyses of F and M mitochondrial types of freshwater bivalves, which show the greatest M-F divergence, suggest that the two genomes have been evolving independently for at least 100 mln years, and the DUI has functioned in Unionidae for at least 200 mln years (HOEH et al. 2002a). The DUI phenomenon is probably even older, and its age is estimated even as 450 MYBP (million years befor present), based on fossil record of Unionidae and Margaritiferidae, with the minimum divergence of 213 MYBP, when they separated in the Triassic (CUROLE & KOCHER 2002).

Initially there were two hypotheses explaining the occurrence of DUI in marine and freshwater bivalves. One assumed that DUI originated independently in three ancestral lineages leading to unionids, *Mytilus* and *Geukensia*. According to the other hypothesis DUI originated once in the ancestral bivalve lineage and was gradually modified, including masculinisation, or lost in some descendant lineages (HOEH et al. 1996a, b, 2002a). Discovery of the phenomenon in consecutive species and families confirmed the hypothesis of the unique origin of DUI in the ancestral autolamellibranch bivalve lineage (DOUCET-BEAUPRÉ et al. 2010). At present it is supposed that DUI arose as a modification of strict maternal inheritance of mtDNA (SMI) through the presence of a suppressor gene which was active during oogenesis, and the process is associated with sex determination (ZOUROS 2000). BRETON et al. (2007) suggest that DUI could first arise in simultaneously hermaphroditic bivalves, in which eggs and spermatozoa were simultaneously produced in the ovotestis (BRETON et al. 2007). Extant bivalves include both dioecious and periodically or permanently hermaphroditic forms (WALKER et al. 2006c, ŁABĘCKA 2009). The incidence of accidental hermaphroditism in bivalves is 1% (ZOUROS 2000), and in *Sinanodonta woodiana* it was estimated as more than 2% (ŁABĘCKA 2009). However, the mechanism of sex determination is not known in these cases. In species with maternal inheritance of mtDNA (SMI), there is a chromosome sex determination and most often it is male heterogamy, and mutations of mtDNA usually have a negative effect on males, especially their fecundity (BRETON et al. 2007). This has been documented for man and mouse (RUIZ-PESINI et al. 2000, NAKADA et al. 2006). It can be supposed that maintaining the male lineage of mtDNA in DUI bivalves makes it possible for the males to increase their fitness (BRETON et al. 2007). Thus DUI may be an alternative way for males of the species where there is no male heterogamy, i.e. male chromosome which could compensate for deleterious mutations taking place in the only, maternal, mtDNA. This is not contradicted by the most recent studies by BRETON et al. (2009), of December 2009, including analysis of all available F and M genomes in Unionidae. The studies have revealed the presence of additional mitochondrial gene which is sex-specific (one F-specific and one M-specific open reading frames) and activity of the proteins has been detected in male or female gonads, depending on sex (BRETON et al. 2009). It is suspected that these F-specific and M-specific genes play transmission and/or gender-specific adaptive roles of M and F mtDNA genomes in unionoid bivalves (BRETON et al. 2009).

Compared to marine bivalves, freshwater species are much less thoroughly studied with respect to DUI. Most researchers of freshwater bivalves use somatic tissues as the source of DNA, and thus obtain exclusively F haplotype of mtDNA, ignoring the possibility of identifying M haplotype and detecting DUI (STĘPIEŃ et al. 1999, GRAF & Ó FOIGHIL 2000a, GIRIBET & WHEELER 2002, LEE & Ó FOIGHIL 2004, ARAUJO et al.

2005, 2009, KÄLLERSJO et al. 2005, SOROKA 2005). In order to obtain M type in Unionidae, DNA should be isolated from male gonads (HOEH et al. 2002b, MOCK et al. 2004, WALKER et al. 2006a, b, SOROKA 2008a, b).

Among unionids, which comprise more than 600 species, DUI has been described in over 50 species based on analyses of single mitochondrial genes, *cox1*, *cox2, cytb* and *16S rRNA* (HOEH et al. 1996b, 2002b, LIU et al. 1996, CUROLE & KOCHER 2002, 2005, KREBS 2004, MOCK et al. 2004, CHAKRABARTI et al. 2006, WALKER et al. 2006a, b, SOROKA 2008a, b). Among these species only *Anodonta anatina* and *Sinanodonta woodiana* (sometimes included in the genus *Anodonta*) occur in Poland and data on these species come from the author's earlier studies (Tables 1, 2) (SOROKA 2008a, b). Only the Chinese mussel *S. woodiana,* introduced in Poland in the 1980s, was earlier studied by researchers from Japan and South Korea, but the data were never published (F and M sequences deposited in GenBank, Table 1). DUI has not been detected in any member of the genus *Unio*, though it is regarded as characteristic of the family Unionidae (WALKER et

Table 1. Sequences available in GenBank for mitochondrial genes *16S rRNA* (synonym of *lrRNA*), *cox1* and *cytb* in seven species of Unionidae. Sequence length in bp given in brackets. F – female haplotype of mtDNA, M – male haplotype of mtDNA. The data do not include own sequences

Species	*16S rRNA*	*cox1*	*cytb*
Unio crassus Philipsson, 1788	DQ060162 (521)[a]	DQ060174 (615)[a]	no data
Unio pictorum (Linnaeus, 1758)	DQ060163 (518)[a]	AF231731 (630)[b]	no data
	EU518634 (371)*	AF156499 (649)[c]	
		DQ060175 (630)[a]	
Unio tumidus Philipsson, 1788	DQ060161 (517)[a]	AF231732 (630)[b]	no data
		DQ060176 (564)[a]	
Anodonta anatina Linnaeus, 1758	DQ060165 (483)[a]	DQ060168 (613)[a]	no data
	EF571332 (487)*	EF571387 (644)*	
	EF571333 (487)*	EF571388 (644)*	
	EF571334 (487)*	EF571389 (657)*	
	EF571335 (377)*	EF571390 (642)*	
	EF571336 (487)*	EF571391 (657)*	
		EF571392 (657)*	
		EF571393 (657)*	
		EF571394 (657)*	
		EF571395 (657)*	
		EF571396 (657)*	
		EF571397 (657)*	
Anodonta cygnea Linnaeus, 1758	DQ060164 (484)[a]	U56842 (613)[e]	no data
	AF232799 (375)[d]	AF232824 (453)[d]	
	EU518635 (400)*	DQ060170 (630)[a]	
		DQ060169 (525)[a]	
		EF571398 (657)*	
Pseudanodonta complanata (Rossmässler, 1835)	DQ060166 (518)[a]	DQ060173 (630)[a]	no data
		DQ060172 (630)[a]	
Anodonta woodiana (Lea, 1834) or *Sinanodonta woodiana* (Lea, 1834)	GQ451855 (454)*	GQ451867 (541)*	GQ451883 (337)*
	DQ073815 (318)*	GQ451868 (541)*	GQ451884 (337)*
	DQ156197 (318)*	AB040832 (309) F*	
	AF389413 (318)[f]	AB040831 (309) M*	
		AB055627 (4582) F*	
		AB055626 (4585) M*	

* – unpublished data, a – KÄLLERSJO et al. 2005, b – BOGAN & HOEH 2000, c – GRAF & Ó FOIGHIL 2000a, d – LYDEARD et al. 2000, e – HOEH et al. 1998, f – HUANG et al. 2002

al. 2006a). Among many sequences available in GenBank (www.ncbi.nlm.nih.gov) for bivalves of the genus *Unio* there is no indication of differentiation into F and M haplotypes (Table 1), which does not preclude the occurrence of DUI in these species; moreover, DUI is to be expected because of its historic origin. Solving the problem of the presence and characteristics of DUI (mainly M haplotype) in various species of freshwater bivalves requires extensive studies, including analyses of several genes and precise isolation of DNA, first of all from male gonads and somatic tissues of both sexes.

Bivalves of the order Unionoida represent the largest freshwater bivalve radiation. They are divided into six families (Etheriidae, Hyriidae, Iridinidae, Margaritiferidae, Mycetopodidae and Unionidae), 181 genera, and about 840 species. These families are distributed across six of the seven continents and represent the most endangered group of freshwater animals alive today (GRAF & CUMMINGS 2007, BOGAN 2008, BOGAN & ROE 2008). Since 1500 42% of the recorded extinctions of animal species are molluscs, including 269 species of gastropods and 31 of bivalves, whereas more than 200 unionoid species are highly imperiled and included in the IUCN Red List of Threatened Species (www.iucnredlist.org): 5 in Eurasia, 5 in Brasil, 1 in Australia and 202 in the United States and Canada (LYDEARD et al. 2004).

Table 2. Own sequences available in GenBank for mitochondrial genes *srRNA*, *tRNA* (for lysine, threonine and tyrosine), *lrRNA*, *cox1* i *cytb* in seven species of Unionidae. Sequence lengths in bp given in brackets. F – female haplotype of mtDNA, M – male haplotype of mtDNA. Unpublished data except the sequences for which references are given

Species	*srRNA – trnK – trnT – trnY - lrRNA*	*cox1*	*cytb*
Unio crassus	GU584009 (1498) F	AF514296 (680) F	GU320055 (410) F
		EU548052 (673) M	
Unio pictorum	GU584005 (1496) F	AF468684 (657) F	GU320056 (410) F
	GU584006 (1497) F	EU548057 (653) F	GU320057 (356) F
	GU584018 (1477) M	EU548056 (665) F	GU320058 (356) F
	GU584019 (1477) M	EU548055 (697) M	
Unio tumidus	GU584007 (1487) F	AY074807 (684) F	GU320059 (410) F
	GU584008 (1487) F	GU230750 (710) F	GU320060 (374) F
	GU584020 (1475) M	EU548053 (662) M	
		EU548054 (662) M	
Anodonta anatina	GU584012 (1347) F	AF494102 (625) F[a]	GU320046 (412) F
	GU584013 (1327) F	EF440346 (709) F[a]	GU320047 (341) F
	GU584014 (1327) F	EF440347 (647) F[a]	GU320048 (408) M
	GU584015 (1464) F	GU230742 (709) F	GU320049 (408) M
		GU230744 (647) F	
		GU230745 (710) F	
		AF462071 (647) M[a]	
		EF440348 (709) M[a]	
		EU252509 (709) M[a]	
		EU252510 (653) M[a]	
		GU230743 (653) M	
		GU230746 (694) M	
		GU230747 (667) M	
Anodonta cygnea	GU584016 (1450) F	AF461419 (684) F	GU320050 (408) F
	GU584017 (1450) F	GU230748 (710) F	GU320051 (408) F
		GU230749 (655) F	
Pseudanodonta complanata		EU734829 (900) F	GU320052 (330) F
Sinanodonta woodiana	GU584010 (1474) F	AF468683 (644) F[b]	GU320053 (407) F
	GU584011 (1474) F	EF440349 (670) F	GU320054 (410) M
	GU584021 (1455) M	EF440350 (711) M	

a – SOROKA 2008a, b – SOROKA 2005

Unionidae are the largest and most widespread of the six families of Unionoida, with 674 species distributed in all geographic regions. The unionid-richest regions are the Nearctic (297 spp.) and Indotropical (217) regions, while Australasia has the fewest species (2). Palaearctic with its 42 species is the poorest in terms of the number of species per surface area (ARAUJO et al. 2005, 2009, GRAF & CUMMINGS 2007). The distribution reflects two major unionid radiations, which are found in the south-eastern USA with 50 genera in North America, and in Asia (from east Iran to Sumatra, the Indo-Malay peninsula, Japan and north to Amur River and Siberia) with 48 genera. These areas are the two oldest uninundated or unglaciated regions of the world (BOGAN & ROE 2008).

Fourteen species of Unionidae occur in Europe (www.faunaeur.org, ARAUJO et al. 2005, 2009, GRAF 2007, GRAF & CUMMINGS 2007), of which seven are found in Poland, three of them being under strict protection (*Unio crassus*, *Anodonta cygnea* and *Pseudanodonta complanata*). All species in Poland are native except the Chinese mussel *Sinanodonta woodiana*, which was introduced in Poland in the 1980s, most probably from Hungary (AFANASIEV et al. 1997, PIECHOCKI & RIEDEL 1997, KRASZEWSKI & ZDANOWSKI 2001). The species penetrated into Europe from Eastern Asia with the introduction of Chinese herbivorous fishes (Silver carp, Bighead carp and Grass carp) from China in 1962 (PETRÓ 1984, KISS 1995).

Because of their unique life cycle and positive effect on aquatic ecosystems through intense water filtration, unionids are popular objects of studies. They have an obligate parasitic larval stage (glochidial larvae) on the gills, fins, sides or body of particuler host fish, using demibranchs for the marsupium and brooding developing glochidia. Genetic studies and characteristics of the doubly uniparental inheritance (DUI) of mtDNA may provide new data on the evolution within the family and higher taxa. Mitochondrial DNA was also used for phylogenetic analyses within the class Bivalvia, where the family Unionidae was represented by single species (HUFF et al. 2004, KREBS 2004). The studies on phylogenetic relations within the family Unionidae did not include species occurring in Poland (WAKLER et al. 2006a, GRAF & Ó FOIGHIL 2000a) and the analyses considering six European species included only F types for genes *16S rRNA* and *cox1* (KÄLLERSJO et al. 2005).

The main objective of this study was search for DUI in the Polish representatives of the family Unionidae through detection of male haplotypes (M) in the analysed species, and characteristics of the phenomenon based on nucleotide divergence of female (F) and male (M) haplotypes. The sequential analyses included seven mitochondrial genes: two protein-coding (cytochrome c oxidase subunit 1, *cox1* and cytochrome b, *cytb*), two rRNA genes, coding RNA for the small and large ribosomal subunit (*srRNA* and *lrRNA*) and three tRNA genes: for lysine (*trnK*), threonine (*trnT*) and tyrosine (*trnY*). The studies aimed also at an estimate of the level of variation of the analysed genes within F and M haplotypes, both intra- and interspecific. Another objective was phylogenetic analysis of unionid bivalves based on female and male haplotypes, using own sequences and sequences available from the international database (GenBank). The studies should considerably expand the knowledge of unionid genetics, characteristics and detection of DUI in the taxon.

MATERIAL AND METHODS

The material included individuals of all the unionid species occurring in Poland (*Unio crassus*, *U. pictorum*, *U. tumidus*, *Anodonta anatina*, *A. cygnea*, *Pseudanodonta complanata* and *Sinanodonta woodiana* (sometimes included in the genus *Anodonta*) collected in 2001–2009.

From 22 to 32 specimens of each species were collected from 2–6 localities (Fig. 1, Table 3). Exceptions were protected species, *U. crassus* and *A. cygnea*, which were collected from single localities, 10 specimens of each (permission from the Minister of Environment, DKFOPogiz-4200/I-12/592/08/aj). Additionally, *A. cygnea* from Ciek Stara Nida was obtained from Dr. K. Zając (PAS, Kraków) (permission DOPweg-4201-01-12/03/jr). Another protected and very rare species, *P. complanata*, was represented by one specimen from the Gulf of Szczecin, obtained alcohol-preserved from Dr. B. Wawrzyniak-Wydrowska (University of Szcze-

Fig. 1. Localities of unionid species in Poland, for more details see Table 3

Table 3. Collecting localities, number and sex of analysed unionids from Poland

Species	Locality	Number of analysed specimens	Sex
Unio crassus	Brda River	10	7 females, 3 males
Unio pictorum	Lake Ślesińskie	3	3 females
	Lake Ińsko	1	–
	Odra River	11	5 females, 6 males
	Lake Hamrzysko	4	3 females, 1 male
	Krutynia River	10	4 females, 6 males
	Szczecin Lagoon	1	1 female
Unio tumidus	Lake Ślesińskie	2	2 females
	Brda River	2	2 females
	Lake Sosnowe	8	3 females, 5 males
	Odra River	5	5 females
	Krutynia River	11	6 females, 5 males
Anodonta anatina	Odra River	8	4 females, 4 males
	Lake Hamrzysko	6	3 females, 3 males
	Lake Wdzydze	5	2 females, 3 males
	Krutynia River	7	3 females, 4 males
Anodonta cygnea	Lake Hamrzysko	10	4 females, 6 males
	Stara Nida River	6	4 females, 2 males
Pseudanodonta complanata	Szczecin Lagoon	1	–
Sinanodonta woodiana	Lake Ślesińskie	12	8 females, 4 males
	Odra River	20	13 females, 7 males

cin)(permission DOPweg-4201-01-13/03/jr). *P. complanata* was not subject to DUI detection since the sex of the only available specimen could not be determined. Gill tissue of the specimen was used for DNA isolation, which made it possible to obtain F form for gene *cox1*. The detailed list of localities and the number of analysed specimens are presented in Table 3 and Fig. 1.

In the case of the Chinese mussel occurring in Europe, two names are applied: initially the name *Anodonata woodiana* (Lea, 1834) was used (PETRÓ 1984, KISS & PEKLI 1988, NAGEL et al. 1996, PIECHOCKI & RIEDEL 1997, WATTERS 1997, KRASZEWSKI & ZDANOWSKI 1997, SOROKA & ZDANOWSKI 2001, SOROKA 2005, 2008b, SOLUSTRI & NARDI 2006), but at present many authors include the species in a separate genus *Sinanodonta* Modell, 1945 (BERAN 1997, 2008, BOHME 1998, FALKNER et al. 2001, YURISHNETS & KORNIUSHIN 2001, GĄBKA et al. 2007, POPA et al. 2007, GRAF 2007, GRAF & CUMMINGS 2007, MUNJIU & SHUBERNETSKI 2008, CAPPELLETTI et al. 2009, ŁABĘCKA 2009). The species has as many as 87 synonyms (GRAF 2007). In the international database GenBank sequences can be submitted only under the name *A. woodiana*. The name *S. woodiana* is used in this paper, and sequences for *A. woodiana* in the GenBank pertain to this species. The taxonomic status of the species will be discussed further in this paper.

Sex determination in bivalves is possible only based on observation of mature gonads. For this reason the bivalves were collected in the spring and summer when their sex could be reliably determined from microscopic slides of their gonads. The presence of large oocytes in the slide indicated female sex, their absence – male sex. Gills of the specimens were also examined since females brood glochidia in the summer. The presence of glochidia in the gills is sufficient to determine the female sex, but their absence does not point unequivocally to the male sex, since the animal in question may be a female prior to brooding which may vary with environmental conditions. Obtaining F haplotype of mtDNA is possible from all somatic tissues, irrespective of the sex of the individual, while M haplotype is obtained only from the male gonad which is the only site of its occurrence. To isolate DNA from male gonads, a small quantity of material was taken from the microscopic slide following examination in light microscope and sex determination as male.

