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Original article

Complete sequences of maternally inherited mitochondrial genomes in mussels *Unio pictorum* (Bivalvia, Unionidae)

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Abstract. Mitochondrial genomes are frequently used to infer phylogenetic relationships. Some taxa are, however, poorly represented. To facilitate better understanding of the potential of mitochondrial genome data in freshwater mussels, we present here, for the first time, the mitochondrial sequences of 4 complete F-type mitochondrial genomes from the European freshwater bivalve *Unio pictorum* (Unionidae). These genomes are very compact (15 761 bp) but have a typical gene complement for bilaterian mitochondrial genomes and a very similar organization to other unionid genomes available in databases. Very low nucleotide diversity within the species suggests a small effective population size of Polish *U. pictorum,* a phenomenon of potential importance for environmental management policies.

Keywords: bivalves, freshwater mussels, mitogenomics, *Unio pictorum*, Unionidae.

Introduction

Standard maternal inheritance of mtDNA is a rule in the animal kingdom. The most notable exception from this rule is the doubly uniparental inheritance (DUI) system discovered in some bivalve species (Skibinski et al. 1994; Zouros et al. 1994; Liu et al. 1996). Under DUI, females pass their mtDNA (here called F-type) to the offspring, but heteroplasmic males pass one of their mtDNAs only to their sons. Thus the paternal mtDNA (M-type) is present mainly in the germline of males. DUI has been so far confirmed in the Mytilidae, Unionidae, Margaritiferidae, Hyriidae, Veneridae, Danacidae, and Solenidae (Skibinski et al. 1994; Zouros et al. 1994; Hoeh et al. 1996; Liu et al. 1996; Passamonti and Scali 2001; Hoeh et al. 2002; Curole and Kocher 2005; Walker et al. 2006; Theologidis et al. 2008; Soroka 2008a, b). This phylogenetic distribution suggests that it may be typical of bivalves. In the superfamily Unionoidea, the divergence of M and F lineages is particularly old and predates the family level radiation (Hoeh et al. 2002).

To date, there are more than 1800 complete metazoan mtDNAs in the RefSeq division of the GenBank (Pruitt et al. 2009). Bivalves are the best-sampled group among molluscs, with 33 complete and 3 nearly complete mitochondrial genomes from 27 species. Marine bivalves represent 2/3 of known sequences, while freshwater species are less represented. Among the freshwater Unionidae, comprising more than 600 species (Graf and Cummings 2007; Bogan and Roe 2008), the 11 complete mtDNAs are known from only 7 species (Table 1). Notably, only 7 sequences have been formally discussed in a publication, while the remaining 4 are "unpublished", even though some of them are in the RefSeq database.

Freshwater mussels are important components of inland aquatic ecosystems, serving as so-called biofiltrators (Lewandowski and Stańczykowska 1975). The most common bivalve in Poland, *Unio pictorum* (known as painter's mussel), is a big contributor to water self-purification, preventing

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Table 1. Complete mitochondrial genomes of the Unionidae available in the GenBank

Species	Accession	Length (bp)	Source
Cristaria plicata, F type	NC_012716	15712	RefSeq
Hyriopsis cumingii, F type	NC_011763	15954	RefSeq
Inversidens japanensis, F type	AB055625	16826	GenBank
I. japanensis, M type	AB055624	16967	GenBank
Lampsilis ornata, F type	AY365193	16060	Serb and Lydeard 2003
Pyganodon grandis, F type	FJ809754	15848	Breton et al. 2009
P. grandis, M type	FJ809755	17071	Breton et al. 2009
Quadrula quadrula, F type	FJ809750	16033	Breton et al. 2009
Q. quadrula, M type	FJ809751	16970	Breton et al. 2009
Venustaconcha ellipsiformis, F type	FJ809753	15976	Breton et al. 2009
V. ellipsiformis, M type	FJ809752	17174	Breton et al. 2009
Unio pictorum, F type	HM014130-3	15761	this study

excessive eutrophication of rivers and lakes (Abraszewska-Kowalczyk 2002). Its documented potential to bioaccumulate harmful substances makes this species also a suitable bioindicator of environmental conditions (Gundacker 2000; Ravera et al. 2005). That is why it has been extensively studied by ecologists (Lewandowski and Stańczykowska 1975; Lewandowski 1990; Abraszewska-Kowalczyk 2002) and biochemists (Stambuk et al. 2008).

