FOLIA MALACOLOGICA
Vol. 13(4): 145–152
ISSN 1506-7629
The Association of Polish Malacologists
& Faculty of Biology, Adam Mickiewicz University
Poznań 2005

MITOCHONDRIAL DNA-BASED DIAGNOSTIC MOLECULAR MARKERS FOR FRESHWATER BIVALVES

MARIANNA SOROKA, EDYTA GRYGIEŃCZO-RAŻNIEWSKA

Department of Genetics, University of Szczecin, al. Piastów 40b, 71-065 Szczecin, Poland,
(e-mail: marianna.soroka@univ.szczecin.pl)

ABSTRACT: The study was carried out on 9 species of freshwater bivalves (*Dreissena polymorpha, D. bugensis, Unio crassus, U. pictorum, U. tumidus, Anodonta anatina, A. cygnea, A. woodiana*, and *Pseudanodonta complanata*). The mitochondrial *COI* gene studied with PCR-RFLP and 6 restriction enzymes (ScrFI, Csp6I, BsiZI, EcoRI, BamHI and AluI) showed the absence of individual variability within each species studied. The genetic variability of the *COI* involved differences at the species, genus, and family level, depending on the restriction enzyme used. Four restriction enzymes (ScrFI, Csp6I, BsiZI and AluI) proved efficient in differentiating between *D. polymorpha* and *D. bugensis* as well as in identifying the three *Unio* species (Csp6I and AluI), *U. crassus*, and *P. complanata* (ScrFI and AluI). EcoRI and AluI made it possible to identify *A. anatina* and *P. complanata*. Two (for EcoRI), 3 (for Csp6I, BsiZI), 5 (for ScrFI) and 9 (for AluI) unique genotypes that occurred in a single species each were observed; the enzymes may therefore be regarded as species-specific markers. Only restriction enzyme AluI can differentiate between *A. cygnea* and *A. woodiana*.

KEY WORDS: mitochondrial DNA, PCR-RFLP, bivalves, *Dreissena, Unio, Anodonta*

INTRODUCTION

The Polish aquatic mollusc fauna consists of about 100 species; freshwater bivalves contribute to this number with about 30 species of three families: Sphaeriidae, Dreissenidae and and Unionidae (PIECHOCKI & RIEDEL 1997). Four freshwater genera include bivalves of the largest size: *Dreissena* (*D. polymorpha*), *Anodonta* (*A. anatina, A. cygnea, A. woodiana*), *Unio* (*U. crassus, U. pictorum, U. tumidus*), and *Pseudanodonta* (*P. complanata*) (PIECHOCKI & RIEDEL 1997). Since 1985, three species: *Unio pictorum*, *Anodonta cygnea* and *Pseudanodonta complanata* have been under legal protection on account of their rarity, although the first is notable for its mass occurrence in Polish water bodies. The Minister's of Environment decision of 26 September 2001 (No. 1456) granted legal protection to all members of the family Unionidae, except for *A. anatina* known for its mass occurrence. Since 2005, only three species of bivalves are legally protected: *Unio crassus*, *Anodonta cygnea* and *Pseudanodonta complanata*. Except *D. polymorpha* and *A. woodiana*, all freshwater bivalve species in Poland are native.

The zebra mussel *Dreissena polymorpha* is a typical invasive species the range of which has gradually expanded from the drainage basins of the Black, Caspian, and Azov Seas into other regions. The bivalve has been present in Poland for almost 200 years (WIKTOR 1969, STAŃCZYKOWSKA 1977). It is presumed to have arrived on the Baltic coast via the River Nemunas which, at the end of the 18th century, was linked with the River Dnieper via the Oginsky Canal (NOWAK 1994). In Poland, the zebra mussel is present mainly in the Masurian Lake District as well as in the Pomerania and Great Poland (Wielkopolska) regions (WIKTOR 1969, STAŃCZYKOWSKA 1977).

Anodonta woodiana is a Chinese bivalve which has been present in Poland for 10 years (PROTASOV et al. 1994). This Far East bivalve, a native of the catchment areas of the rivers Amur and Jangtse, arrived in Europe from China along with cyprinids that have been stocked since 1962 (KISS 1995). *A. woodiana* reached Poland from Hungary in the early 1980s along with imported silver and bighead carp, intended for stocking (ZDANOWSKI 1994). So far, the bivalve is known in Poland from two heated localities: the Konin Lakes, channels, and cooling reservoirs of the Konin-Pątnów power station in central Poland (ZDANOWSKI 1994,

PIECHOCKI & RIEDEL 1997, KRASZEWSKI & ZDANOWSKI 2001, SOROKA & ZDANOWSKI 2001) and the Dolna Odra power station cooling water channel, located near the city of Szczecin in north-western Poland (since 2003, DOMAGAŁA et al. 2003).