MOLECULAR TECHNIQUES

Total DNA was isolated from the gills of the males and females or gonads of the females using the standard phenol/chloroform extraction and ethanol precipitation. Column method of Qiagen company

Table 4. List of primers used

Gene	Name and sequence (5' to 3')	L	Reference
cox1	LCO1490 GGTCAACAAATCATAAAGATATTGG	710	FOLMER et al. 1994
	HCO2198 TAAACTTCAGGGTGACCAAAAAATCA		
cytb	UCYTB151F TGTGGRGCNACYGTWATYACTAA	400	MERRITT et al. 1998
	UCYTB270R AANAGGAARTAYCAYTCNGGYTG		
srRNA-trnK-trnT-trnY-lrRNA	LR-PCR for F type	ca. 16000	SOROKA & BURZYŃSKI 2010
	DUF GGTTTGCGACCTCGATGTTGG		
	UR1 TTCCTAGTCTTGCCATTCACTGGC		
	LR-PCR for M type	ca. 5000	
	UP.co1.02 CGGGGAAACGCTATATCAGGACA		
	UR1 TTCCTAGTCTTGCCATTCACTGGC		
	re-PCR for F and M types	1500	
	UP.srn.01 TGGTGCCAGCAGTCG		
	UP.lrn.04 CACGCTCACGCTAACG		

L – Length of PCR product in bp

(DNeasy Tissue Kit, Germany) was used to isolate small quantities of DNA from the male gonads. PCR was carried out in order to detect F (from somatic tissues and female gonads) and M (from male gonads) haplotypes for seven mitochondrial genes using universal primers listed in Table 4. Details of DNA extraction and conditions of PCR for fragments of gene *cox1* have been described by SOROKA & GRYGIEŃCZO-RAŹNIEWSKA (2005). Procedures and primers according to MERRITT et al. (1998) were used to obtain the fragment of gene *cytb*. For the remaining five genes, amplified as a whole, a different procedure and universal primers were used (SOROKA & BURZYŃSKI 2010). Studies on *U. pictorum* showed that universal primers (UP.srn.01 & UP.lrn.04, Table 4) amplified a fragment of mtDNA including the gene encoding RNA for the small ribosomal subunit (*srRNA,* 3' end), 3 *tRNA* genes for lysine (*trnK*), threonine (*trnT*) and tyrosine (*trnY*), and the gene encoding RNA for the large ribosomal subunit (*lrRNA,* 5' end). In order to amplify this fragment of mtDNA, first Long Range PCR (LR-PCR) was used (with Phusion High-Fidelity DNA polymerase, Finnzymes), till obtaining the whole molecule of mtDNA of F type and 1/3 mtDNA of M type; this was followed by re-PCR reactions with universal primers, the source of DNA being LR-PCR product diluted as 1:800 (Table 4). PCR and re-PCR reactions were conducted in a volume of 10 µl containing: 1 µl DNA (or 1:800 dilution of LR-PCR), 0.7 µM of each primer, 200 µM of each dNTP, 2.0 mM $MgCl_2$, 0.1 U of DyNAzyme^{TM} EXT DNA polymerase (Finnzymes) and appropriate reaction buffer and water. After an initial 2-min denaturation at 94°C, 30 cycles were used with denaturation at 94°C for 40 sec, annealing for 30 sec at 51 or 55°C, and extension at 72°C for 60 sec with a final 5 min extension at 72°C. After 1.5% agarose gel electrophoresis, the amplification products were viewed in UV light. The results were saved and the sizes of the PCR products were analysed with, respectively, BioCapt and Bio1D programmes (Vilbert Lourmat, France). PCR products were purified using Clean-up columns (A&A Biotechnology) or according to ExoSAP procedure (WERLE et al. 1994). The samples were sequenced with BigDye Terminator chemistry at the Molecular Biology Techniques Laboratory, Faculty of Biology, Adam Mickiewicz University in Poznań (Poland) or in companies Oligo in Warsaw (Poland, http://oligo.pl/) and Macrogen in Seoul (South Korea, http://dna.macrogen.com/eng/). Both DNA strands were sequenced and then assembled into consensus using DNAMAN 5.2.9 software (Lynnon Corporation, Canada). The obtained different haplotypes for analysed genes and species were submitted to GenBank and their accession numbers are shown in Table 2.

DATA ANALYSIS

Sequences divergence, measured as uncorrected p-distance (Hamming distance, *K*) was calculated using MEGA4 (TAMURA et al. 2007). The divergences in the protein-coding genes both in synonymous (K_s) and non-synonymous (K_a) sites were calculated with the modified Nei-Gojobori method with Jukes-Cantor correction (NEI & GOJOBORI 1986), in MEGA4 (TAMURA et al. 2007). This programme was also used to

calculate amino acid compositions. Homology tree was generated with DNAMAN 5.2.9 software (Lynnon Corporation, Canada). Secondary structures of tRNA genes were reconstructed with ARWEN software (LASLETT & CANBACK 2008).

Sequences obtained from gender-associated mitochondrial genomes available for four unionid species from Asia: *Inversidens japanensis* (female-AB055625 and male-AB055624), and USA: *Pyganodon grandis* (female-FJ809754 and male-FJ809755), *Quadrula quadrula* (female-FJ809750 and male-FJ809751) and *Venustaconcha ellipsiformis* (female-FJ809753 and male-FJ809752) were used for comparative analyses of both *rRNA* genes and three *tRNA* genes. Programmes of the EMBOSS package were used to manipulate the sequences (RICE et al. 2000). Comparative and phylogenetic analyses were done with MEGA4 software (TAMURA et al. 2007) and MrBayes3 (RONQUIST & HUELSENBECK 2003), using sequences obtained in this study and those available in GenBank, partly presented in Table 1. Many GenBank entries show identical sequences for different localities. For the sake of legibility of the phylogenetic tree, one or two sequences of female and male haplotype were selected for each species, depending on the observed variation among the sequences. At the variation not exceeding 3% one own sequence was selected, when the variation was greater, two sequences were used, one own one and one from GenBank, to reflect the variation level. Phylogenetic analysis for gene *cox1* included also sequences of the bivalves from the south of Europe: *U. mancus* (AY522858) and *Potomida litoralis* (EF571400). DNA sequences for calculations of parameters K, K_s, K_a and construction of phylogenetic trees were aligned using ClustalW programme, being a part of MEGA4 software (TAMURA et al. 2007). Alignment of sequences may be the greatest source of error during phylogenetic reconstructions (FALNIOWSKI 2003). For protein-coding genes phylogenetic trees based on protein alignment are more precise than those obtained through direct alignment of DNA sequences (HALL 2005). Therefore DNA sequences for all protein-coding genes were translated to amino acids, using invertebrate mitochondrial code, and aligned with ClustalW. Subsequently, the amino acid alignment was used to align the nucleotide sequences. Prior to phylogenetic analyses credibility of the alignment was assessed by checking the mean degree of identity of amino acids for protein-coding genes and DNA sequences for non-protein-coding genes, expressed as p-distance. Alignment was regarded as reliable when $p<0.8$ for protein sequences or $p<0.33$ for DNA sequences (HALL 2008). All the phylogenetic analyses met these criteria.

Phylogenetic trees for nucleotide sequences were generated with Neighbor Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) using programme MEGA4 and Bayesian method in MrBayes3 software. Prior to constructing a phylogenetic tree with NJ method the data were tested for adequacy for tree-building with this method. When the mean distance between the sequences calculated with the formula of Jukes-Cantor (JC) exceeds 1, the data are not adequate for tree-building with NJ method (HALL 2008). NJ method was used for phylogenetic analyses since in each case the value for the sequences used was smaller than 1, and the method is regarded as the best algorithmic technique (FALNIOWSKI 2003). The best model of nucleotide substitutions for phylogenetic analysis with NJ method for the studied genes was selected with jModelTest (http://darwin.uvigo.es/software/modeltest.html) (GUINDON & GASCUEL 2003, POSADA 2008). In the case of gene *cox1* Jukes and Cantor (JC) model was proposed, assuming equal transition rates as well as equal equilibrium frequencies for all bases. Methods NJ and ME used model substitution, JC and bootstrap value of 2000. MP used Close-neighbor-interchange (CNI), search level 3, Random Addition with 10 repeats and bootstrap value 500. For each phylogenetic analysis outgroup sequences with their accession numbers are given directly on the tree.

The Bayesian approach combines the relative reliability of ML with the fast scanning of the parameter landscape by the Markov Chain Monte Carlo (MCMC) approach implemented in the programme MrBayes3 (RONQUIST & HUELSENBECK 2003). The Bayesian tree reveals significant support for main branches and is a significant confirmation for other methods (especially NJ), allowing to resolve the deep branching pattern. All analyses were performed using the sequence alignment for family representatives, with a gamma distribution of substitution rates, using the approximation of six rate classes for each. The General Time Reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites was used to provide substitution priors for the sequence partition of the data. A Metropolis-coupled Markov-chain Monte-Carlo analysis was performed with 5,000,000 generations, two runs and eight chains (four per run). The Markov chain was sampled every 100 generations. Convergence of runs was confirmed by average standard split deviation factor that falls under the recommended value of 0.01. Every calculation was repeated. Phylogenetic analyses done with MrBayes (RONQUIST & HUELSENBECK 2003) used different evolution models depending on the analysed gene, selected with jModelTest programme (GUINDON & GASCUEL 2003, POSADA 2008). The model used for gene *cox1* and the sequence including two ribosomal genes and three tRNAs was GTR (General Time Reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites), and for *cytb* – HKY+I+G (Hasegawa, Kishino, and Yano).

RESULTS

ANALYSIS OF GENE FOR CYTOCHROME C OXIDASE SUBUNIT 1, *cox1*

Based on the fragment of gene *cox1*, F and M haplotypes were distinguished in seven and five bivalve species, respectively. This showed that doubly uniparental inheritance of mitochondrial DNA (DUI) occurred in *U. crassus*, *U. pictorum*, *U. tumidus*, *A. anatina* and *S. woodiana*. In *A. cygnea*, despite analysis of eight males, no M type was detected, and because of the species protection further specimens could not be obtained. Male gonads of the species yielded only F type which was identical with that from somatic tissue.

F and M haplotypes for sequences of gene *cox1* of mean length of 684 bp showed no gaps associated with insertion/deletion events (indels) in any of the studied species.

Specimens of *U. crassus* collected from one locality, Brda River (Table 3), showed no variation of F and M haplotypes; the divergence between them was 29% and included 193 nucleotide substitutions, of which more than 1/4 resulted in amino acid substitutions (K_a, Table 5).

Analysis of the total of 30 sequences of *cox1* in specimens of *U. pictorum* from six localities revealed the presence of three F haplotypes, while examination of 13 male gonads detected only one M haplotype. The

Table 5. Frequencies of F and M haplotypes and variation in sequence of gene *cox1* fragment in seven species of Unionidae

Species	N	Haplotype and Accession number		Frequence	Distance (SE)		
					K	K_s	K_a
U. crassus	7	F-type	AF514296	1.00	0.00	NA	NA
	3	M-type	EU548052	1.00	0.00	NA	NA
		F/M			0.287 (0.015)	0.670 (0.077)	0.260 (0.031)
U. pictorum	17	F-type	AF468684	0.88	0.004 (0.002)	0.014 (0.007)	0.000
			EU548057	0.06			
			EU548056	0.06			
	13	M-type	EU548055	1.00	0.00	NA	NA
		F/M			0.317 (0.018)	0.815 (0.105)	0.286 (0.043)
U. tumidus	18	F-type	AF074807	0.86	0.002 (0.002)	0.005 (0.004)	0.000
			GU230750	0.14			
	10	M-type	EU548053	0.50	0.002 (0.002)	0.005 (0.005)	0.000
			EU548054	0.50			
		F/M			0.283 (0.016)	0.592 (0.073)	0.273 (0.036)
A. anatina	12	F-type	GU230742	0.30	0.002 (0.001)	0.007 (0.004)	0.000
			GU230744	0.30			
			GU230745	0.40			
	14	M-type	GU230746	0.56	0.002(0.001)	0.007 (0.004)	0.000
			GU230747	0.07			
			EF440348	0.30			
			EU252509	0.07			
		F/M			0.298 (0.017)	0.749 (0.096)	0.263 (0.034)
A. cygnea	16	F-type	GU230748	0.96	0.002 (0.001)	0.000	0.002 (0.002)
			GU230749	0.04			
P. complanata	1	F-type	EU734829	1.00	–	–	–
S. woodiana	21	F-type	EF440349	1.00	0.00	NA	NA
	11	M-type	EF440350	1.00	0.00	NA	NA
		F/M			0.305 (0.017)	0.736 (0.085)	0.280 (0.035)
Mean	92	F			0.127 (0.009)	0.564 (0.037)	0.012 (0.003)
	51	M			0.147 (0.010)	0.505 (0.041)	0.067 (0.067)
		F/M			0.310 (0.015)	0.775 (0.058)	0.281 (0.031)

NA – not applicable

most frequent F haplotype (AF468684) occurred in all localities, the remaining haplotypes were detected in single specimens and in separate localities, i.e. haplotype EU548056 in a specimen from the Odra River, EU548057 in a specimen from Lake Hamrzysko. F haplotypes differed in four polymorphic sites, with silent mutations of transition type (2×T/C, C/T and G/A, K_a=0.00, Table 5). Divergence between F and M haplotypes was greater than in *U. crassus,* and was nearly 32%, at a high K_s level (Table 5).

In *U. tumidus* analysis of 28 sequences of gene *cox1* from five localities in Poland revealed the presence of two F haplotypes and two M haplotypes (Table 5). The variation within the two haplotypes was similar (0.2%), the divergence between them was 28%. The more frequent F haplotype (AF074807) occurred in all the localities, the second only in the Odra River.

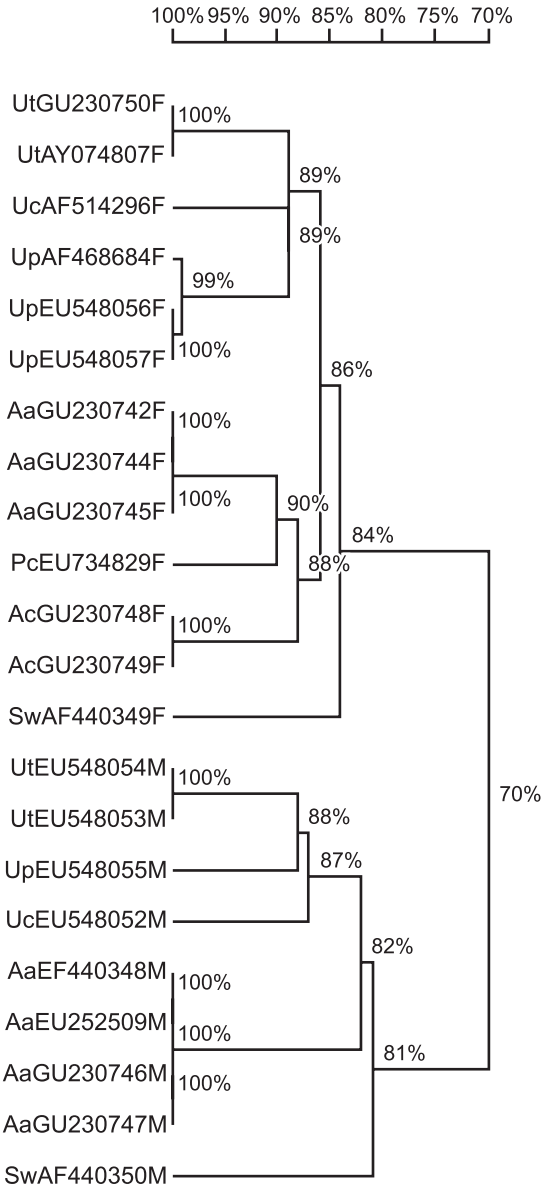

Fig. 2. Homology tree for species of Unionidae based on observed divergence into partial *cox1* sequences

Both M haplotypes had equal total frequencies, but their frequencies varied among the localities. F haplotypes differed in one transversion (C/A) in the third position of the codon, which did not change the encoded amino acid, proline. Similarly M haplotypes differed in one transition (T/C) in the third position, without changing the amino acid (asparagine).

Three F haplotypes were detected in *A. anatina,* with two polymorphic sites (C/T and A/C), whose substitutions were neutral, and the difference was 0.2% (Table 5). Variation among the four identified M haplotypes was similar (0.2%), where all three substitutions in the third position in the codon were silent. The divergence between F and M types was nearly 30%, with three times as many synonymous substitutions (K_s), as non-synonymous substitutions (K_a) (Table 5).

Two F haplotypes were detected in *A. cygnea*, one with high frequency (0.96) the other with low frequency, present only in one specimen. The difference was one G/A transition, which involved the first position in the codon, resulting in amino acid substitution, glycine for serine. In this case K_a value was higher than K_s, with 0.2% nucleotide difference between the sequences (Table 5).

The greatest number of sequences (over 30) was analysed for *S. woodiana* introduced in Poland in the 1980s. In spite of this, identical sequences of F and M haplotypes were found in the two localities, and the divergence between the haplotypes was 30% (Table 3, 5).

Variation in F haplotypes within the genus was smaller for *Unio* (ca. 12%) and somewhat greater for *Anodonta*, up to 13%. Divergence between the genera was over 14%, except *Pseudanodonta* and *Anodonta*, which differed less. For M haplotypes variation within *Unio* was ca. 13%, and between *Unio* and *Anodonta* was over 18% (Fig. 2). Variation of all F haplotypes within species of unionids, at 16%, was smaller than that observed for M haplotypes (19%) and they formed separate groups in the homology tree, with divergence of 30% (Fig. 2). Species of the genera *Unio* and *Anodonta/Pseudanodonta* clustered together in both groups, while *S. woodiana* was genetically the most remote (Fig. 2).