The high phenotypic plasticity of freshwater mussels is a well-known phenomenon. Molecular techniques are needed to elucidate the genetic basis of phenotypic differences. Mitochondrial DNA markers are frequently the method of choice, because primers for amplification of certain fragments of mtDNA are readily available, e.g. through barcoding initiatives, such as The Barcode of Life (Hebert et al. 2003; Ratnasingham and Hebert 2007). In the Unionidae the F type is usually studied for that purpose, because it is present in all tissues of every mussel, regardless of the gender of the animal (Stepien et al. 1999; Graf and Ó Foighil 2000; Giribet and Wheeler 2002; Lee and Ó Foighil 2004; Araujo et al. 2005; Soroka 2005; Araujo et al. 2009). To date only fragments of 3 genes (*cox1, cytb* and *lrRNA*) were used in population and phylogenetic studies. The usually highly conserved *cox1* showed up to 0.3% diversity within *U. pictorum* and *U. tumidus* (Källersjo et al. 2005) or even the complete lack thereof in *Anodonta* sp., *A. cygnea*, *Pseudanodonta complanata*, and *Sinanodonta woodiana* (synonym *A. woodiana*) (Mock et al. 2004; Källersjo et al. 2005; Soroka 2005; Soroka 2008b). Somewhat higher diversity indices were obtained for *A. anatina* and *U. gibbus* (Soroka 2008a; Araujo et al. 2009). These numbers must be interpreted cautiously, since intraspecies estimates were frequently based on very few sequences. The interspecies diversity in *lrRNA* and *cox1* is quite high in the freshwater Unionidae: 5–14% and 10–16%, respectively (Krebs 2004; Källersjo et al. 2005). This is consistent with the relatively old age of Unionidae. These data are, however, very scarce, and there is clearly a need for more systematic coverage. It is important to assess the genetic status of *U. pictorum*, as well as other Unionidae, some of which (e.g. *U. crassus*, *Anodonta cygea, Pseudanodonta complenata*) are considered threatened across Europe.

Here we contribute novel, complete mtDNA sequences of four F genomes from the freshwater mussel *U. pictorum.* The presented data will allow the use of mtDNA markers of higher variability in population and phylogenetic studies of freshwater mussels. We also provide preliminary estimates of nucleotide diversity across the whole mtDNA in Polish *U. pictorum*.

Materials and methods

The mussels were sampled in 2005–2006 from 3 locations in Poland: one specimen from the Szczecin Lagoon (female 109), one from Lake Pątnowskie in central Poland (female 132), and two (female 147 and male 149) from the Odra (Oder) river close to Szczecin. Total DNA was isolated from the mussel mantle tissue by using the phenol/chloroform method. For all specimens, nearly full-length mtDNA (ca. 16 kb in length) was obtained from a long-range polymerase chain reaction (LR-PCR) by using universal primers DUF1 and UR1 (Table 2). Subsequently, the LR-PCR product was sequenced by the primer

Table 2. Primers used in LR-PCR, re-PCR, and sequencing of the complete mitochondrial genome of *Unio pictorum*