Unequivocal identification of the freshwater bivalves of the genera *Unio*, *Anodonta* and *Pseudanodonta*, based on shell morphology and body anatomy, is very difficult, both with respect to adults and juveniles; in the latter, the key diagnostic characters are partly absent. Numerous diagnostic characters, such as colour or shell shape, are affected by environmental variables, which poses additional difficulties in ultimate identification. Molecular techniques, successfully used recently in molluscan taxonomy, offer new possibilities of correct identification (FOLMER et al. 1994, BALDWIN et al. 1996, VOSS et al. 1999, RYBSKA et al. 2000, FALNIOWSKI & WILKE 2001, WOOD et al. 2003).

The present work was aimed at finding molecular markers that would make it possible to objectively identify freshwater bivalve species. This, in turn, should facilitate monitoring of their distribution and protection in Poland and worldwide.

The study involved the polymerase chain reaction--restriction fragment length polymorphism (PCR-RFLP) technique for cytochrome c oxidase subunit I (*COI*) mitochondrial gene. The gene codes for conservative protein and evolves very slowly, as a result of which the analysis of its DNA sequence makes it possible to compare species, genera, and even individual organisms that are phylogenetically distinct (BROWN 1985).

MATERIAL AND METHODS

MATERIAL

The study focused on eight species of freshwater bivalves from Poland and involved also *D. polymorpha* and *D. bugensis* from the River Dnieper (Ukraine). Individuals of *D. polymorpha* were also collected in lakes Ińsko and Wicko (Pomeranian Lake District) and in Lake Mamry (Masurian Lake District); *U. crassus* was obtained from the River Brda (Pomeranian Lake District); *U. pictorum* from lakes Ślesińskie (central Poland), Spore and Marianowo (Pomeranian Lake District); *U. tumidus* from Lake Ślesińskie (central Poland), Lake Wicko and River Brda (Pomeranian Lake District); *A. anatina* was obtained from Lake Ślesińskie (central Poland), lakes Wicko and Marianowo (Pomeranian Lake District), and from the Szczecin Lagoon; *A. woodiana* was collected from Lake Licheńskie (central Poland).

The bivalves from Poland were obtained in 1997–2001 (spring and summer), while those from the Dnieper (Ukraine) were collected in September 2000. Individuals (alcohol-preserved) of legally protected species *A. cygnea* and *P. complanata* were kindly made available by Dr KATARZYNA ZAJĄC, of the Polish Academy of Sciences' Institute of Nature Conservation in Cracow. Altogether, the analyses involved 16 individuals of *D. polymorpha* (7 from Ukraine), 6 individuals of *D. bugensis*, 3 individuals of *U. crassus*, 5 individuals of *U. pictorum*, 6 individuals of *U. tumidus*, 10 individuals of *A. anatina*, 3 individuals of *A. cygnea*, 8 individuals of *A. woodiana,* and 4 individuals of *P. complanata*.

DNA EXTRACTION

Genomic DNA was extracted from 3 mm × 3 mm somatic tissue fragments (from gills), removed with a scalpel and transferred onto microscopic slides; the tissue fragments were placed in 50 µl of the homogenisation buffer solution STE 100 (0.1 M NaCl, 0.1 M EDTA, 0.05 M Tris-HCl, pH 8.0). The homogenate was subsequently transferred to an Eppendorf tube; its content was brought to 600 µl with STE 100, and the nucleic acid was extracted by lysis in SDS (10% sodium dodecyl sulphate). The nucleic acid was purified by phenol/chloroform extraction. An isopropanol precipitation was then carried out at room temperature; the precipitate was washed with 70% and absolute alcohol, dried, and dissolved in TE buffer (1 mM EDTA, 0.01 M Tris-HCl, pH 8.0).

POLYMERASE CHAIN REACTION (PCR)

Double-stranded DNA was generated via the polymerase chain reaction (PCR) using the primers LCO1490 and HCO2198 (FOLMER et al. 1994). These primers produced a 735 bp long fragment of DNA. The PCR reaction was carried out in a volume of 20 µl which included: 0.8 to 1 µl of the isolated DNA as well as 0.5 unit of Taq polymerase (Fermentas), 2 µl of 10 × buffer, 50 µM of nucleotide mixture, 5 pmol of each of the two primers, and 12.1 µl of sterilised distilled water. The final concentration of $MgCl_2$ in the PCR reaction was 2.5–3 mM. PCR was carried out for 5 cycles at denaturation temperature of 95°C (for 30 s, but first for 1 min 30 s prior to all the remaining cycles), at annealing temperature of 45°C (for 1 min), and extension temperature of 72°C (for 1 min 30 s). Subsequently, 27 cycles were run at denaturation temperature of 95°C (for 30 s), annealing temperature of 55°C (for 45 s), and extension temperature of 72°C (for 1 min, but 7 min in the final cycle).