Table 6 presents mean nucleotide frequencies in DNA sequences of gene *cox1* for F and M haplotypes in seven species of unionid bivalves. The nucleotide composition of F and M haplotypes was similar, almost identical for G base, with a slight prevalence of T base in male genomes and C in female genomes. The situation with amino acid frequencies in the encoded protein, cytochrome c oxidase subunit 1, was different: 1/3 of amino acids had similar values for both haplotypes (Table 7). Cysteine did not occur in female genotypes of any of the species (it constituted 1.4% in M haplotypes), but they contained two times more alanine and ca. 1.5 times more leucine and glutamine than male genomes. The remaining amino

Table 6. Mean nucleotide frequencies for *cox1* fragment in species of Unionidae. F – female haplotype, M – male haplotype

Species and Haplotype	Mean Length (bp)	Mean Base Composition (%)			
		T	C	A	G
U. crassus F	680	41.9	15.1	20.9	22.1
U. crassus M	673	42.6	14.6	20.7	22.1
U. pictorum F	658.3	40.6	16.4	19.8	23.3
U. pictorum M	697	42.9	13.3	20.8	23.0
U. tumidus F	697	42.5	14.3	20.6	22.6
U. tumidus M	662	43.4	13.2	19.6	23.7
A. anatina F	688.7	41.3	15.5	21.0	22.2
A. anatina M	694.8	41.7	13.6	22.9	21.7
A. cygnea F	682.5	40.9	14.7	20.6	23.8
P. complanata F	709	42.0	14.4	21.0	22.6
S. woodiana F	670	40.9	15.5	20.6	23.0
S. woodiana M	711	41.2	13.4	23.8	21.7
Mean F	681.5	41.3	15.3	20.6	22.8
Mean M	687.1	42.3	13.6	21.8	22.3

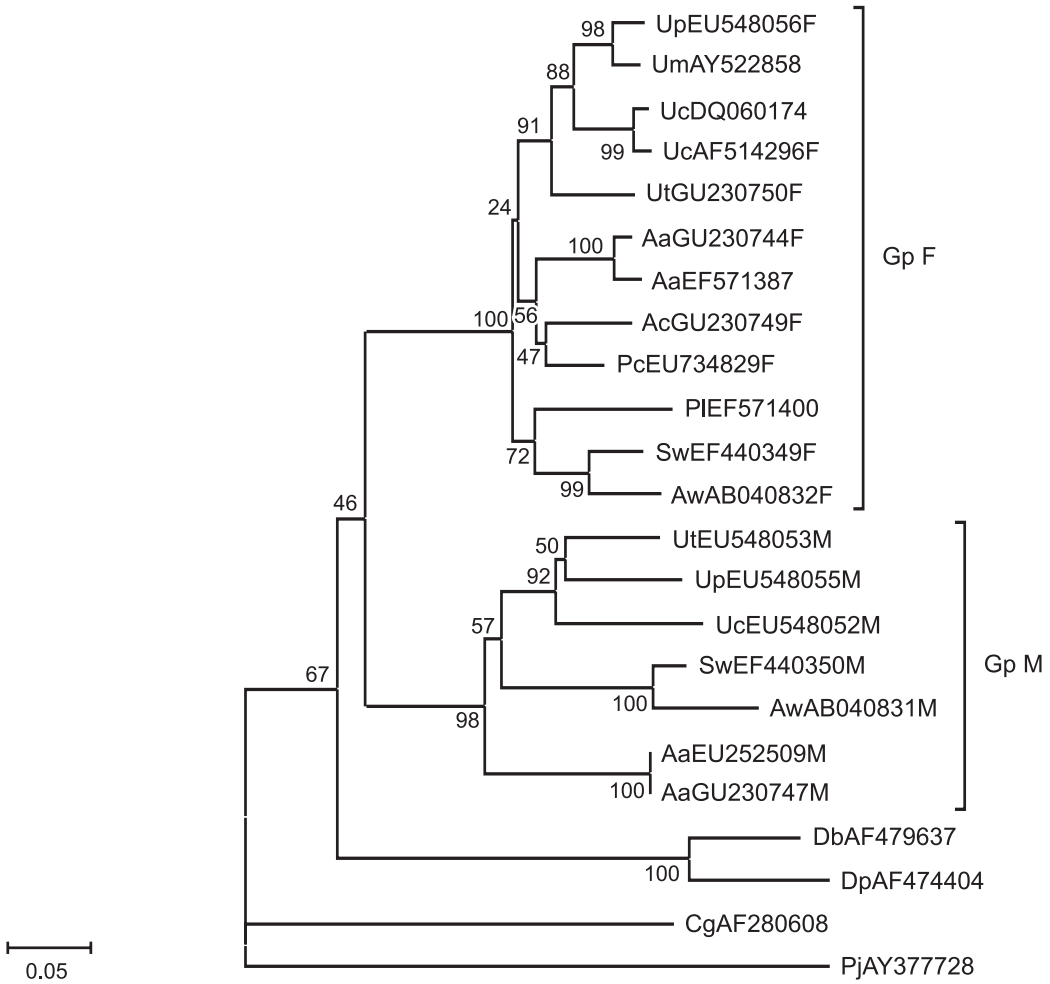

Fig. 3 a. Neighbour Joining tree for Unionidae species – partial *cox1* sequences. Abbreviations: Aa – *Anodonta anatina*, Ac – *A. cygnea*, Aw, Sw – *Sinanodonta woodiana*, Pc – *Pseudanodonta complanata*, Pl – *Potomida litoralis*, Uc – *Unio crassus*, Um – *U. mancus*, Up – *U. pictorum*, Ut – *U. tumidus*. *Dreissena polymorpha* (Dp), *D. bugensis* (Db), *Crassostrea gigas* (Cg) and *Pecten jacobaeus* (Pj) are used as outgroup. Values above branches represent bootstrap suport (2,000 replicates)

acids which were more frequent in F haplotypes were aspartate and proline. In male haplotypes lysine occurred four times more frequently than in female haplotypes. Other amino acids which were more frequent in M haplotype were glutamate, phenylalanine, methionine, serine, valine and tryptophan (Table 7). Leucine was the most frequent amino acid in both haplotypes.

Phylogenetic analysis of own sequences of gene *cox1* and European unionid species available in GenBank is presented in Fig. 3a and b. Male and female sequences formed clearly separated clades though

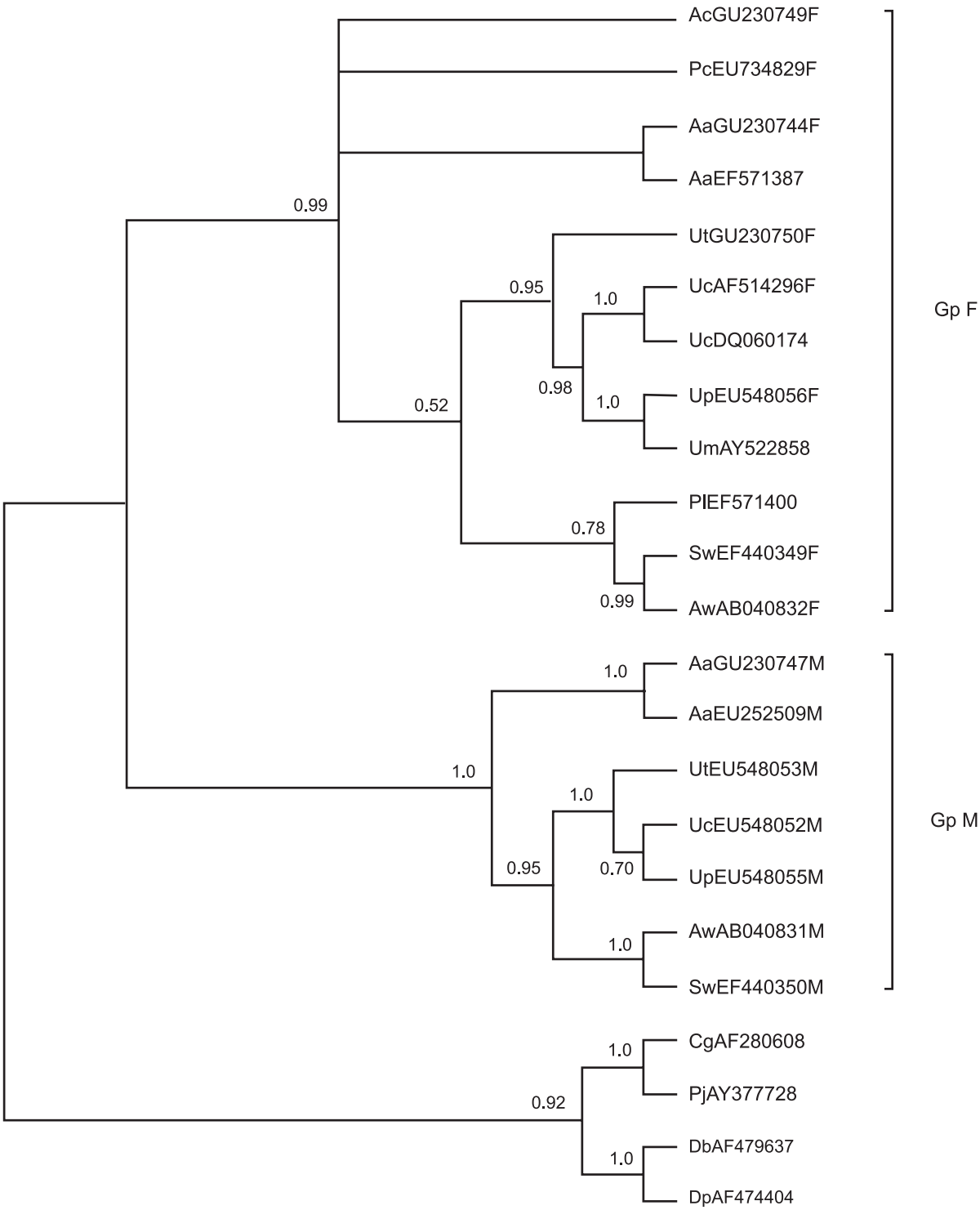

Fig. 3 b. Bayesian method (GTR model of evolution) tree for species of Unionidae – partial *cox1* sequences. Abbreviations: Aa – *Anodonta anatina*, Ac – *A. cygnea*, Aw, Sw – *Sinanodonta woodiana*, Pc – *Pseudanodonta complanata*, Pl – *Potomida litoralis*, Uc – *Unio crassus*, Um – *U. mancus*, Up – *U. pictorum*, Ut – *U. tumidus*. *Dreissena polymorpha* (Dp), *D. bugensis* (Db), *Crassostrea gigas* (Cg) and *Pecten jacobaeus* (Pj) are used as outgroup. Values above branches represent posterior probabilities

Table 7. Amino acid frequencies (in percent) for *cox1* fragment in Unionidae. F – female haplotype, M – male haplotype, Uc – *U. crassus*, Up – *U. pictorum*, Ut – *U. tumidus*, Aa – *A. anatina*, Ac – *A. cygnea*, Pc – *P. complanata*, Sw – *S. woodiana*

Species and Haplotype	Ala (A)	Cys (C)	Asp (D)	Glu (E)	Phe (F)	Gly (G)	His (H)	Ile (I)	Lys (K)	Leu (L)	Met (M)	Asn (N)	Pro (P)	Gln (Q)	Arg (R)	Se (S)	Thr (T)	Val (V)	Trp (W)	Tyr (Y)	Total
UcAF514296-F	9.29	0.00	3.10	1.77	6.64	10.18	2.21	7.08	0.00	18.14	4.42	3.98	6.19	0.88	2.21	7.52	4.42	7.08	3.10	1.77	226
UcEU548052-M	4.93	1.79	3.59	1.79	9.42	10.31	2.24	6.73	0.45	12.11	6.28	3.59	4.93	0.45	3.14	8.97	5.38	8.97	3.59	1.35	223
UpAF468684-F	9.63	0.00	3.21	1.38	5.50	10.09	1.83	6.88	0.00	19.27	4.59	4.13	5.96	0.92	2.29	7.80	5.05	7.34	2.29	1.83	218
UpEU548056-F	9.50	0.00	3.17	1.81	6.79	10.41	2.26	6.79	0.00	17.19	4.52	4.07	6.33	0.90	2.26	7.69	4.52	7.69	2.71	1.36	221
UpEU548057-F	9.22	0.00	3.23	1.84	6.91	10.60	2.30	6.91	0.00	17.05	4.61	4.15	6.45	0.92	2.30	7.37	4.61	7.83	2.30	1.38	217
UpEU548055-M	5.19	1.73	3.90	1.73	8.66	10.39	2.16	6.06	0.87	13.42	6.93	3.90	4.76	0.87	2.60	8.23	5.19	8.23	3.46	1.73	231
UtAY074807-F	9.25	0.00	3.08	1.76	6.61	10.13	2.20	6.17	0.00	18.50	4.41	3.96	6.17	0.88	2.20	7.49	4.85	7.93	2.64	1.76	227
UtGU230750-F	8.90	0.00	3.39	1.69	6.36	10.17	2.54	6.36	0.42	17.80	4.24	4.24	5.93	0.85	2.12	8.05	5.08	7.63	2.54	1.69	236
UtEU548053-M	5.45	2.27	3.18	2.27	8.64	10.45	2.27	6.36	0.45	13.18	5.91	4.09	5.45	0.91	2.27	7.73	4.55	9.55	3.64	1.36	220
UtEU548054-M	5.45	2.27	3.18	2.27	8.64	10.45	2.27	6.36	0.45	13.18	5.91	4.09	5.45	0.91	2.27	7.73	4.55	9.55	3.64	1.36	220
AaGU230742-F	9.79	0.00	3.40	1.70	6.38	10.21	2.55	6.38	0.43	17.87	4.26	4.26	5.96	0.85	2.13	7.23	4.68	7.66	2.55	1.70	235
AaGU230744-F	10.23	0.00	3.26	1.86	6.98	10.23	2.33	6.51	0.00	16.74	4.65	4.19	6.51	0.93	2.33	6.98	4.19	8.37	2.33	1.40	215
AaGU230745-F	9.75	0.00	3.39	1.69	6.36	10.17	2.54	6.36	0.42	17.80	4.24	4.24	5.93	0.85	2.12	7.20	4.66	7.63	2.97	1.69	236
AaGU230746-M	3.91	0.87	2.61	1.74	8.70	11.74	2.61	6.09	0.87	12.61	7.39	4.78	4.78	0.43	3.04	8.70	4.35	9.57	3.48	1.74	230
AaGU230747-M	4.07	0.90	2.26	1.81	9.05	11.76	2.26	5.88	0.45	12.67	7.69	4.52	4.98	0.45	3.17	8.60	4.52	9.95	3.62	1.36	221
AaEF440348-M	3.83	0.85	2.55	2.13	8.51	11.49	2.55	5.96	0.85	12.34	7.23	4.68	5.11	0.43	2.98	8.94	4.68	9.79	3.40	1.70	235
AaEU252509-M	3.83	0.85	2.55	2.13	8.51	11.49	2.55	5.96	0.85	12.34	7.23	4.68	5.11	0.43	2.98	8.94	4.68	9.79	3.40	1.70	235
AcGU230748-F	8.90	0.00	3.39	1.69	6.36	10.17	2.54	6.36	0.42	17.80	4.24	4.24	5.93	0.85	2.12	7.63	5.08	7.63	2.97	1.69	236
AcGU230749-F	9.22	0.00	3.23	1.84	6.91	10.14	2.30	6.45	0.00	17.05	4.61	4.15	6.45	0.92	2.30	7.83	4.61	8.29	2.30	1.38	217
PcEU734829-F	8.94	0.00	3.40	1.70	6.38	10.21	2.55	6.38	0.43	17.87	4.26	4.26	5.96	0.85	2.13	7.66	5.11	7.66	2.55	1.70	235
SwEF440349-F	9.46	0.00	3.15	1.80	6.76	10.36	2.25	6.31	0.00	17.57	4.50	4.05	6.31	0.90	2.25	7.66	4.50	8.11	2.70	1.35	222
SwEF440350-M	4.66	1.27	2.54	2.12	8.47	10.17	2.12	5.93	0.85	13.14	7.63	4.66	5.51	0.85	2.54	9.32	4.24	8.47	3.39	2.12	236
Mean F	9.38	0.00	3.26	1.73	6.53	10.23	2.35	6.53	0.17	17.75	4.42	4.15	6.15	0.88	2.21	7.55	4.73	7.75	2.62	1.60	226.2
Mean M	4.58	1.41	2.93	2.00	8.73	10.92	2.34	6.14	0.68	12.77	6.92	4.34	5.12	0.63	2.78	8.58	4.68	9.31	3.51	1.61	227.9

clustering within them was not identical. Except *S. woodiana* all sequences obtained from GenBank, without clear indication to F or M haplotypes, represent F haplotypes, i.e. irrespective of sex of the specimen they were obtained from somatic tissues. Detailed analyses of DNA isolation techniques for the published sequences showed that DNA for the studies was isolated from the foot or the mantle (GRAF & Ó FOIGHIL 2000a, ARAUJO et al. 2005, KÄLLERSJO 2005). Bayesian method produced identical phylogenetic relationships for F and M sequences. Phylogenetic analyses with NJ and ME methods yielded the same topologies with slightly different bootstrap values. Variation within F and M haplotypes involved clustering of three species of *Unio* and *A. anatina*. Within F types three groups could be distinguished: *Unio*, *Potomida/Sinanodonta* and *Anodonta/Pseudanodonta*, the last with the lowest bootstrap value. Phylogenetic relations among species of *Unio* were very reliable with all the methods (high bootstrap values) and placed *U. pictorum* and *U. mancus* as sister species, closest related to *U. crassus*, and the most remote species was *U. tumidus*. Different relations followed only from M sequences in NJ method where *U. tumidus* was the closest to *U. pictorum* (but with a smaller bootstrap value 50%), and *U. crassus* was fairly remote. *S. woodiana* was phylogenetically closer to *Unio* than to *Anodonta* within F and M haplotypes, except for the grouping of F sequences with NJ method. Within M haplotypes also three groups could be distinguished: *Unio*, *Sinanodonta* and *Anodonta*, the last group being the most remote phylogenetically, like with female sequences. In phylogenetic analysis with MP method groupings of the species of *Unio* were identical for F and M clades, and they agreed with the relations obtained with the other methods, except M sequences with NJ method. The greatest differences for MP method pertained to the grouping of *A. anatina* and *P. complanata*. With a small bootstrap value, *A. anatina* clustered with species of *Unio*, while *P. complanata* had the least distinct sequence among F haplotypes (at a high bootstrap value).

ANALYSIS OF CYTOCHROME B GENE, *cytb*

F haplotypes of all the species and male haplotypes of two species, *A. anatina* and *S. woodiana*, were detected based on sequential analyses of the fragment of gene *cytb*. For the remaining four species F haplotypes were obtained from male gonads instead of M haplotypes. M sequences could not be obtained for *P. complanata* because only one preserved specimen of indeterminate sex was available.

Table 8. Frequencies of F and M haplotypes and variation in sequence of gene *cytb* fragment in seven species of Unionidae

Species	N	Haplotype and Accession number		Frequence	Distance (SE)		
					K	K_s	K_a
U. crassus	7	F-type	GU320055	1.00	0.00	NA	NA
U. pictorum	18	F-type	GU320056	0.83	0.006 (0.003)	0.012 (0.009)	0.003 (0.003)
			GU320057	0.11			
			GU320058	0.06			
U. tumidus	12	F-type	GU320059	0.58	0.005 (0.004)	0.017 (0.012)	0.000
			GU320060	0.42			
A. anatina	7	F-type	GU320046	0.71	0.015 (0.006)	0.030 (0.017)	0.009 (0.006)
			GU320047	0.29			
	4	M-type	GU320048	0.50	0.003 (0.003)	0.008 (0.008)	0.000
			GU320049	0.50			
		F/M			0.337 (0.025)	1.004 (0.196)	0.298 (0.049)
A. cygnea	6	F-type	GU320050	0.83	0.005 (0.003)	0.017 (0.012)	0.000
			GU320051	0.17			
P. complanata	1	F-type	GU320052	1.00	–	–	–
S. woodiana	12	F-type	GU320053	1.00	0.00	NA	NA
	6	M-type	GU320054	1.00	0.00	NA	NA
		F/M			0.354 (0.022)	1.148 (0.244)	0.309 (0.044)
Mean	63	F			0.181 (0.013)	0.737 (0.077)	0.070 (0.013)
	10	M			0.145 (0.015)	0.544 (0.108)	0.071 (0.018)
		F/M			0.352 (0.021)	1.007 (0.121)	0.334 (0.047)

NA – not applicable

No gaps were observed after alignment in any of F and M sequences of gene *cytb*, with average length of 397 bp. One F haplotype was obtained for each *U. crassus* and *P. complanata* (Table 8). *U. pictorum* had three F haplotypes, and the most frequent of them occurred in all the localities, the remaining two only in Lake Hamrzysko. Three polymorphic sites (K=0.006) pertained to one transition (G/A) and two transversions (T/G and C/A), two changes in the third position in the codon were synonymous (K_s=0.012), in the second position they caused substitution of glycine for valine (K_a=0.003) (Table 8). Analysis of 12 specimens of *U. tumidus* revealed two different F haplotypes differing in two polymorphic sites, where transitions in the third position of the codon were neutral at protein level (Table 8). Genotype GU320060 was characteristic of the locality in the Odra River. Two F and two M haplotypes were distinguished for *A. anatina;* their divergence was nearly 34%. The difference between the F haplotypes was 1.5%, with five polymorphic sites, of which two were changes in the first and second position of the codon resulting in amino acid substitutions (K_a=0.009), the remaining ones were neutral (Table 8). M haplotypes within the species were less varied (0.3%), with one transition (G/A) in the third position of the codon, without the change of the encoded amino acid. The two F haplotypes distinguished in *A. cygnea* had two polymorphic sites and they were neutral changes at protein level, pertaining to the third position in the codon. F and M haplotypes in *S. woodiana* showed 35% divergence and more than 1/3 of the changes caused amino acid substitutions (Table 8). No variation was observed within F and M haplotypes of the species.