Name	Sequence (5' to 3')
DUF1	GGTTTGCGACCTCGATGTTGG
UR1	TTCCTAGTCTTGCCATTCACTGGC
ND6for	TATCTATACTTCAATTACCTACC
NDrevend	ATGTATTATAGTAACCAGAGGAG
NDfor29	CCCTGTATCTTCCAATTATCTTC
ATPfor29	TTTGGTAGCCTATTCGTCTGTTG
UP.nd6.01	CTTTCCCCTAACATAACTT
UP.nd4.03	TGACTATTTTGATTTCTGG
UP.nd4.01	AAACCAACACAAAGCAA
UP.at6.02	TCGTCCAATTACTTTAGG
UP.nd4.04	TGATTAACAAAATAGGAATAAG
UP.co3.01	TTGTGTATTTATTGATGAGG
UP.at6.01	CCTAAAGTAATTGGACGAA
UP.co1.01	TTAATTTCTTTTGTTTCTGTT
UP.co3.02	ATCAATAAATACACAAATACAAA
UP.co1.07	TGGCTACCATTCATGG
UP.co1.05	AACAGAAACAAAAGAAATTAA
UP.co1.04	CTACTAATCATAAGGATATTGG
UP.co1.08	CATGAATGGTAGCCAAC
UP.co2.01	TTGGTTTCGTCTTTTGT
COendUnio	GATCATCGCCTAATAACCTCCCAG
CO-6	AACATAGTGTTTGGTTAGTAAG
UP.co2.02	ACAAAAGACGAAACCAA
UP.nd2.01	CTCCTAAAGAAAGGATTGT
CO2-4	GCTAATATGTGGCTTCTAACTAC
CO2-3	AAGATTATGGTTTGTTGAGAC
CO-7	GTTCAATAAATTCGCTCCTAATC
CO2-2	CAATTCTTTTAGGTCCTTAGGTG
UP.srn.01	TGGTGCCAGCAGTCG
UP.lrn.04	CACGCTCACGCTAACG
UP.trn.03	CCGAGAACAGGCATCGAGCTTTT
UP.lrn.01	AAGGAGATAGAAACCAACCTAGCTT
UP.lrn.02	CAACCGCTATGACAGG
UP.trn.02	TTTAACATTTTCAGTGTTATG
UP.lrn.03	TGCCTGTTTACCAAAAACATCG
UP.cob.03	GTCTTCTACTGGTTGTTTACCAATTCA
UP.cob.01	TAAACAACCAGTAGAAGACC
UP.nd5.01	TTGGGGATGCTTTGTT
NAD5	AAAAATCGACTTAACCTTCC
ND6rev	AGGTGCTAGAATATAAGAAGG
NADfornew	TAACTGACTCAAAGTAGACAAAG
NDrev29	GGAATAATTGTCATAACCTGAGG

walking method (female 147). The remaining 3 genomes were sequenced by re-PCR approach, as described previously (Zbawicka et al. 2007). The strategy was based on re-amplification of 20 overlapping fragments and direct sequencing of PCR products, as outlined in Figure 1, using primers listed in Table 2. The short fragment not present in any LR-PCR product was obtained in a separate PCR with the specific primers. For LR-PCR, Phusion High-Fidelity DNA polymerase (Finnzymes) was used, according to the manufacturer's protocol. All the re-amplifications were carried out in a 10-μL reaction volume containing 1:800 dilution of LR-PCR product as a template, 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 the appropriate reaction buffer. An initial 2-min denaturation at 94°C, was followed by 25 cycles of denaturation at 94°C for 40 s, annealing for 30 s at 51–55°C (depending on the primers), and extension at 72°C for 55 s, with a final 5-min extension at 72°C. All PCRs were performed in a Mastercycler gradient from Eppendorf. PCR products were purified by the ExoSAP procedure (Werle et al. 1994) and sequenced directly using BigDye Terminator chemistry. Sequences were read on an ABI multicapillary sequencer. Individual reads were assembled into contigs by using Staden package (Staden et al. 2001) and annotated as described previously (Zbawicka et al. 2007). ClustalW was used to align the obtained sequences (Thompson et al. 1997). Sequence composition and diversity indices were calculated in MEGA4 (Tamura et al. 2007) and DnaSP (Rozas et al. 2003). The amino acid substitutions were evaluated by the SIFT (Sorting Intolerant From Tolerant) method (Ng and Henikoff 2001). Several programs from the EMBOSS package (Rice et al. 2000) were used for sequence analysis. The genetic map was generated by CGView (Stothard and Wishart 2005). The 4 complete mtDNA sequences of *U. pictorum* obtained in this study have been deposited in the GenBank (accession numbers HM014130-3).