Mean variation within F and M haplotypes for gene *cytb* in Unionidae was 18 and 14.5%, respectively, and the divergence between them was 35% (Table 8, Fig. 4). The variation within M haplotype of *cytb*, by 4% smaller, did not result from an exceptionally small variation of male sequences of the gene, but probably from the small number of available sequences. The interspecific variation of F haplotypes of gene *cytb* ranged from 15 to 21% and lower values were characteristic of the genus *Unio*, higher – of *Anodonta*. M haplotypes showed a slightly greater variation which between *A. anatina* and *S. woodiana* was 23% (Fig. 4).

The nucleotide composition of F and M haplotypes of gene *cytb* was very similar (Table 9), some differences involved the amino acid composition of the encoded protein, cytochrome b (Table 10). In F haplotypes of *U. crassus, U. pictorum* and *S. woodiana* methionine did not occur and in the remaining species it constituted 0.8–3.5%; on average it occurred 2.5 times more frequently in M haplotypes. Also glutamine was almost twice as frequent in M genomes, while tyrosine was twice more frequent in F haplotypes (Table 10). Like in *cox1* gene, in both haplotypes leucine was the most frequent amino acid.

Among the analysed unionids, in the international database GenBank *cytb* sequences are available only for *S. woodiana* (Table 1). Two sequences coming from South Korea, though somewhat shorter, represent the same fragment of the gene. Comparative analysis of all the sequences of *S. woodiana* showed a 10% divergence between the Polish and Korean specimens, with a typical 0.6% intraspecific difference of the two Korean sequences.

Because of the lack of sequences of gene *cytb* for the remaining unionid species in GenBank, the phylogenetic analysis was based on the results of own studies, two Korean sequences of *S. woodiana* (Table 1), and species of *Dreissena* as outgroup (Fig. 5). The analysis showed that the available sequences of *S. woodiana* from South Korea (GQ451883 and GQ451884) represented female genotypes and formed one clade with the Polish sequences of *S. woodiana*, at a maximum bootstrap value. However, the arm lengths and their estimated difference at 10% suggest species distinctness of these sequences. Phylogenetic analyses of gene *cytb* with methods NJ, ME, MP and

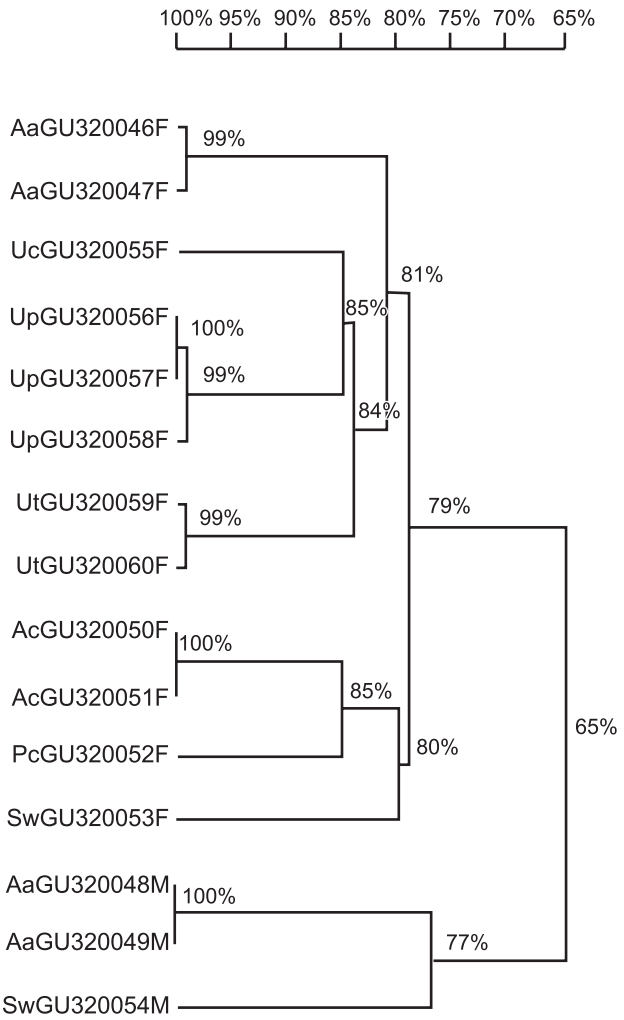

Fig. 4. Homology tree for species of Unionidae based on observed divergence into partial *cytb* sequences. Abbreviations – see Fig. 3

Table 9. Mean nucleotide frequencies for *cytb* fragment in species of Unionidae. F – female haplotype, M – male haplotype

Species and Haplotype	Mean Length (bp)	Mean Base Composition (%)			
		T	C	A	G
U. crassus F	410	38.3	21.2	26.8	13.7
U. pictorum F	374	37.1	22.1	27.3	13.5
U. tumidus F	392	36.0	23.0	27.3	13.8
A. anatina F	376.5	35.8	21.7	29.0	13.6
A. anatina M	408	32.6	24.3	30.0	13.1
A. cygnea F	408	34.1	26.3	25.2	14.3
P. complanata F	330	34.2	24.5	29.1	12.1
S. woodiana F	407	36.6	23.6	25.8	14.0
S. woodiana M	410	36.8	20.7	28.8	13.7
Mean F	385.1	36.0	23.1	27.2	13.7
Mean M	408.7	34.0	23.1	29.6	13.3

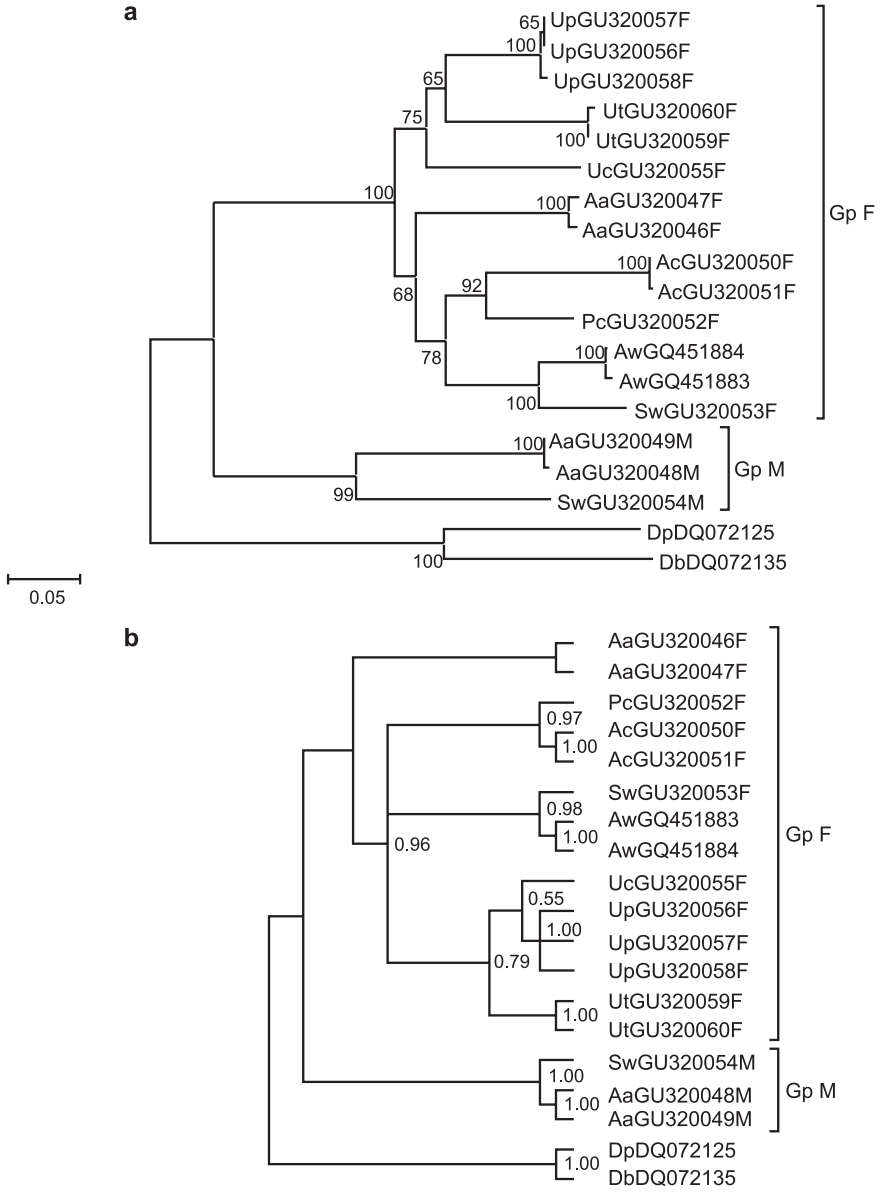

Fig. 5. Phylogenetic trees for species of Unionidae based on partial *cytb* sequences for Neighbour Joining (NJ) method – a, and Bayesian method (HKY+I+G model of evolution) – b. For abbreviation see caption to Fig. 3. As outgroup are used *Dreissena polymorpha* (Dp) and *D. bugensis* (Db). Values above branches represent bootstrap support (2,000) for NJ and posterior probabilities for Bayesian method

Bayesian yielded separate clades for F and M sequences, with internal topologies differing in the position of *A. anatina* in the F lineage (Fig. 5a, b). NJ and ME analyses yielded identical topologies where F sequences formed two clades: *Unio* and *Anodonta/Pseudanodonta/Sinanodonta*. Bayesian and MP analyses yielded three clades and the most remote species was *A. anatina*. In Bayesian analysis of gene *cytb U. pictorum* and *U. crassus* formed sister clades, and *U. tumidus* was the most remote among *Unio*, while with the remaining methods (NJ, ME and MP) the most remote member of *Unio* was *U. crassus*.

ANALYSIS OF mtDNA FRAGMENT INCLUDING GENES *srRNA, trnK, trnT, trnY* AND *lrRNA*

A fragment of mtDNA, 1,327–1,498 bp long, including parts of the two ribosomal RNA genes (*srRNA* and *lrRNA*) and three whole *tRNA* genes (for lysine, *trnK*; threonine, *trnT* and tyrosine, *trnY*) was used to detect F and M genomes in the seven unionid species. Ribosomal RNA genes of mtDNA are highly conservative which made it possible to amplify the region with universal primer pairs for all analysed species (Table 4). F haplotypes of this mtDNA region were obtained for all species except *P. complanata*, in which the poor quality of DNA precluded the first LR-PCR reaction. M haplotypes for this fragment of mtDNA were obtained for three species: *U. pictorum*, *U. tumidus* and *S. woodiana*. M haplotype results for *U. crassus* and *A. anatina* were illegible, while in the case of *A. cygnea* F haplotype was obtained despite the isolation of DNA from the male gonad.

Indels were common in the alignment for all the analysed species in ribosomal RNA genes, and only few gaps were observed in *tRNA* genes.

RIBOSOMAL RNA GENES, *rRNA*

The analysed fragment of gene coding for RNA of the small mitochondrial ribosome subunit (*srRNA*), was 539 to 589 bp long and constituted ca. 2/3 of the length of the gene in Unionidae (SERB & LYDEARD 2003, BRETON et al. 2009, SOROKA & BURZYŃSKI 2010). The gene showed a greater proportion of Thymine (T) and smaller of Guanine (G) in male haplotypes of most species (Table 11). No intraspecific variation of the gene was observed, either in F or in M haplotypes in all the unionids from Poland. The number of polymorphic sites distinguished in F haplotypes of these bivalves was 150, resulting in the mean variation of 10%. However, variation within the genera *Unio* and *Anodonta* was smaller, up to 9% (Table 12). A similar level of variation was observed in M haplotypes, where 102 variable sites were detected for three species. Divergence between the female and male sequences of the Polish unionids in the fragment of gene *srRNA* exceeded 23% and was smaller than in other Unionidae (Table 11). The variation within female and male sequences of the gene estimated based on the analysis of all the data was 13 and 15%, respectively, and the divergence between them was 24%.

The studied fragment of the gene coding for RNA of the large subunit of mitochondrial ribosome (*lrRNA*) had an average length of 680 bp, which is ca. 1/2 of its initial length in Unionidae (SERB & LYDEARD 2003, BRETON et al. 2009, SOROKA & BURZYŃSKI 2010). The nucleotide composition of F and M sequences of gene *lrRNA* was similar, with a tendency to a smaller Thymine content in male haplotypes. Male haplotypes of this gene fragment in the unionid species from Poland showed no variation, whereas female haplotypes showed intraspecific variation (Table 13). Except for *U. crassus* all the studied species had one or three polymorphic sites, with substitutions of transition type and one insertion of T in *U. pictorum*. The greatest intraspecific variation was observed in *U. tumidus* (0.4%), followed by *A. anatina* (0.3%) and the remaining species with 0.1% in each (Table 13). Interspecific variation of F haplotypes among the studied species ranged from 10 to 22%, at the mean value of 16%, while for M sequences the respective values were 14 to 20%, with the mean of 18% (Table 12). The divergence between F and M haplotypes was on average 33% and thus somewhat greater than in other unionids from the USA and Asia (Tables 11 and 12). The overall analysis of all the data showed variation within female and male sequences of gene *lrRNA* of 15 and 20% respectively, and the divergence between them was 34%.

The sequences of the beginning of gene *lrRNA* from the six unionid species from Poland obtained in this study were also compared with other European specimens of these species and with *A. woodiana* from China, represented by F haplotypes (Table 1). All the sequences in GenBank included the further fragment of gene *lrRNA* and for this reason half of them had no nucleotides in common with the sequences obtained in this study; the other half shared only 40–50 bp which made it impossible to perform comparative analyses. Comparative analyses including only sequences from GenBank were aimed at characterising this part of the gene in the available species. They showed a greater intraspecific variation than the beginning of gene *lrRNA* in the Polish specimens of the studied species, which probably results from a greater geographical variation of the compared sequences. The difference between the Swedish and Italian specimens of *U. pictorum* and *A. cygnea* was 2.6% and 0.2% respectively, and between the Swedish and Spanish specimens of *A. anatina* – nearly 1%. The four Asian specimens of *A. woodiana* showed a surprisingly high variation (Table 1). Sequences DQ073815 and DQ156197 from China, differing from each other by 0.7%, were very remote from the remaining ones: 7.8% compared to AF389413 (from Lake Poyang in

Table 10. Amino acid frequencies (in percent) for *cytb* fragment in Unionidae. F – female haplotype, M – male haplotype, Uc – *U. crassus*, Up – *U. pictorum*, Ut – *U. tumidus*, Aa – *A. anatina*, Ac – *A. cygnea*, Pc – *P. complanata*, Sw – *S. woodiana*

Species and Haplotype	Ala (A)	Cys (C)	Asp (D)	Glu (E)	Phe (F)	Gly (G)	His (H)	Ile (I)	Lys (K)	Leu (L)	Met (M)	Asn (N)	Pro (P)	Gln (Q)	Arg (R)	Se (S)	Thr (T)	Val (V)	Trp (W)	Tyr (Y)	Total
UcGU320055-F	5.15	1.47	1.47	1.47	9.56	5.15	2.94	7.35	1.47	16.91	0.00	6.62	8.09	1.47	0.74	7.35	5885	10.29	2.21	4.41	136
UpGU320056-F	5.15	1.47	1.47	2.21	8.82	5.15	3.68	8.82	1.47	17.65	0.00	6.62	7.35	0.74	0.74	5.88	6.62	9.56	2.21	4.41	136
UpGU320057-F	4.24	0.85	1.69	2.54	10.17	4.24	4.24	7.63	0.85	18.64	0.00	6.78	7.63	0.85	0.85	5.93	5.93	10.17	2.54	4.24	118
UpGU320058-F	4.24	0.85	1.69	2.54	10.17	5.08	4.24	7.63	0.85	18.64	0.00	6.78	7.63	0.85	0.85	5.93	5.93	9.32	2.54	4.24	118
UtGU320059-F	5.88	1.47	1.47	1.47	9.56	5.15	3.68	6.62	1.47	15.44	1.47	5.15	5.15	0.74	0.74	7.35	8.09	9.56	2.21	4.41	136
UtGU320060-F	4.84	0.81	1.61	1.61	10.48	4.84	4.03	7.26	1.61	15.32	0.81	4.84	4.84	0.81	0.81	7.26	7.26	9.68	2.42	4.84	124
AaGU320046-F	4.44	1.48	1.48	2.22	8.15	5.19	3.70	8.15	1.48	16.30	2.96	6.67	7.41	0.74	0.74	6.67	6.67	8.15	2.22	5.19	135
AaGU320047-F	3.54	0.88	1.77	3.54	9.73	4.42	4.42	9.73	0.88	15.93	3.54	7.08	7.96	0.88	0.88	7.08	5.31	6.19	2.65	3.54	113
AaGU320048-M	5.19	0.74	2.22	2.96	11.11	5.19	4.44	5.19	2.96	14.81	3.70	6.67	6.67	1.48	0.74	6.67	8.15	7.41	2.22	1.48	135
AaGU320049-M	5.19	0.74	2.22	2.96	11.11	5.19	4.44	5.19	2.96	14.81	3.70	6.67	6.67	1.48	0.74	6.67	8.15	7.41	2.22	1.48	135
AcGU320050-F	5.19	1.48	1.48	2.22	8.89	5.19	3.70	8.89	1.48	15.56	1.48	7.41	7.41	0.74	0.74	5.93	6.67	8.89	2.22	4.44	135
AcGU320051-F	5.19	1.48	1.48	2.22	8.89	5.19	3.70	8.89	1.48	15.56	1.48	7.41	7.41	0.74	0.74	5.93	6.67	8.89	2.22	4.44	135
PcGU320052-F	5.50	0.92	1.83	2.75	10.09	4.59	4.59	7.34	0.92	16.51	1.83	7.34	8.26	0.92	0.92	7.34	5.50	8.26	0.92	3.67	109
SwGU320053-F	3.70	1.48	1.48	2.22	8.15	5.19	3.70	9.63	1.48	17.04	0.00	5.93	7.41	0.74	0.74	8.15	6.67	10.37	2.22	3.70	135
SwGU320054-M	3.68	0.74	1.47	2.21	10.29	5.15	4.41	8.09	2.21	16.18	2.94	6.62	6.62	1.47	0.74	6.62	8.09	7.35	2.21	2.94	136
Mean F	4.77	1.24	1.57	2.35	9.35	4.97	3.86	8.17	1.31	16.60	1.11	6.54	7.65	0.85	0.78	6.73	6.47	9.15	2.22	4.31	127.5
Mean M	4.68	0.74	1.97	2.71	10.84	5.17	4.43	6.16	2.71	15.27	3.45	6.65	6.65	1.48	0.74	6.65	8.13	7.39	2.22	1.97	135.3

Table 11. Length, base composition and sequence divergence of F and M haplotypes in two *rRNA* genes (*srRNA*, *lrRNA*) and *tRNA*s for lysine (*trnK*), threonine (*trnT*), tyrosine (*trnY*) of seven species of Unionidae. *U. pictorum*, *U. tumidus* and *S. woodiana* – own data, data for *Pyganodon grandis, Quadrula quadrula, Venustaconcha ellipsiformis* and *Inversidens japanensis* obtained from GeneBank

Gene	Species and Haplotype		Length (bp)	Base Composition (%)				Divergence (SE)
				T	C	A	G	K
rRNA genes								
srRNA	*U. pictorum*	F	588	21.9	22.8	38.9	16.3	0.236 (0.017)
		M	578	26.3	20.9	37.4	15.4	
	U. tumidus	F	589	24.4	20.4	38.9	16.3	0.231 (0.017)
		M	581	26.0	21.7	36.5	15.8	
	S. woodiana	F	581	25.5	20.3	37.5	16.7	0.255 (0.017)
		M	568	26.6	19.5	36.8	17.1	
	P. grandis	F	847	25.5	20.7	35.4	18.4	0.295 (0.016)
		M	841	27.1	20.1	36.1	16.6	
	Q. quadrula	F	857	24.4	22.2	37.1	16.3	0.284 (0.016)
		M	865	23.7	22.1	38.7	15.5	
	V. ellipsiformis	F	865	24.6	22.4	36.3	16.6	0.293 (0.016)
		M	859	25.4	20.8	36.9	16.9	
	I. japanensis	F	845	22.1	25.6	34.4	17.9	0.290 (0.015)
		M	867	21.7	25.0	35.3	18.0	
lrRNA	*U. pictorum*	F	705.5	29.0	20.4	39.4	11.2	0.320 (0.018)
		M	704	27.0	20.7	41.1	11.2	
	U. tumidus	F	693	32.1	17.7	39.4	10.8	0.350 (0.018)
		M	702	27.6	20.1	40.2	12.1	
	S. woodiana	F	695	29.6	18.0	40.9	11.4	0.333 (0.018)
		M	697	30.0	18.2	39.6	12.2	
	P. grandis	F	1271	29.3	19.8	35.4	15.5	0.311 (0.013)
		M	1287	27.2	20.5	36.4	15.9	
	Q. quadrula	F	1297	26.2	21.0	37.8	15.0	0.316 (0.013)
		M	1306	21.4	24.3	38.4	15.8	
	V. ellipsiformis	F	1271	25.6	21.7	38.6	14.1	0.318 (0.013)
		M	1290	24.6	22.3	37.6	15.5	
	I. japanensis	F	1304	23.7	25.5	34.6	16.3	0.317 (0.013)
		M	1334	21.0	22.2	36.4	15.7	
tRNA genes								
trnK	*U. pictorum*	F	65	26.2	23.1	30.8	20.0	0.225 (0.055)
		M	66	31.8	19.7	29.5	18.9	
	U. tumidus	F	69	33.3	18.8	29.0	28.8	0.200 (0.050)
		M	63	31.1	19.0	31.7	17.5	
	S. woodiana	F	67	29.9	22.4	28.4	19.4	0.220 (0.057)
		M	61	31.1	21.3	27.9	19.7	
	P. grandis	F	67	22.4	25.4	29.9	22.4	0.267 (0.057)
		M	62	37.1	17.7	27.4	17.7	
	Q. quadrula	F	64	25.0	25.0	29.7	20.3	0.271 (0.058)
		M	61	24.6	21.3	34.4	19.7	
	V. ellipsiformis	F	63	27.0	23.8	30.2	19.0	0.190 (0.051)
		M	63	27.0	23.8	31.7	17.5	

Table 11. continued

Gene	Species and Haplotype		Length (bp)	Base Composition (%)				Divergence (SE)
				T	C	A	G	K
	I. japanensis	F	65	23.1	30.8	24.6	21.5	0.238 (0.054)
		M	68	26.5	22.1	35.3	16.2	
trnT	*U. pictorum*	F	64	26.6	18.8	37.5	17.2	0.180 (0.049)
		M	62	22.6	25.8	29.0	22.6	
	U. tumidus	F	64	26.6	20.3	35.9	17.2	0.230 (0.054)
		M	62	21.0	29.0	25.8	24.2	
	S. woodiana	F	62	24.2	19.4	38.7	17.7	0.207 (0.053)
		M	63	15.9	30.2	30.2	23.8	
	P. grandis	F	63	22.2	22.2	34.9	20.6	0.207 (0.051)
		M	64	18.8	31.3	26.6	23.4	
	Q. quadrula	F	63	20.6	22.2	34.9	22.2	0.230 (0.052)
		M	70	20.0	25.7	35.7	18.6	
	V. ellipsiformis	F	67	25.4	20.9	37.3	16.4	0.306 (0.059)
		M	68	17.6	27.9	30.9	23.5	
	I. japanensis	F	62	22.6	24.2	35.5	17.7	0.304 (0.061)
		M	65	24.6	23.1	32.3	20.0	
trnY	*U. pictorum*	F	62	24.2	22.6	29.0	24.2	0.230 (0.056)
		M	62	22.6	17.7	37.1	22.6	
	U. tumidus	F	62	24.2	22.6	30.6	22.6	0.169 (0.050)
		M	61	23.0	16.4	39.3	21.3	
	S. woodiana	F	62	25.8	22.6	29.0	22.6	0.176 (0.051)
		M	64	20.3	20.3	39.1	20.3	
	P. grandis	F	62	25.8	21.0	30.6	22.6	0.276 (0.059)
		M	64	21.9	17.2	42.2	18.8	
	Q. quadrula	F	62	27.4	21.0	29.0	22.6	0.246 (0.054)
		M	69	21.7	23.2	36.2	18.8	
	V. ellipsiformis	F	62	25.8	24.2	27.4	22.6	0.246 (0.059)
		M	62	21.0	22.6	33.9	22.6	
	I. japanensis	F	62	19.4	25.8	30.6	24.2	0.196 (0.053)
		M	63	19.0	25.4	34.9	20.6	

Table 12. Values of p-distance between F and M haplotypes of *srRNA* gene (below diagonal) and *lrRNA* gene (above diagonal) in six species of Unionidae

			1	2	3	4	5	6	7	8	9
1	*U. crassus*	F		0.105	0.127	0.147	0.168	0.180	0.310	0.331	0.331
2	*U. pictorum*	F	0.069		0.160	0.192	0.199	0.222	0.314	0.326	0.357
3	*U. tumidus*	F	0.094	0.071		0.150	0.151	0.174	0.327	0.352	0.332
4	*A. anatina*	F	0.112	0.108	0.110		0.126	0.174	0.317	0.335	0.353
5	*A. cygnea*	F	0.131	0.113	0.129	0.073		0.170	0.349	0.363	0.361
6	*S. woodiana*	F	0.119	0.123	0.121	0.087	0.087		0.308	0.335	0.339
7	*U. pictorum*	M	0.223	0.225	0.221	0.248	0.246	0.231		0.143	0.182
8	*U. tumidus*	M	0.219	0.229	0.215	0.238	0.246	0.238	0.081		0.200
9	*S. woodiana*	M	0.229	0.233	0.223	0.252	0.250	0.244	0.106	0.121	

Table 13. Polymorphic sites in the fragment of RNA gene for the large ribosomal subunit (*lrRNA*) in female haplotypes of six species of Unionidae. Number of polymorphic site corresponds to its position during alingnmet of all sequences over the length of 817 bp. Polymorphic sites of the species marked as shaded boxes

Species	N	Haplotype	Length (bp)	Frequecy	Distance (SE)	Polimorphic sites								
						43	363	368	369	392	492	524	596	744
U. crassus	2	GU584009	709	1.00	0.00	T	T	T	A	G	T	A	T	–
U. pictorum	5	GU584005	705	0.80	0.00	A	T	T	A	A	C	T	A	–
		GU584006	706	0.20		A	T	T	A	A	C	T	A	T
U. tumidus	4	GU584007	693	0.75	0.004	G	C	T	A	A	T	C	T	–
		GU584008	693	0.25	(0.002)	G	T	T	A	A	C	T	T	–
A. anatina	5	GU584012	589	0.40	0.003	T	T	A	A	A	C	T	T	–
		GU584013	589	0.40	(0.002)	T	T	A	A	G	C	T	T	–
		GU584015	695	0.20		T	T	A	G	A	C	T	C	–
A. cygnea	6	GU584016	687	0.83	0.001	T	T	T	A	G	T	C	A	–
		GU584017	687	0.17	(0.002)	T	T	C	A	G	T	C	A	–
S. woodiana	5	GU584010	695	0.60	0.001	A	C	C	–	–	C	A	T	–
		GU584011	695	0.40	(0.001)	G	C	C	–	–	C	A	T	–

Jiangxi Province, China, HUANG et al. 2002) and 13% compared to GQ451855 (from South Korea). The above parameters of variation of gene *lrRNA* render it doubtful if the sequences are conspecific with a single taxon, *A. woodiana* under which name they were submitted to GenBank. If they represent two species, the question arises which of them occurs in Poland? Studying the sequence of the whole gene *lrRNA* in Polish specimens of the Chinese mussel (SOROKA 2010) made it possible to compare it with the available fragments of the gene in Asian specimens (Table 1). The studies confirmed the occurrence of two genetically different taxa under the name *A. woodiana*. Variation parameters were 10%, compared to sequences DQ073815 and DQ156197 and up to 4%, compared to the remaining two sequences (Table 16).

TRANSFER RNA GENE, *tRNA*

Between ribosomal RNA genes, mtDNA of unionid bivalves contains three transfer genes *tRNA* transporting lysine (*trnK*), threonine (*trnT*) and tyrosine (*trnY*), which form standard cloverleaf structure (Figs 6, 7). Between genes *trnT* and *trnY* occurs a non-coding region of 3–12 bp in F haplotypes and a somewhat shorter region in M haplotypes (2–6 bp). The length of *tRNA* genes ranged from 59 to 69 bp depending on the gene and species (part of the results in Table 11 and Figs 6, 7) and the values for F and M haplotypes were similar, at mean lengths of 64 and 63 bp, respectively. No intraspecific variation was observed for these genes, except one A/G substitution in M haplotype of *trnK* in *U. pictorum* (Fig. 7a).

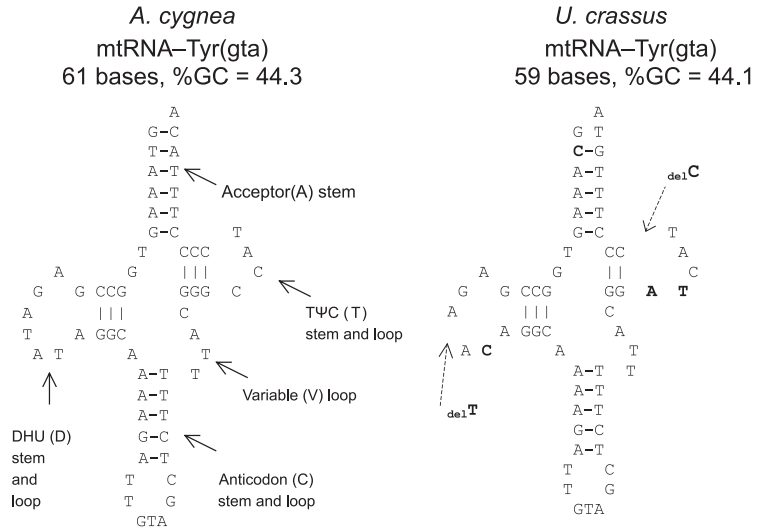

Fig. 6. Comparison of the potential cloverleaf secondary structure for F inferred tRNA transporting tyrosine in *A. cygnea* and *U. pictorum*. Different nucleotides indicated in the sequence for *U. crassus*. Features of RNA secondary structure are illustrated on one tRNA for *A. cygnea*

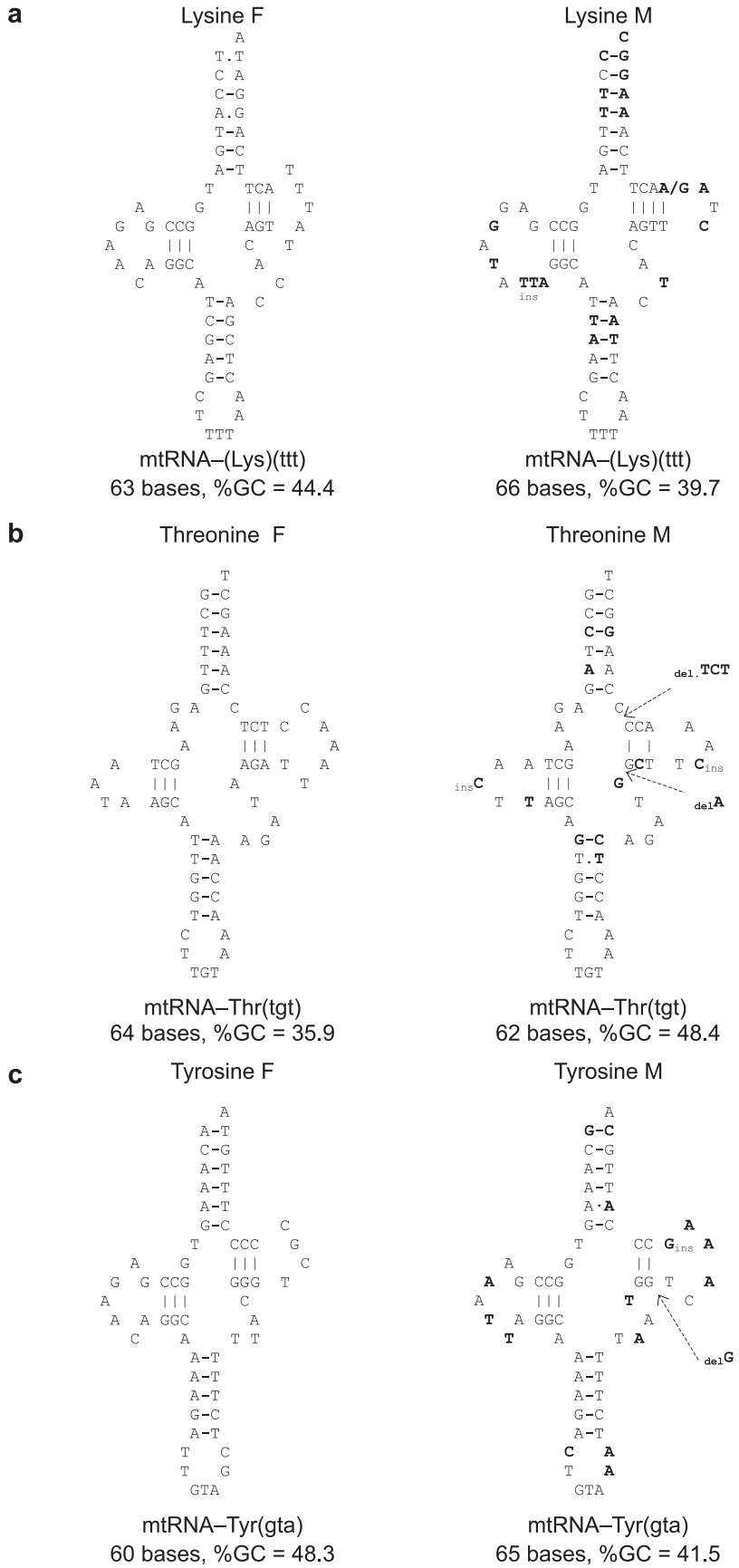

Fig. 7. Potential secondary structure for three inferred tRNAs of *U. pictorum*: a – for F and M tRNA transporting lysine, b – for F and M tRNA transporting threonine and c – for F and M tRNA transporting tyrosine. Different nucleotides indicated for M sequence

The observed interspecific variation involved single substitutions and insertions/deletions, most often within both loops (D and C loop), less often in the base-pair stems. For example, interspecific differences within F sequences between *A. cygnea* and *U. crassus* (causing 11.5% variation) for the most variable transfer gene, *trnY*, are shown in Fig. 6.

For female haplotypes the smallest interspecific differences were observed in gene *trnT* (1.6–8.1%), the greatest – up to 16.4% – in gene *trnY* (Table 14a, b, c). For male haplotypes the interspecific differences were smaller in genes *trnK* (each 3.3%) and *trnY* (up to 6.6%) and higher in *trnT* (up to 11.7%). The mean variation for the three transfer genes in the Polish Unionidae was ca. 5 and 7% for F and M haplotypes respectively, and the divergence between the haplotypes was 22%. The divergence between F and M haplotypes included additionally substitutions within the stems, frequent insertions of single bases, or even insertions or deletions of three base pairs (Fig. 7). Comparison of the present data with four other unionid species for which F and M haplotypes for the analysed *tRNA* genes were available showed a considerably greater variation within M haplotypes (12%), compared to F haplotypes (8%) and a comparable (23%) divergence between the haplotypes (Table 15).