Results and discussion

The 4 complete F-type mitochondrial genomes of *U. pictorum* are 15760, 15761 or 15762 bp long. This length polymorphism is caused by 2 single-bp indels within either the *trnH* or *lrRNA* (Table 3). There is an 8-bp overlap between *nad4l* and *nad4* genes, and there are only few and relatively short non-coding sequences, ranging in size from 2 bp to 259 bp, and totalling 771 bp (Figure 2, Table 4). The 3 non-coding regions were named NCRα (259 bp between *nad5* and *trnQ*), NCRβ (36 bp between *trnF* and *nad5*), and NCRγ (137 bp between *nad3* and *trnA,* excluding *trnH*). The NCRα, NCRβ, and NCRγ correspond to the respective regions shared by other known F genomes in the Unionidae. Interestingly, the change in transcription orientation occurs within

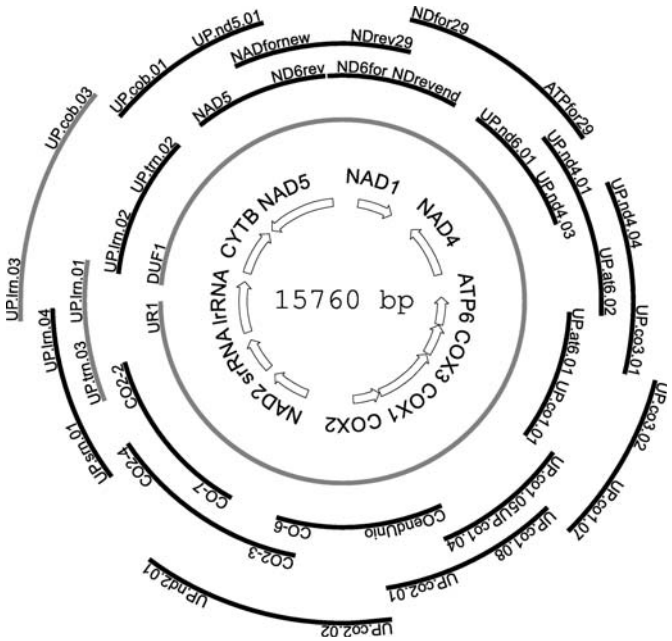

Figure 1. Scheme of the sequencing strategy. One LR-PCR was performed with primers DUF1 and UR1, giving a 15-kb-long product (long grey arc). This product then served as a template in a series of PCRs yielding shorter products (black arcs) used in direct sequencing. The fragment not contained in the LR-PCR product was sequenced from 2 short, direct PCR products spanning this region (short grey arcs). The location and orientation of selected genes is given for reference (open arrows).

Table 3. Comparison of 4 mitochondrial genomes of *Unio pictorum*

Individual no.	Length (bp)	Variable sites											
		NC 1341	NC 1342	*nad4L* 3472	*atp6* 4317	*atp6* 4378	*cox2* 7151	*trnH* 8092	*trnA* 8236	*nad2* 9328	*srRNA* 10305	*lrRNA* 11650	*trnL* 12318
109	15762	**A**	**T**	C	**G**	**G**	**A**	T	G	T	C	**T**	C
132	15761	T	A	C	A	A	G	T	G	C	C	–	C
147	15760	T	A	**T**	A	A	G	–	**A**	T	C	–	**T**
149	15761	T	A	C	A	A	G	T	G	T	**T**	–	C

all of them (Serb and Lydeard 2003; Breton et al. 2009), separating parts of genomes with different compositional biases (Figure 2). The remaining non-coding regions were small (2-53 bp long). The F genome of *U. pictorum* is one of the most compact genomes described to date in the Unionidae. It is 86 bp shorter than that of *Pyganodon grandis* (Breton et al. 2009), 298 bp shorter than *Lampsilis ornata* (Serb and Lydeard 2003), and 1064 bp shorter than *Inversidens japanensis* (Table 1). Only the genome of *Cristaria plicata* (15712 bp) is 50 bp shorter than that of *U. pictorum* (Table 1). All 37 genes typically found in the metazoan mitochondrial genome are present in the *U. pictorum* genome (Figure 2). Genes are arranged in the same order as in 5 of the 7 known unioidean F genomes: *C. plicata*, *L. ornata* (Serb and Lydeard 2003) *P. grandis*, *Q. quadrula*, and *V. ellipsiformis* (Breton et al. 2009), even though their sequences differ by 22–30%. Perhaps not surprisingly, the lowest divergence is observed in *cox1* (18%). Despite a high level of synonymous substitutions (K_s = 0.712) and variable length, this gene is very conserved at the amino acid level (K_a = 0.025). The most divergent part is the F-specific open reading frame (F ORF) recently identified by Breton et al. (2009). Despite the lack of appreciable homology (~80% predicted amino acid p-distance), ORF of similar length (82 amino acids) and localization (between *trnE* and *nad2*) are also present in *U. pictorum*, suggesting that this extremely variable protein is present in all unionids.

Intraspecies nucleotide diversity (π) is estimated at 0.03%, due to 12 polymorphic sites (Table 3). The emerging pattern of polymorphism is characterized by one reference (consensus) sequence and single substitutions at different sites in each studied haplotype. Two substitutions are in

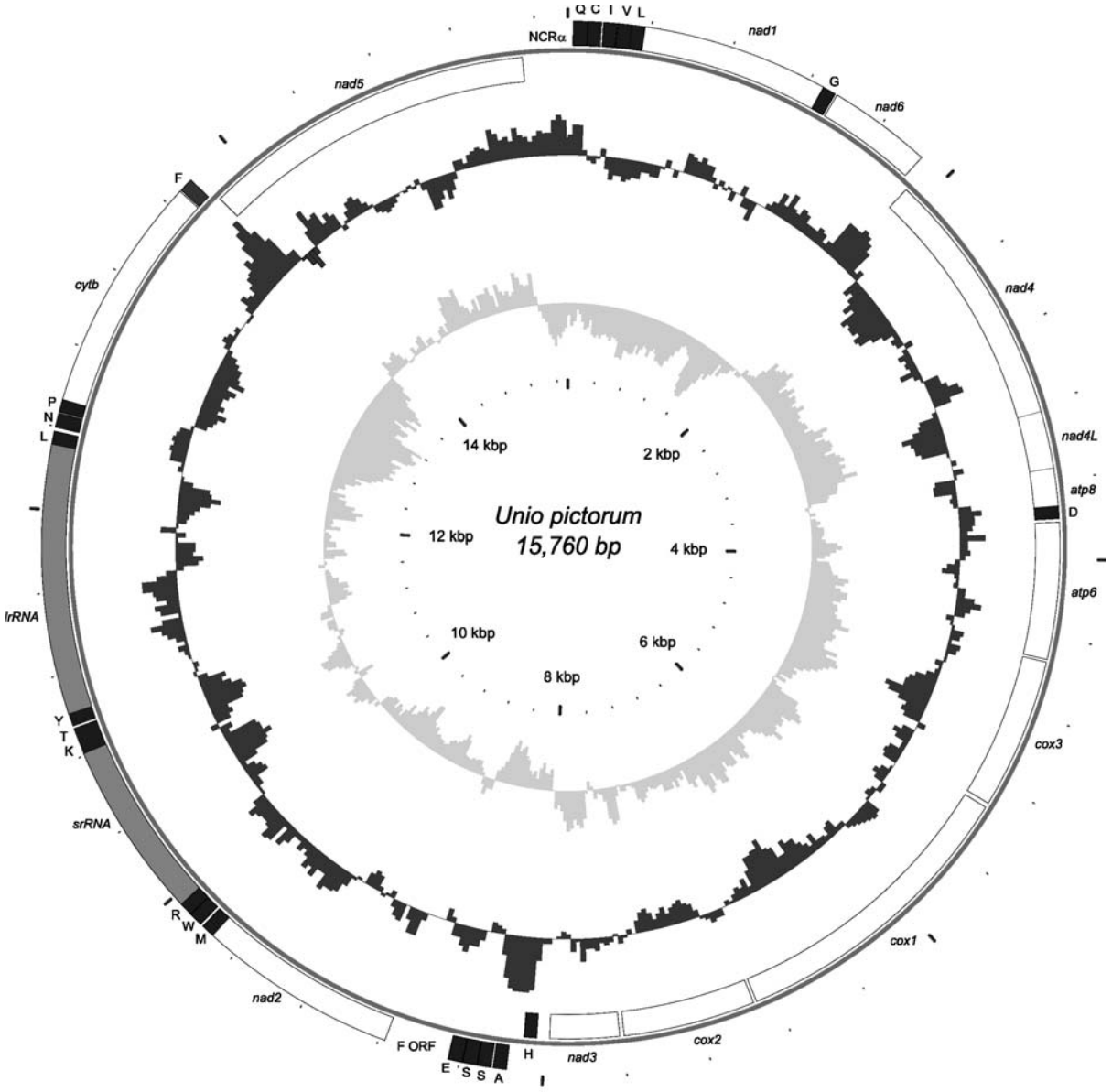

Figure 2. Genetic map of the mitochondrial F genome of *Unio pictorum*. Open boxes represent protein-coding genes. Black boxes represent tRNA genes (labelled with the one-letter code of their corresponding amino acid) and the grey boxes represent rRNA genes. The genes transcribed clockwise are shown on the outside of the circle, the genes transcribed anticlockwise are on the inside. The inner black plot shows local deviations in AT content, related to the average AT content. The inner grey plot illustrates the local deviations from the average AT skew. Both plots were calculated in a sliding window of 200 bp with a 50-bp step size.

non-coding regions, while 8 within genes, of which 2 are non-synonymous: I/T in *atp6* (4317) and Y/H in *nad2* (9328) (Table 3). Both non-synonymous substitutions were scored as potentially tolerated, as expected for neutral or nearly-neutral evolution. Even though the number of sequences is small, and therefore the results must be interpreted cautiously, the observed level of polymorphism is unexpectedly low. The 0.3% diversity reported in conserved *cox1* of this species (Källersjo et al. 2005) shows that substitutions do accumulate in mitochondrial sequences of *U. pictorum.