Phylogenetic analysis was performed for the whole mtDNA fragment of the mean sequence length of 1,467 bp, including the two *rRNA* genes and the three *tRNA* genes located between them in six unionid species from Poland (Fig. 8a, b). No additional outgroup was used, since F and M haplotype

Table 14. Values of p-distance between F (below diagonal) and M (above diagonal) haplotypes of three *tRNA* genes in six species of Polish Unionidae

a) Gene *tRNA* for lysine – *trnK*							
		1	2	3	4	5	6
1	*U. crassus*		–	–	–	–	–
2	*U. pictorum*	0.032		0.033	–	–	0.033
3	*U. tumidus*	0.032	0.048		–	–	0.033
4	*A. anatina*	0.063	0.048	0.063		–	–
5	*A. cygnea*	0.095	0.095	0.063	0.111		–
6	*S. woodiana*	0.064	0.063	0.064	0.063	0.111	
b) Gene *tRNA* for threonine – *trnT*							
1	*U. crassus*		–	–	–	–	–
2	*U. pictorum*	0.016		0.117	–	–	0.083
3	*U. tumidus*	0.016	0.032		–	–	0.100
4	*A. anatina*	0.065	0.081	0.048		–	–
5	*A. cygnea*	0.048	0.032	0.032	0.048		–
6	*S. woodiana*	0.016	0.000	0.032	0.081	0.032	
c) Gene *tRNA* for tyrosine – *trnY*							
1	*U. crassus*		–	–	–	–	–
2	*U. pictorum*	0.066		0.033	–	–	0.066
3	*U. tumidus*	0.016	0.082		–	–	0.066
4	*A. anatina*	0.098	0.131	0.082		–	–
5	*A. cygnea*	0.115	0.148	0.098	0.049		–
6	*S. woodiana*	0.131	0.164	0.115	0.066	0.016	

Table 15. Values of p-distance between F (below diagonal) and M (above diagonal) haplotypes for three *tRNA* genes jointly, in seven species of Unionidae

		1	2	3	4	5	6	7
1	*U. pictorum*		0.069	0.074	0.103	0.149	0.149	0.120
2	*U. tumidus*	0.039		0.069	0.063	0.160	0.171	0.160
3	*S. woodiana*	0.073	0.056		0.051	0.149	0.160	0.126
4	*P. grandis*	0.096	0.090	0.056		0.189	0.189	0.160
5	*Q. quadrula*	0.112	0.096	0.101	0.124		0.149	0.171
6	*V. ellipsiformis*	0.084	0.090	0.107	0.135	0.101		0.149
7	*I. japanensis*	0.107	0.101	0.101	0.135	0.084	0.112	

Table 16. Values of p-distance between female sequences of *S. woodiana* from Poland and *A. arcaeformis*, *A. arcaeformis flavotincta*, and *A. woodiana* from Asia and *A. beringiana* for three mitochondrial genes. Numbers of isolates given for taxa from South Korea; their accession numbers are presented in Fig. 9 and Table 1

Species and accession number or isolate	*S. woodiana* from Poland		
	cox1	*cytb*	*lrRNA*
A. arcaeformis – c8	0.057	0.098	0.024
A. arcaeformis – c82	0.062	0.083	0.024
A. arcaeformis flavotincta – c9	0.163	0.226	0.116
A. arcaeformis flavotincta – c92	–	0.228	–
A. woodiana – c7	0.062	0.101	0.041
A. woodiana – c72	0.062	0.101	–
A. woodiana AB040832 (Japan)	0.062	–	–
A. woodiana DQ073815 (China)	–	–	0.102
A. woodiana DQ156197 (China)	–	–	0.102
A. beringiana DQ272370 (Canada)	0.126	–	–
A. beringiana EU327357 (USA)[a]	0.126	–	–

[a]CHONG et al. 2008

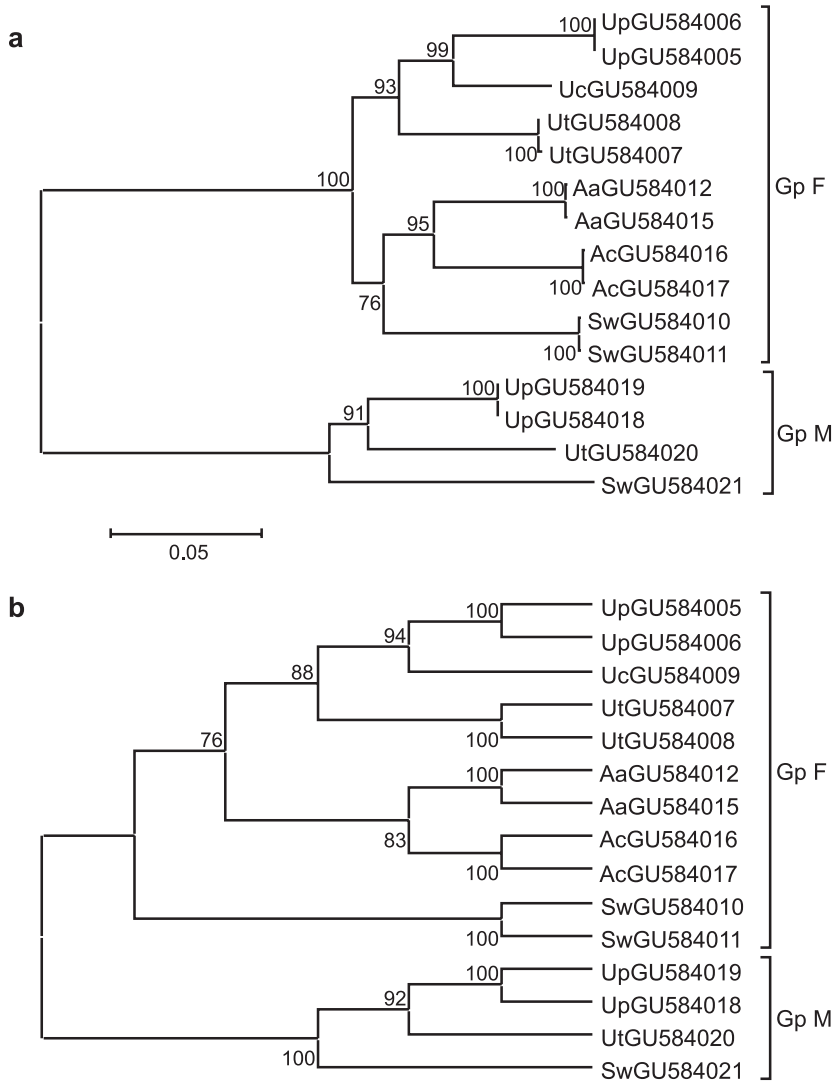

Fig. 8. Neighbour Joining (a) and Maximum Parsimony (b) trees for species of Unionidae – partial *srRNA*, *lrRNA* and complete three *tRNA*s (*trnK-trnT-trnY*) sequences. Abbreviations as in Fig. 3. Values above branches represent bootstrap support (2,000 and 500 replicates for NJ and MP analyses, respectively)

could be treated as outgroups to each other. In phylogenetic reconstruction with NJ, ME, MP and Bayesian methods male and female sequences of the analysed fragment of mtDNA formed separate clades and the clustering within the clades was identical for NJ, ME and Bayesian methods and slightly different for MP method. In NJ, ME and Bayesian methods trees in each F clade one group included the genus *Unio*, the other *Anodonta* and *Sinanodonta*, whereas in MP tree *Sinanodonta* formed a separate clade, and *Unio* clustered with *Anodonta*. In male sequences of this mtDNA fragment the topologies were identical, with the absence of data for the genus *Anodonta*.

DISCUSSION

Among freshwater unionid bivalves DUI was first observed in *Pyganodon grandis* (synonym of *Anodonta grandis grandis*) in 1996, based on population studies of mtDNA with RFLP method (Restriction Fragment Length Polymorphisms) (LIU et al. 1996). In the same year HOEH et al. (1996b) reported on identification of DUI in another two unionid species (*P. fragilis* and *Fusconia flava*) based on sequential analyses of COI gene (synonym of *cox1*). In 2002–2009 the list was extended to include further 48 unionid species with descriptions of M haplotypes in single genes *cox1*, *cox2*, *cytb* or *16S rRNA* (HOEH et al. 2002b, CUROLE & KOCHER 2002, 2005, KREBS 2004, MOCK et al. 2004, WALKER et al. 2006a, b, CHAKRABARTI et al. 2006, SOROKA 2008a, b); whole female mtDNA genomes were studied in three species (*Cristaria plicata* NC_012716, *Hyriopsis cumingii* NC_011763, GenBank unpublished data and *Lampsilis ornata* SERB & LYDEARD 2003), and both female and male genomes – in four species (*Inversidens japanensis* AB055625-4, GenBank unpublished data, *P. grandis*, *Quadrula quadrula* and *Venustaconcha ellipsiformis*, BRETON et al. 2009). Current paper reports also on the occurrence of M haplotypes in another three species of *Unio* (*U. crassus*, *U. pictorum* and *U. tumidus*), which brings the total number of unionid species with documented DUI to 54. Many sequences available in GenBank for Unionidae (Tables 1 and 16) have no specified appurtenance to F or M haplotype. In phylogenetic analyses these sequences cluster with F haplotypes (Fig. 3a, b) indicating that they represent mtDNA from somatic tissue and that the species have also M haplotypes occurring in male gonads. Examples are *U. mancus*, *A. cygnea*, *Potomida litoralis*, or *A. arcaeformis* and *A. beringiana*.

Among the studied genes *cox1* was the best marker for detection of M haplotypes in unionids; it amplifies both forms of mtDNA with universal primers, depending on the kind of tissue used for DNA isolation (FOLMER et al. 1994). Gene *cox1* was used in more than 50% of papers on DUI in Unionidae (HOEH et al. 1996b, 2002b, MOCK et al. 2004, WALKER et al. 2006a, SOROKA 2008a, b). It is also commonly studied in many other animals, since it has been found that more than 95% of animals have characteristic sequences, and its 5' end, 648-bp long, is used to create the huge database, The Barcode of Life (HEBERT et al. 2003a, b, RATNASINGHAM & HEBERT 2007).

Cytochrome b proved to be the least effective gene for DUI detection; its M haplotypes were obtained for two species (*A. anatina* and *S. woodiana*). Likewise, using the same primers (Table 4) male haplotypes were obtained only for the genus *Anodonta*: *A. californiensis*, *A.* cf. *oregonensis*, *A.* cf. *wahlamentensis* (MOCK et al. 2004). The primers used, UCYTB151F and UCYTB270R, are universal for bivalves, gastropods and cephalopods, but the amplified fragment of gene *cytb* revealed nucleotide diversity in small samples within several of these taxa (MERRITT et al. 1998). In the case of Unionidae these primers are also universal for F haplotypes, but for M haplotypes – only for the genus *Anodonta*. Intra- and interspecific variation of F and M haplotypes in this gene is higher than in the case of *cox1* (Tables 5 and 8, Figs 2, 4), which may be the reason for the lack of specificity of these primers for M sequences of the genus *Unio*.

This study is the first to report on unionid male and female sequences including several consecutive mitochondrial genes. The technique used was effective for F and M haplotypes in 100% and 50% cases, respectively; it required Long-Range PCR, followed by re-PCR. The resulting fragment, ca. 1,500 bp long, included three whole *tRNA* genes and the flanking fragments of both mitochondrial *rRNA* genes in the following order: 3' end of *srRNA* – *trnK* – *trnT* – *trnY* – 5' end of *lrRNA*. The genes are encoded on one, light strand of mtDNA, and their arrangement is identical in F and M haplotypes, as well as in all completely known mitochondrial genomes of Unionidae (SERB & LYDEARD 2003, BRETON et al. 2009, SOROKA & BURZYŃSKI 2010). Based on this marker, M haplotypes were obtained for three species: *U. pictorum, U. tumidus* and *S. woodiana,* and female haplotypes for all six analysed species.

Because of the different tissue localisation of F and M genomes in unionids, generally DUI detection should be easy but in practice it is not. In this study the failure to detect M haplotypes in the case of all molecular markers used in *A. cygnea*, in spite of precise DNA isolation from gonads of eight males, and the experience of the author who had obtained M haplotypes from five other species of the genera *Anodonta* (SOROKA 2008a, b) and *Unio* (this study), was surprising. In the case of all used pairs of primers for amplification of mitochondrial genes in *A. cygnea* F

haplotypes were obtained from male isolates, and they were identical with those obtained from females, or the sequences were illegible. In this situation it is likely that during isolation, apart from male gonads, also somatic tissues of the males were sampled, where F genomes prevailed and underwent amplification or caused contamination, or both forms of mtDNA became amplified, resulting in illegible sequences. The presence of both F and M forms in male gonads was observed in the mytilid *Geukensia demissa* and a unionid *Fusconia flava* (HOEH et al. 1996a, b). Difficulty to detect both mtDNA genomes in various species may also result from their great divergence; in such a case the DNA primers used may reveal only one, most often female haplotype (THEOLOGIDIS et al. 2008). Less variable female genomes offer a greater chance of obtaining similar sequences in many taxa with the use of the same PCR primers. For male genomes, with their higher rate of substitutions, the universal PCR primers are often ineffective and only designing specific M primers yields satisfactory results, like e.g. in species of Ambleminea (CUROLE 2004, WALKER et al. 2006b). M haplotypes in *Geukensia* were obtained only for one gene, *cox1*, and attempts at obtaining M forms for gene *16SrRNA*, using universal primers, failed (HOEH et al. 2002b). DUI identification based on one gene is sufficient, but ascertaining the absence of DUI based on one gene is not. The lack of M haplotypes in other genes, after its detection in only one, suggests a great divergence of M and F sequences and thus difficulties in studying the whole problem.

For more than 30 years mitochondrial DNA has been intensively used in taxonomic, evolutionary, phylogeographic or molecular ecological studies, and for 20 years it has been an interesting object of studies on several families of marine and freshwater bivalves, mainly Mytilidae and Unionidae, after identification of doubly uniparental inheritance of mtDNA (DUI) in these taxa (FISHER & SKIBINSKI 1990, BOORE & BROWN 1994b, HOEH et al. 1996b, LIU et al. 1996). DUI identification through sequenctial analyses of various mitochondrial genes in a total of 54 species of Unionidae did not reveal masculinisation in this family, though it appeared several times in the history of *Mytilus* (ZOUROS et al. 1992, HOEH et al. 1996a, 1997, 2002a, QUESADA et al. 1999). An additional DNA fragment on 3' end of gene *cox2* (ca. 555 bp or ca. 185-codon extension) found exclusively in males was observed in Unionidae, but it is absent in females of this family, and in male and female genomes in *Mytilus* (CUROLE & KOCHER 2002, 2005, CUROLE 2004, MIZI et al. 2005, ZBAWICKA et al. 2007, 2010, BRETON et al. 2009). This male-specific region evolves more rapidly than the male-female homologous region and reveals no significant similarity with any sequences in GenBank during blast analyses (CUROLE & KOCHER 2002). It is conjectured that this fragment of mtDNA may play a part during reproduction or be responsible for the lack of masculinisation in Unionidae. Gene M *cox2* produces an active protein which is present in testes and sperm and, to a lesser extent, in other male tissues, but not detected in female tissues (CUROLE & KOCHER 2002, CHAKRABARTI et al. 2006, 2007). In 2009 BRETON et al. (2009) discovered a new F and M-specific gene (F and M-specific open reading frames, ORFs) in all available unionid genomes, which was then confirmed for another species, *U. pictorum* (SOROKA & BURZYŃSKI 2010). These genes are characterised by variation of their length and amino acid sequence, for which very low similarity was observed, of 20 and 60% for M and F ORFs, respectively (BRETON et al. 2009). It was found that in *V. ellipsiformis* the F-specific and M-specific proteins were effectively expressed in female and male gonads, respectively (BRETON et al. 2009). The gender-specific new genes cound be responsible for the different mode of transmission of the mtDNAs and/or gender-specific adaptive functions of M and F mtDNA genomes in Unionidae (BRETON et al. 2009).

In bivalve species with DUI type inheritance of mtDNA characteristics of mitochondrial genes should include female and male lineages. Divergence of entire F and M genomes in the genus *Mytilus* exceeds 20% (STEWART et al. 1995, MIZI et al. 2005, ZBAWICKA et al. 2007, 2010), and an even greater divergence, up to 52%, has been observed in Unionidae (THEOLOGIDIS et al. 2008, BRETON et al. 2009, DOUCET-BEAUPRÉ et al. 2010), while for single genes in unionids it ranges from 20 to 35% (HOEH et al. 1996a, b, SOROKA 2008a, b). Both forms of mtDNA show a great variation, not only with respect to nucleotides but also in the genome length, male genomes being longer (MIZI et al. 2005, ZBAWICKA et al. 2007, BRETON et al. 2009, DOUCET-BEAUPRÉ et al. 2010). The elongation of male mitochondrial genomes in unionids is a result of the presence of additional sequences ca. 555 bp long on 3' end of gene *cox2*, which are absent from female forms (CUROLE & KOCHER 2002, 2005, CHAKRABARTI et al. 2006, BRETON et al. 2009). The length of the region is conservative in 92% of the studied species and continues coding for the functional protein which however shows a decidedly greater rate of synonymous mutations, compared to nonsynonymous substitutions (CUROLE & KOCHER 2005, CHAKRABARTI et al. 2006).

Characteristics of F and M sequences of seven mitochondrial genes in all the unionid species occurring in Poland, presented here, showed a similar level of divergence of the two haplotypes within species, and differences between the genes were observed. The smallest (ca. 20%) nucleotide divergence between F and M haplotypes was observed for *tRNA* genes, followed by the *RNA* gene for the small ribosomal subunit (24%) and then much greater (over 30%) for protein-coding genes (*cox1*, *cytb*) and the *RNA* gene for the large ribosomal subunit (*lrRNA*)

(Tables 5, 8, 11). A similar level of divergence of F and M haplotypes (32%) was observed for gene *lrRNA* in *P. grandis* (KREBS 2004). In this study examination of entire mitochondrial genes of both forms included three *tRNA* genes in three species, where also a variation in length was observed. Most often male haplotypes were longer, though only by 1–2 bp, which was up to 3% of length; an exception was *tRNA* for lysine (*trnK*) (Table 11). However, with the number of 22 transfer genes present in mtDNA, even their small elongation may globally cause a certain total elongation of M genomes. The tendency is more pronounced in four other unionid species (DOUCET-BEAUPRÉ et al. 2010), where longer M haplotypes were observed in analysed *tRNA* genes (up to 7 bp, except *trnK*) and both *rRNA* genes, even up to 30 bp in *I. japanensis* (on average by 17 bp). Though the difference in the length of the compared genes in favour of longer male sequences is slight in terms of percent (up to 3%, except *tRNA* genes in *Q. quadrula*), their nucleotide divergence is decidedly great. For seven species of unionids from Poland, Japan and USA it ranges from 18 to 31% for *tRNAs* genes, from 23 to 30% for *srRNA* and from 31 to 35% for *lrRNA* (Table 11).

Despite the large sequential differences, mitochondrial rRNAs and tRNAs have a very conservative secondary structure, and the differences in this respect are very small. For example, secondary structures of both *rRNA* genes in *Drosophila yakuba* are much similar to those in mouse (CLARY & WOLSTENHOLME 1985b); also most helices in the structure of *rRNA* of *Escherichia coli* have their counterparts in other species (HIGGS & ATTWOOD 2008). The increased content of G+C bases in the sequence, because of the lower free energy of the helices with their higher content, is an essential factor affecting the stability of the secondary structure of RNA (HIGGS 2000, HIGGS & ATTWOOD 2008). Generally pairs GC are more stable than AU, and the remaining types of pairs (GU, UG and mismatched MM) occurring in the internal part of the helices are usually unstable (HIGGS & ATTWOOD 2008). In the analysed unionid species the G+C content in the sequences of the two ribosomal RNA genes, *srRNA* and *lrRNA* and jointly in the three transfer genes is ca. 37, 30 and 41% respectively, and also protein-coding genes are within this range of values (ca. 37% G+C for *cox1* and *cytb*, Tables 6, 9). The lowest values were observed in *D. yakuba* in *srRNA* and *lrRNA* (21 and 17%, respectively), where the GC content was uneven along the whole length of these genes, with extremely low values (15 and 10%, respectively) in 5' end (CLARY & WOLSTENHOLME 1985b). The results of this study pertaining to the 5' end of *lrRNA* and data available from GenBank (Table 1) for 3' end of this gene made it possible to analyse the distribution of G+C bases on its whole length in six bivalve species. In all species of unionids, like in *Drosophila*, the proportion of G+C was much smaller in 5' end of *lrRNA* than in 3' and the mean values for all species were 30 and 39%, respectively. The greatest differences, of 10%, in the G+C content in these two regions of *lrRNA* were observed in *U. crassus*, *U. tumidus*, *A. anatina* and *S. woodiana*. Among the analysed species, the whole sequence of *lrRNA* gene, 1,302 bp long and containing 34% G+C bases, was ascertained only for *U. pictorum* (SOROKA & BURZYŃSKI 2010). Regions 5' (620 bp) and 3' (620 bp) of the gene had 31 and 38% G+C, respectively. For M haplotypes of *lrRNA* it was possible to determine the G+C content only for 5' end in three unionid species (Table 11), and it was somewhat lower than that estimated for F haplotypes and amounted to 31.5%. However, the greatest content of G and C bases was observed in fragments forming helices of *tRNA* genes where they constituted ca. 43% (Figs 6, 7), the value of 45% being reported as typical of mitochondrial *tRNA* genes (HIGGS 2000). Bacterial and nuclear *tRNA* genes have an even higher G+C content, with 68% in helical fragments (HIGGS 2000).