* Under the neutral model, $\theta = 2N_e\mu$, where μ is the substitution rate, and N_e is the effective population size. The estimated θ for our data is 3.5×10^{-4} (SE 2.1×10^{-4}), therefore the substitution rate would have to be more than one per mitochondrial genome per generation for N_e to be greater than one. Thus we can conclude that the observed low nucleotide diversity indicates a very low effective population size, possibly caused by a recent population bottleneck. Since the sampling

Table 4. Average length and base composition of genes and non-coding regions of 4 haplotypes of *Unio pictorum*

Gene or region		Coding strand	Base composition (%), L strand			
			T	C	A	G
Non-coding regions	NCRα		37.1	23.9	35.9	3.1
	NCRβ		30.6	11.1	55.6	2.8
	NCRγ		34.3	18.2	44.5	2.9
All non-coding regions			36.0	21.2	39.6	3.2
All tRNA genes		20L 2H	30.6	17.3	34.7	17.4
rRNA genes	*srRNA*	L	23.5	21.4	39.1	15.9
	lrRNA	L	28.7	19.5	36.9	14.9
All rRNA genes		L	26.7	20.2	37.8	15.3
Protein-coding genes	*nad1*	L	34.5	21.6	30.3	13.6
	nad6	L	35.1	22.8	32.9	9.2
	nad4	H	43.1	10.5	20.3	26.1
	nad4L	H	45.1	7.4	17.9	29.6
	atp8	H	37.4	5.1	26.2	31.3
	atp6	H	44.7	9.1	20.7	25.5
	cox3	H	44.3	11.2	17.8	26.7
	cox1	H	42.8	14.0	20.3	22.9
	cox2	H	41.1	10.5	22.3	26.1
	nad3	H	44.6	8.1	20.4	26.9
	F ORF	L	26.5	27.4	37.4	8.7
	nad2	L	32.3	21.7	34.9	11.1
	cytb	L	36.5	21.8	27.8	13.9
	nad5	H	42.5	9.7	23.6	24.2
All protein-coding genes		5L 9H	40.1	14.4	24.5	21.0
Complete genome			**26.5**	**23.1**	**38.6**	**11.8**

NCR = non-coding region; H = genes encoded on the heavy strand (anticlockwise); L = genes encoded on the light strand (clockwise)

was limited to the Odra river basin, the effect may be local and more data are required to draw a firm conclusion.

The presented sequences of F type mitochondrial genome of *U. pictorum* add new data to the existing mitogenomic data set, allowing the exploration of new mitochondrial markers both within and between species.

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