The mitochondrial transfer RNAs of many metazoan species are often degenerate in sequence and structure, compared to tRNAs in their bacterial ancestral and nuclear tRNAs, and these mitochondrial genes are referred to as 'bizarre' (HELM et al. 2000, LASLETT & CANBACK 2008). Compared to the typical secondary structure, mitochondrial tRNAs have smaller dihydrouridine (D) stems and loops, as well as TΨC (T) stems and loops, and elongated anticodon (C) stems (Fig. 6). However, the greatest observed differences involve the absence of the whole D-arm or T-arm or variable (V) loop, where there are short sequences instead (BRETON et al. 2006, LASLETT & CANBACK 2008). In bivalves of the genera *Lampsilis* and *Mytilus* both *trnS1* and *trnS2* (for serine) encode shorter D arms without paired bases (SERB & LYDEARD 2003, MIZI et al. 2005, BRETON et al. 2006). This atypical (unpaired D arm) form of gene *trnS1* occurs also in several other mollusc species (BOORE & BROWN 1994a, YAMAZAKI et al. 1997, BOORE et al. 2004). The absence of loop in T arm was observed only in *Lampsilis* in tRNA transporting cysteine (SERB & LYDEARD 2003), while the genera *Mytilus* (bivalve), *Katharina* (chiton) and *Graptacme* (scaphopod) had its typical form (BOORE & BROWN 1994a, BOORE et al. 2004, MIZI et al. 2005). A wide variation in the length of T arm was observed in snails, including its reduction or complete absence (YAMAZAKI et al. 1997). Three mitochondrial tRNAs for lysine, threonine and tyrosine in six unionid species adopted the typical secondary structure. All C and D arms had 5 and 3 base-pair (bp) stem, respectively, and always 7 bp in C loop, where three bases formed anticodon. In contrast, the loop of D arm was variable and had 4–8 bp or exceptionally 10 in the male form of tRNA(L) in *U. pictorum* (Figs 6, 7). The remaining T and A arms dis-

played variation in their stems and the length of T loop. The variation of mitochondrial tRNA genes in unionid bivalves described above is interspecific, while within species the genes are characterised by very high stability.

The mean length of analysed tRNAs was similar in all the species: 64 and 63 bp for F and M haplotypes, respectively. The values were close to the mean values for these genes in the studied unionid species (64 and 65 bp for F and M haplotypes, respectively; Table 11). The presented length values for unionid *tRNAs* are somewhat smaller than the mean values for the marine genus *Mytilus* (66 bp for F and M genomes) (HOFFMANN et al. 1992, MIZI et al. 2005, ZBAWICKA et al. 2007) and for the insect *Drosophila yakuba* (67 bp) (CLARY & WOLSTENHOLME 1985a), but larger than those observed in three species of pulmonate gastropods (61 bp) (YAMAZAKI et al. 1997). The very small length of tRNAs in *Euhadra herklotis*, *Cepaea nemoralis* and *Albinaria coerulea* results from the reduction of D and T loops which is correlated with small mitochondrial genomes (14.1–14.5 kbp) and nucleotide overlap with unstable acceptor stems of tRNAs in downstream flanking genes (YAMAZAKI et al. 1997). The overlap of 3' ends of *tRNA* genes was observed also in *D. yakuba* (CLARY et al. 1983), but not in marine or freshwater bivalves (this study and HOFFMANN et al. 1992, SERB & LYDEARD 2003, BOORE et al. 2004, MIZI et al. 2005, ZBAWICKA et al. 2007).

Base pairing departing from the Watson-Crick type is frequently observed in cloverleaf structures of *tRNAs* of various animal species, both vertebrate and invertebrate. GU pairs, though less stable than the Watson-Crick pairs, occur rather often in helical fragments of RNA (up to a few percent of all pairs) and are regarded as normal base pairs (HIGGS & ATTWOOD 2008). In the three tRNAs of all the analysed bivalve species mismatched nucleotide pairs in the stems were observed, most of all in the acceptor (A) stem, and they were: G·T, T·T, C·A, A·G and A·A (Figs 6, 7). G·T mismatched in the acceptor stem of *trnY* was observed once in the same three tRNAs of the freshwater bivalve of the genus *Lampsilis*, while in the marine *M. galloprovincialis* one case of mispairing was observed in each of these genes, and even two in *trnY,* and in all cases they were of G·T type (MIZZI et al. 2005). The greatest number of mismatched nucleotide pairs (up to 11 per molecule) was observed in four additional tRNA-like structures in a recently masculinised mt genome of *M. trossulus* (BRETON et al. 2006). When comparing F and M cloverleaf structures of the same *tRNA* genes in one unionid species, it can be seen that normal pairs in the stem of one form have mispairing in the same site in the other form, and this involves mainly the acceptor stem. For example in *U. pictorum* in genes *trnT* and *trnY* (Fig. 7) it can be observed that M forms compared to F forms have mispairing and different nuleotides already paired according to the Watson-Crick rule in the acceptor stem. It can be conjectured that the first mutation causing mispairing disturbs the molecule structure, and the second compensates for the first error. The phenomenon is termed compensatory substitution and provides evidence for forming pairs by the nucleotides in the spatial structure of RNA and correlated evolution of two ends of the RNA molecule (HIGGS & ATTWOOD 2008).

This study provides the first characteristics of female and male haplotypes of both *rRNA* genes (partial sequences) and three *tRNA* genes (complete sequences) in the Polish species of Unionidae. Whole *rRNA* and *tRNA* genes were first identified in mitochondrial female and male genomes of *I. japanensis* (2001, unpublished data), and then in female genomes of *L. ornata* (SERB & LYDEARD 2003), *C. plicata* and *H. cumingii* (2009, unpublished data), and recently both forms of mtDNA in *P. grandis, Q. quadrula* and *V. ellipsiformis* (BRETON et al. 2009). All these data pertain to specimens from Asia and USA, and only single specimens were used, which enables gene characteristics at the interspecific but not intraspecific level. In this study up to six specimens per species were analysed which makes it possible to make comparisons at both these levels. Variation of up to 0.4% (1, 2 or 3 female haplotypes per species) was observed for *lrRNA* in six species of Polish unionids (Table 13). Two female haplotypes of the gene were also detected in American populations of *P. grandis,* as well as many more male haplotypes (5 haplotypes, KREBS 2004). The variation for *lrRNA* gene observed among the Polish species of Unionidae (Table 12) was comparable with that found for male sequences of American unionids (up to 20%) (KREBS 2004). For the Polish female haplotypes it was slightly higher than among the American unionids; it was up to 20 and 16%, respectively (KREBS 2004). No Polish unionid species showed intraspecific variation of *srRNA* and short *tRNAs*, except the single polymorphic site (A/G) in T loop of male form of *trnK* in *U. pictorum*, causing variation of 1.5% (Fig. 7a). Genes *tRNA* of the Polish unionids displayed a wide range of interspecific divergence: from 1.6% (within the genus *Unio*) to 16% for F haplotypes and from 3 to 12% for M haplotypes (Table 14), with mean values of 7 and 9% for F and M haplotypes, respectively. The sequences available from GenBank (Table 11) showed a greater interspecific variation, of 18 and 26% for F and M types, respectively. This wider variation results from the fact that the sequences represented single species of different genera, from both USA and Asia.

Among the three analysed *tRNA* genes only male haplotypes of *trnT* varied more than female haplotypes among the studied species (Table 14). Among the sequences available from GenBank (*P. grandis*, *Q. quadrula* and *V. elipsiformis*) male forms of these genes were more varied, except one *trnY*, where the variation

was the same for both haplotypes, 8.5% each. The same range of divergence between F and M haplotypes was observed in the three *tRNA* genes analysed jointly in the Polish unionids; it was 21% and thus comparable to *trnY* gene in the remaining species from the USA and Asia, and smaller compared to *trnK* (30%) and *trnT* (32%). In the marine members of *Mytilus* male and female *tRNA* genes, 23 of each, were analysed (22 and additional *tRNA* for methionine) and showed divergence of 11%; they are the least variable mitochondrial genes, compared to other mitochondrial rRNA or protein-coding genes (MIZI & ZOUROS 2005, ZBAWICKA et al. 2007). In freshwater Unionidae, both in the Polish species and those available from GenBank, the divergence between female and male haplotypes in the three *tRNA* genes was more than twice higher than in marine bivalves (Table 11).

Among the 13 mitochondrial protein-coding genes, two are most often used for molecular studies: *cox1* (HOEH et al. 1998, 2002a, GRAF 2000, GRAF & Ó FOIGHIL 2000a, LYDEARD et al. 2000, HUFF et al. 2004, MOCK et al. 2004, ARAUJO et al. 2005, 2009, CAMPBELL et al. 2005, KÄLLERSJO et al. 2005, SOROKA 2005, 2008a, b, GRAF & CUMMINGS 2006, SERB 2006, WALKER et al. 2006a, CHONG et al. 2008) and *cox2* (CUROLE & KOCHER 2002, 2005, CUROLE 2004, CHAKRABARTI et al. 2006, WALKER 2006b), as well as *nad1* (SERB et al. 2003, CAMPBELL et al. 2005, SERB 2006) and *cytb* (MOCK et al. 2004). Often the sequences used for phylogenetic analyses originate from single specimens of various species which precludes estimates of intraspecific variation, for example analysis of *cox1* gene in 26 species of freshwater mussels in North America (GRAF & Ó FOIGHIL 2000a), or *lrRNA* in six species of Unionidae (KÄLLERSJO et al. 2005). In phylogenetic analyses interspecific variation, though possible to estimate, is not directly relevant and thus ommited (LYDEARD et al. 1996, GIRIBET & WHEELER 2002, HUANG et al. 2002, GRAF & CUMMINGS 2006, WALKER et al. 2006a, HOEH et al. 2009). Though DUI in Unionidae was described in 1996 (HOEH et al. 1996b, LIU et al. 1996), studies on mitochondrial DNA in the members of the family most often pertain to female (F) sequences, because they are easily obtained from somatic tissues, disregarding the specimen's sex (STĘPIEŃ et al. 1999, GRAF & Ó FOIGHIL 2000a, GIRIBET & WHEELER 2002, LEE & Ó FOIGHIL 2004, ARAUJO et al. 2005, 2009, KÄLLERSJO et al. 2005, SOROKA 2005). However, in DUI bivalves complete characteristics of genes at the population, intra- and inter-specific and evolutionary level should include both male and female sequences.

The first population studies considering F and M haplotypes pertained to *Anodonta californiensis* from the USA (MOCK et al. 2004). They showed the absence of or a slight variation in mitochondrial genes *cox1* and *cytb* among F haplotypes and a slightly greater variation in their male counterparts. Interpopulation variation in *A. californiensis* for *cox1* gene was 0 to 5% for both F and M types (MOCK et al. 2004). A small interpopulation variation was also observed for female sequences of *cox1* and *nad1* in American populations of *Cyprogenia aberti*; it was up to 0.7 and 0.9%, respectively (SERB 2006). The previous studies on European unionid species revealed great differences in the level of intraspecific variation. A small variation among female haplotypes of *cox1* gene was observed in *U. pictorum* (0.3%) and *U. tumidus* (0.2%) (KÄLLERSJO et al. 2005) as well as *U. gibbus* (0.4%) (ARAUJO et al. 2009) and *A. anatina* (0.3%) (SOROKA 2008a). No variation was observed in species under strict protection, such as *A. cygnea* and *P. complanata* (KÄLLERSJO et al. 2005) or in *S. woodiana* which had been introduced in Poland in the 1980s (SOROKA 2008b). The analysis of the Polish specimens of these species showed a greater variation in female sequences of *cox1* in *U. pictorum* and *A. cygnea*, up to 0.4% and 0.2%, respectively (Table 5). In the case of *U. pictorum* it is known that the genetic relationships within populations from central Europe are connected with palaeogeographical relationships between river systems during the Pliocene and Pleistocene (NAGEL 2000). A decidedly greater intraspecific variation of *cox1* gene (3%) was observed in species where the sequences were derived from geographically remote specimens (Sweden, Poland and Iberian Peninsula), and these were *A. anatina* and *U. crassus*. A comparable variation was recorded for species of the genus *Potamilus* for *cox1* gene (2.6%) (ROE & LYDEARD 1998) and of the genus *Quadrula* for *nad1* gene (up to 3.3%) (SERB et al. 2003).

The Polish specimens of *S. woodiana* displayed a wide genetic variation compared to the sequences available from GenBank for this species. For *cox1* gene it exceeded 6%, compared to female haplotypes of Japanese and South Korean specimens (Table 16). The difference in *cytb* and *lrRNA* for female sequences was 10 and 4% respectively between specimens of *S. woodina* from Poland and South Korea (Table 16). The presented values are the highest among the observed differences, and they are surprising, considering the absence of variation in all the analysed genes in the Polish specimens of *S. woodiana*, except one A/G substitution in gene *lrRNA* (Table 13), with the total of 56 analysed sequences. The observed differences between the Polish, Japanese and South Korean populations of *S. woodiana* are greater than the differences between European species *U. pictorum* and *U. mancus* within *cox1* gene, of 4.2% and 11% among the remaining species of *Unio*, or *Anodonta* (Fig. 2). In view of the above values, either *S. woodiana* shows an extremely great intraspecific variation, or the compared sequences originate from specimens representing different species living in Poland and Asia.

Comparison of sequences from the Polish specimens of *S. woodiana* within three mitochondrial genes

(*cox1*, *cytb* and *lrRNA*) with the available sequences from Asian *Anodonta* showed the greatest similarity to Asian sequences from *Anodonta arcaeformis*, with *A. arcaeformis* and *A. arcaeformis flavotincta* showing great genetic differences, at the level of distinct species (Fig. 9a, b, c). Besides, Asian specimens identified as *A. woodiana* sometimes cluster with *A. arcaeformis,* testifying to a difficulty or imprecision of taxonomic identification of the species. However, clustering of the same specimen of *A. woodiana* (isolate c7) with *A. arcaeformis* in the case of gene *lrRNA*, and with *A. woodiana* in the case of the remaining genes (at high bootstrap value), suggests a necessity of analysis of the greatest possible number of genes in order to avoid computer artefacts. In the light of the available data the Polish specimens of the Chinese mussel are genetically closer to *A. arceaformis* than to *A. woodiana.* Their mean level of variation for *lrRNA* gene is 2.4% and is the lowest compared to the remaining Asian species of *Anodonta* (Table 16). The differences in this gene between the Polish specimens of the Chinese mussel and *A. woodiana* have a wide range, from 4%, which is possible due to the wide geographical range of *A. woodiana* in the Palaearctic and Indotropical regions, to 10%, suggesting two distinct species one of which may be misidentified. For genes *cox1* and *cytb* the differences between the Polish specimens and *A. arcaeformis* and *A. woodiana* are comparable, at over 6 and 9%, respectively (Table 16). If the Polish specimens represented one of these species, it would anyway show a wide intraspecific variation which can be explained by the existence of a single large species or cryptic biological diversity (SERB 2006). Some bivalve species show such a wide intraspecific variation. Sympatric individuals of *Cyprogenia aberti* from Ouachita and St. Frances River Drainage (USA) showed surpris-

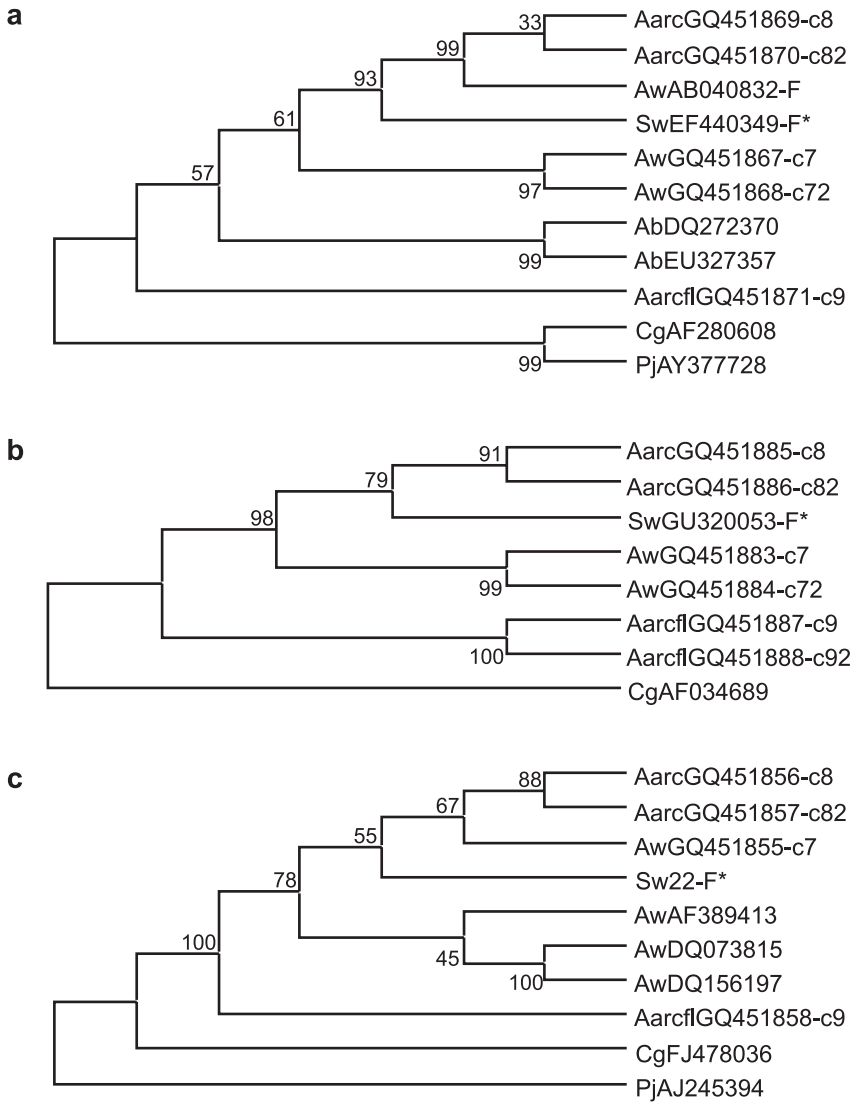

Fig. 9. Maximum Parsimony trees for *cox1* (a), *cytb* (b) and *lrRNA* (c), comparing *S. woodiana* (Aw, Sw), *Anodonta arcaeformis* (Aarc), *A. arcaeformis flavotincta* (Aarcfl) and *A. beringiana* (Ab). *Crassostrea gigas* (Cg) and *Pecten jacobaeus* (Pj) are used as outgroup. GenBank Accession numbers are given for all sequences and isolates for sequences from South Korea. Own sequences marked with asterisk. Values above branches represent bootstrap support greater than 40% (2,000 replicates)

ingly high genetic distances for *cox1* (15%) and *nad1* (20%). These two very different mitochondrial lineages in the species correlate with sympatric red and brown conglutinate forms, with no correlation with the host fish preferences (SERB 2006, SERB & BARNHART 2008). In the light of the previous studies it is suggested that *C. aberti* is a species complex that contains cryptic biological diversity (SERB & BARNHART 2008). It can not be excluded however that the Chinese mussels occurring in Europe, despite their morphological similarity to *A. woodiana* (PETRÓ 1984, GIRARDI & LEDOUX 1989) and genetic similarity to *A. arcaeformis*, represent yet another species of *Anodonta* occurring in Asia, one whose DNA sequences are still unknown and thus unavailable from GenBank.

Unfortunately, Asian members of *Anodonta*, numbering eight species (GRAF 2007, GRAF & CUMMINGS 2007) are genetically little known (HUANG et al. 2002) and sequences for only three of them (*A. arcaeformis*, *A. beringiana* and *A. woodiana*) are available in GenBank, making it impossible to attempt a comprehensive comparative analysis. Ultimate solution of the taxonomic status of the Polish specimens of the Chinese mussel requires anatomical and molecular examination of a greater number of specimens from more European localities, and most of all Asian species of *Anodonta*, considering the Asian origin of the species (PETRÓ 1984, KISS & PEKLI 1988, KISS 1995). There are numerous reports on molecular verification of species identifications based on great genetic variation or close genetic similarity between the earlier distinguished taxa. For example, the wide genetic variation of *Dreissena* from American Great Lakes revealed the presence of two sympatric introduced species: *D. polymopha* and *D. bugensis* (MAY & MARSDEN 1992, SPIDLE et al. 1994). Two snail species, occurring allopatrically in Europe, showed a great genetic and morphological uniformity which strongly supports their conspecificity; they all represent *Marstoniopsis insubrica* (FALNIOWSKI & WILKE 2001). Another molecular study failed to confirm the earlier reports on the presence of *Bythinella austriaca* (Gastropoda) in Romania and Bulgaria (FALNIOWSKI et al. 2009a, b).

Comparative analysis of all available sequences of *cox1* gene for the protected species, *U. crassus, P. complanata* and *A. cygnea* (Table 1), makes it possible to estimate the intraspecific variation which correlates with geographical distances between the localities and is 3.1% for *U. crassus* and 0.1% for the remaining two species. All the analysed protected species showed a small or no variation within localities. The loss of variation certainly has a negative effect on the adaptive potential of the species and may result in decrease in abundance when the habitat changes. On the other hand, deleterious mutations accumulate faster in smaller populations as a result of drift, while in large populations the same mutations are eliminated (BERGSTROM & PRITCHARD 1998, BALLARD & WHITLOCK 2004).

This study is the first to report on the intra- and interspecific variation of male haplotypes of *cox1* gene in European Unionidae. Intraspecific variation of male haplotypes was observed in *U. tumidus* and *A. anatina* out of the five studied species for which 10–14 male sequences of *cox1* gene were analysed, except the protected *U. crassus*. The variation was 0.2% (Table 5). The absence of variation among male haplotypes of *cox1* gene in *U. pictorum*, despite the analysis of 13 male sequences and the highest level of variation among the female forms of the gene (0.4%), are surprising.

The intraspecific variation of *cox1* gene observed in the analysed unionid species is greater than in other molluscs or other animals in which no DUI was detected. For example in *Dreissena polymorpha* the variation of this gene is up to 1.1% (THERRIAULT et al. 2004), 0.3% in the freshwater snail *Marstoniopsis insubrica* (FALNIOWSKI & WILKE 2001) or 2.1 and 1.1% in two slug species, *Arion lusitanicus* and *A. rufus* respectively (SOROKA et al. 2009). This confirms the earlier reports on faster evolution in F and M lineages in bivalves, compared to mitochondrial genomes of SMI animals (HOEH et al. 1996a, b). The interspecific variation for female haplotypes of *cox1* gene in Unionidae is 10–16% (KÄLLERSJO et al. 2005). Similar values were observed for the studied Polish specimens (11–17%), and higher values – for their male haplotypes (13–20%). A similar interspecific variation of *cox1* gene (14%) was recorded among *Hyridella*, which also corresponds to the mean variation among Pleurobemini and Lampsilini (GRAF & Ó FOIGHIL 2000b).

Since it is impossible to watch evolution directly, it is reconstructed using deduction, initially mainly based on fossil record. However, a considerable part of evolutionary history was reconstructed not on the basis of not fossil but extant organisms. The possibilities are offered by comparative analyses of morphological and anatomical characters and, increasingly often, by molecular methods. The development of molecular methods, mainly polymerase chain reaction techniques (PCR) and DNA sequencing, revolutionised phylogenetic analyses and the wide access to databases and software contributed to their popularity (KIM 2001). In spite of this, accessibility of data is still the main problem, because though the data are abundant, in order to be useful for phylogenetic analysis they should pertain to the same genes or morphological characters of all analysed species, including outgroups. At present phylogenetic analyses are performed by biologists representing different disciplines, while earlier they were within the domain of systematists. Facility of constructing phylogenetic trees is not tantamount to their reliability. Phylogeny reconstructions are only hypotheses on a taxon, and not ultimate solutions. Organisms with complex life histories and unusual modes of genome inheritance can

present challenges for phylogenetic reconstruction and accurate assessment of biological diversity. The difficulties pertain especially to Unionidae, where species occur in riverine habitats with complex hydrogeological histories, and the two main processes of unionid radiations reflect the history of cyclic glaciations of our planet and tectonic movements (NAGEL et al. 1996, NAGEL 2000, BOGAN & ROE 2008, SERB & BARNHART 2008). Species of Unionidae have complex life cycles that include a parasitic larva and obligate fish host. Last but not least these species have an unusual mode of mitochondrial inheritance and possess both female and male mitochondrial genome that is transmitted throuth doubly uniparental inheritance (DUI). Besides, mitochondrial DNA has proved particularly useful for phylogenetic analyses of animals, mainly because of its haploid character, absence of introns and recombination (with some exceptions, see Introduction) and faster rate of accumulating mutations, compared to nuclear DNA. Furthermore, most models of mtDNA evolution consider neutral evolution and purifying selection as the major processes that shape sequence divergence. Thus, mtDNA variation should reflect the species' natural history. However, modifications in mitochondrial inheritance could affect phylogenetic inferences from these genes, making them incongruent with other lines of evidence (HOEH et al. 1996a, 1997).

Mitochondrial genes most often used in molecular phylogeny of unionids are 1) *cox1* (HOEH et al. 1998, 2001, GRAF 2000, GRAF & Ó FOIGHIL 2000a, LYDEARD et al. 2000, HUFF et al. 2004, MOCK et al. 2004, CAMPBELL et al. 2005, KÄLLERSJO et al. 2005, GRAF & CUMMINGS 2006, SERB 2006, WALKER et al. 2006a, CHONG et al. 2008, ARAUJO et al. 2009); 2) *cox2* (CUROLE & KOCHER 2005, WALKER et al. 2006b); 3) *nad1* (SERB et al. 2003, CAMPBELL et al. 2005, SERB 2006) and 4) *16S rRNA* (LYDEARD et al. 1996, 2000, KANDL et al. 2001, CAMPBELL et al. 2005, KÄLLERSJO et al. 2005). At present, because of the availability of sequences, phylogenetic analyses include at least one or two genes and many morphological characters (LYDEARD et al. 1996, GRAF & Ó FOIGHIL 2000a, GIRIBET & WHEELER 2002, KÄLLERSJO et al. 2005, GRAF & CUMMINGS 2006, ARAUJO et al. 2009). Nuclear genes such as histone (*H3*) as well as *18* and *28S rRNA* are also used for phylogeny reconstruction, but they represent regions which evolve too slowly to be useful in phylogenetic analyses of Unionoida (GRAF & Ó FOIGHIL 2000b, GRAF 2002, HUFF et al. 2004, BOGAN & ROE 2008). The order Unionoida includes bivalves which are widely distributed in all continents except Antarctic, representing six families, Unionidae with their more than 600 species being the most speciose (GRAF & CUMMINGS 2006, 2007, GRAF 2007, BOGAN 2008, BOGAN & ROE 2008). The members of this family are most numerous in the Nearctic (nearly 300 species) and Indotropical region (over 200 species), while Palaeartic has only 42 species, 14 of them found in Europe (www. faunaeur.org; ARAUJO et al. 2005, 2009, GRAF 2007, GRAF & CUMMINGS 2007, ARAUJO 2008). Most of the previous phylogenetic analyses using mitochondrial genes within Unionidae deal with species of North America (LYDEARD et al. 1996, GRAF & Ó FOIGHIL 2000a, GIRIBET & WHEELER 2002, GRAF 2002, SERB & LYDEARD 2003, CAMPBELL et al. 2005, WALKER et al. 2006a, b, CHONG et al. 2008, BOGAN & ROE 2008), fewer pertain to Europe (KÄLLERSJO et al. 2005, ARAUJO et al. 2009) and China (HUANG et al. 2002). Most of these studies include only female haplotypes which are easily obtainable from somatic tissues. It was found that the evolution of mtDNA in DUI bivalves was faster compared to SMI organisms, and the variation of male genes was greater than that of female genes (STEWART et al. 1995, HOEH et al. 1996a, b, 2002a, b, ZOUROS 2000, KREBS 2004, WALKER et al. 2006a, DOUCET-BEAUPRÉ et al. 2010). Since 1996 the exceptional way of inheritance – DUI – has been documented in more than 50 unionid species (for details and references see above), including only one native European species, *A. anatina* (SOROKA 2008a) and one introduced from China, *S. woodiana* (SOROKA 2008b), and in another three – members of *Unio*, discussed in this paper.

First morphological and molecular phylogenetic analyses of unionids were not unambigous and indicated paraphyletic (BOGAN & HOEH 2000, GRAF 2000, HOEH et al. 2001, 2002a) or monophyletic character of the family (ROE & HOEH 2003, GRAF & CUMMINGS 2006). With improvement of molecular analyses with respect to the number of taxa, sequence length, DNA saturation and outgroup selection, the monophyly of the family Unionidae is gaining support. GRAF & CUMMINGS (2006), analysing 59 morphological characters, mitochondrial gene *cox1* and nuclear gene *28S rRNA*, showed that the whole order Unionida was monophyletic on the basis of eight synapomorphies, including larval parasitism, brood protection and restriction to freshwaters, and that the families Unionidae and Margaritiferidae were sister clades. Three main subfamilies are distinguished within Unionidae: Ambleminae, Anodontinae and Unioninae, the last two being more closely related (HOEH et al. 2001, GRAF 2002, HUANG et al. 2002) and occurring in Europe. Phylogenetic analyses conducted independently for all analysed mitochondrial genes of European unionids grouped sequences of F and M haplotypes in separate clades according to the literature data (HOEH et al. 1996a, 2002a, CUROLE & KOCHER 2002, 2005, SOROKA 2008a, b, THEOLOGIDIS et al. 2008). This suggests that the origin of DUI precedes speciation. Likewise, separate F and M clades are distinguished when the phylogenetic analysis in extended to include the families Margaritiferidae and Hyriidae, members of Unionoidea (HOEH et al. 2002a, b, CUROLE & KOCHER 2005).

This study is the first to present a phylogenetic analysis of European unionid species based on F and M haplotypes. No doubt their evolution took place only once, and the phylogenetic analyses are only attempts at its reconstruction. Thus the reconstructed phylogeny should be identical for F and M genomes of the DUI species. However, topologies within the F and M sequences sometimes differ within one gene (Fig. 3a), or between genes (Figs 5, 8), which considerably complicates phylogenetic inferences. Previous phylogenetic analyses of unionid bivalves based on F haplotypes alone, without confirmation from M haplotypes, can be regarded as very likely but not ultimate. The differences within F and M groups observed in this study are not an exception (HOEH et al. 2002a). Two possible topologies, depending on the analysed fragment of mtDNA, F or M sequence and the kind of phylogenetic algorithm applied were observed within *Unio*. In the first, more frequent clustering, *U. pictorum* (with *U. mancus* for gene *cox1*) and *U. crassus* are sister clades, while *U. tumidus* is phylogenetically the most remote (Fig. 3 – NJ F group, – Bayesian method F and M groups, Fig. 5 – Bayesian method F group and Fig. 8 – all F groups). In the second case *U. pictorum* (with *U. mancus* for gene *cox1*) and *U. tumidus* are sister clades, and *U. crassus* is phylogenetically the most remote (Fig. 3 – NJ M group, Fig. 5 – NJ F group). However, always *U. pictorum* and *U. mancus* cluster together. The first clustering is in accordance with phylogenetic relations within the genus *Unio* based on F sequences of genes *cox1* and *srRNA* (KÄLLERSJO et al. 2005, ARAUJO et al. 2009) and their joint analysis with the nuclear ribosomal internal transcribed spacer region (*ITS1*, *5.8S*, *ITS2*) (KÄLLERSJO et al. 2005). However these molecular data are not compatible with morphological characters for the three species of *Unio*; according to such characters *U. crassus* is rather remote from *U. pictorum* and *U. tumidus*, and the last two are morphologically very similar and sometimes difficult to distinguish. Molecular studies on Rissooidea snails also undermined the reliability of phylogeny reconstructions based on numerous morphological characters, which often showed homoplasies (WILKE et al. 2001,

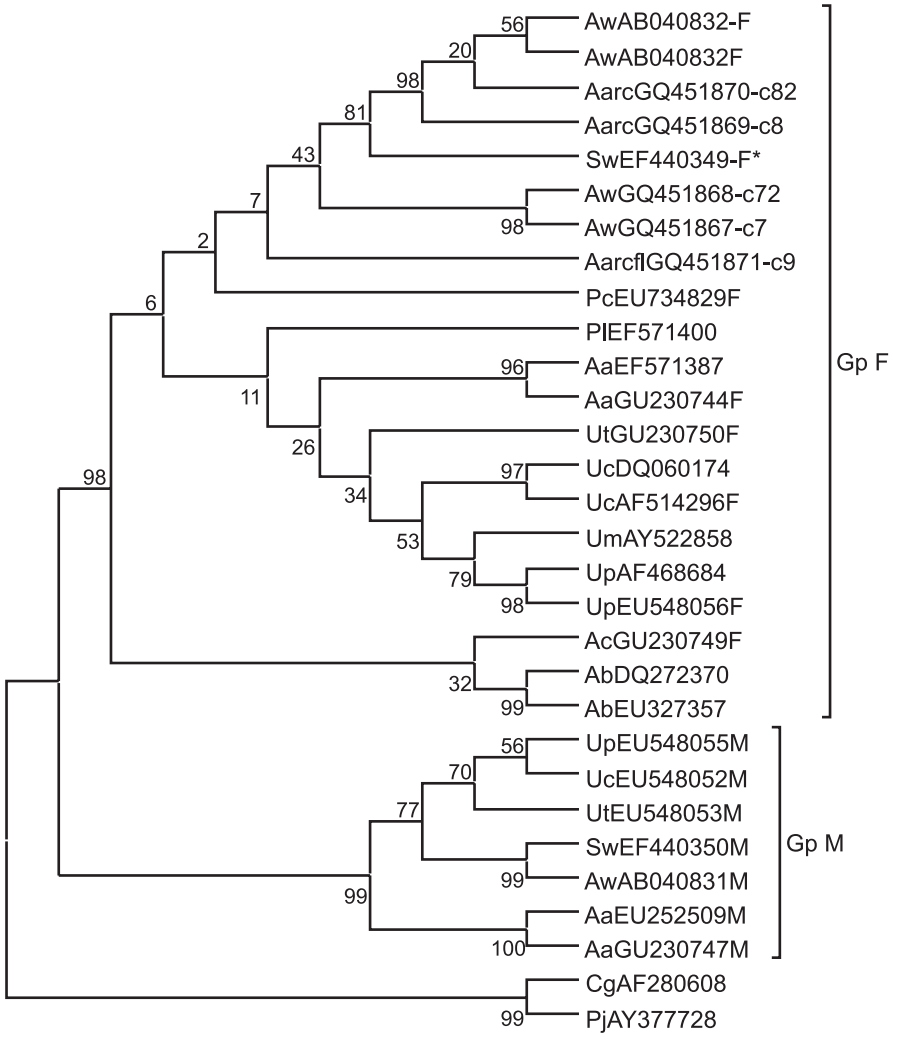

Fig. 10. Maximum Parsimony tree for *cox1* comparing European and Asian species of Unionidae. For abbreviations see caption to Fig. 3

SZAROWSKA 2006). Also molecular distinctness of six species of *Bythinella* (Rissooidae) from Romania found no confirmation in their variable and overlapping morphological characters (FALNIOWSKI et al. 2009b). It is likely that all morphological character states in Rissooidae appeared early in phylogeny and were then lost or sometimes regained, which may be associated with regulatory genes and their role in macroevolution (SZAROWSKA 2006).

Two species of the genus *Anodonta* (*A. anatina* and *A. cygnea*) of very similar shell morphology, are very remote from *Pseudanodonta complanata* in both adult morphology and glochidium structure (PEKKARINEN & ENGLUND 1995a, b). The data were confirmed by enzyme electrophoresis of European Anodontinae, where *Pseudanodonta* generally exhibited the highest values of genetic distances from all other investigated species (NAGEL et al. 1996). Different relations among these three species were indicated by analyses of nuclear and mitochondrial DNA sequences. In this study and in the analysis by KÄLLERSJO et al. (2005) *A. anatina* was the next genetically most remote after *S. woodiana*, while *A. cygnea* and *P. complanata* were more closely related to each other than either was to *A. anatina*. Based on male sequences of *cox1 S. woodiana* was closer to *Unio* than to *Anodonta* (Fig. 3a and b). Joint analysis of European and Asian sequences of *cox1* gene showed completely different relations. Within female haplotypes *A. anatina* clustered with *Unio*, *P. complanata* with Asian species of *Anodonta*, and *A. cygnea* with American specimens of *A. beringiana*, forming a group which was the most remote from the remaining taxa (Fig. 10). No unambiguous phylogenetic relations were obtained for western North American *Anodonta* and other anodontine female mitochondrial *cox1* sequences (CHONG et al. 2008). It is very difficult to draw ultimate phylogenetic conclusions considering the great observed discrepancies among European, Asian and American species of *Anodonta*. Most probably in the future male mitochondrial sequences of these species or analyses of entire mitochondrial genomes will provide necessary data to solve this problem.

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