PCR products were checked by electrophoresis in TBE buffer and in 2% agarose gel containing ethidium bromide (1.5 µl/120 ml); the gels were visualised under UV.

RESTRICTION ENDONUCLEASE DIGESTION

To perform the restriction analysis of the amplified 735 bp *COI* gene fragment, the following six restriction endonucleases were used: ScrFI, Csp6I, BsiZI, EcoRI, AluI and BamHI.

Restriction enzyme digests consisted of 3 µl of the PCR product, 1 µl of 10 × buffer, 4 U of the appropriate enzyme, and 5.6 µl of sterilised distilled water. The reaction was run for 12 h at temperatures appropriate for each restriction enzyme. Subsequently, the whole reaction volume was loaded on 2% agarose gel with ethidium bromide. Electrophoresis was carried out for 2 h at 60 V in standard TBE buffer (pH 8.0). The gels were visualised under UV using BioCapt MW (Vilber Lourmat, France). The results were analysed with the computer programme Bio1D (Vilber Lourmat, France).

RESULTS

The primers LCO1490 and HCO2198 (FOLMER et al. 1994) applied to amplify fragments of the *COI* gene yielded identical, about 735 bp-long products, in all ten bivalve species analysed (Fig. 1).

RFLP and different restriction enzymes applied to the previously amplified *COI* gene fragments resulted in similar, in terms of the number of base pairs, total lengths of the restriction fragments to those obtained as a result of amplification (about 735 bp). Certain discrepancies could have resulted from very small DNA fragments that were formed as a result of DNA digestion by restrictases and did not appear on the gel during visualisation.

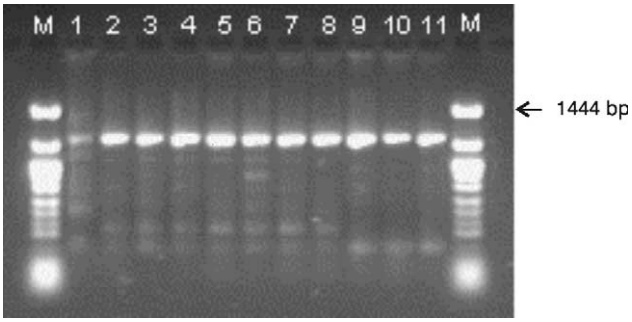

Fig. 1. Agarose gel of PCR-generated *COI* products. Lanes M, 1444 ladder. Lane 1, *Dreissena polymorpha* from Poland; lane 2, *D. polymorpha* from Ukraine; lane 3, *D. bugensis*; lane 4, *Unio crassus*; lane 5, *U. pictorum*; lane 6, *U. tumidus*; lane 7, *Anodonta anatina*; lane 8, *A. cygnea*; lane 9, *A. woodiana*; lanes 10 and 11, *Pseudanodonta complanata*

Restriction analysis of the mitochondrial *COI* gene with enzyme ScrFI identified 5 different genotypes (A–E) within the nine bivalve species studied (Table 1). The endonuclease used revealed from two to three restriction sites in the freshwater species (Table 1; Fig. 2). Differences in restriction fragment patterns between the genera *Dreissena*, *Unio*, and *Anodonta* were observed. In addition, different restriction patterns occurred within the genus *Dreissena*. *D. polymorpha* from Poland and Ukraine showed the presence of an identical genotype A with three restriction fragments (400, 220, and 130 bp), while *D. bugensis* from Ukraine produced four fragments (300, 220, 130, and 50 bp), referred to as genotype B (Table 1). The two *Anodonta* species as well as *Unio pictorum* and *U. tumidus* showed identical 400, 220, and 130 bp restriction patterns, called genotype A. Four species: *D. bugensis*, *U. crassus*, *A. anatina* and *P. complanata*, had unique genotypes, labelled B, C, D and E, respectively.

The enzyme Csp6I identified a single restriction site within the *COI* gene in all the bivalve species analysed, except *U. pictorum*. The sequence recognised (G/TAC), however, occurred at different sites within

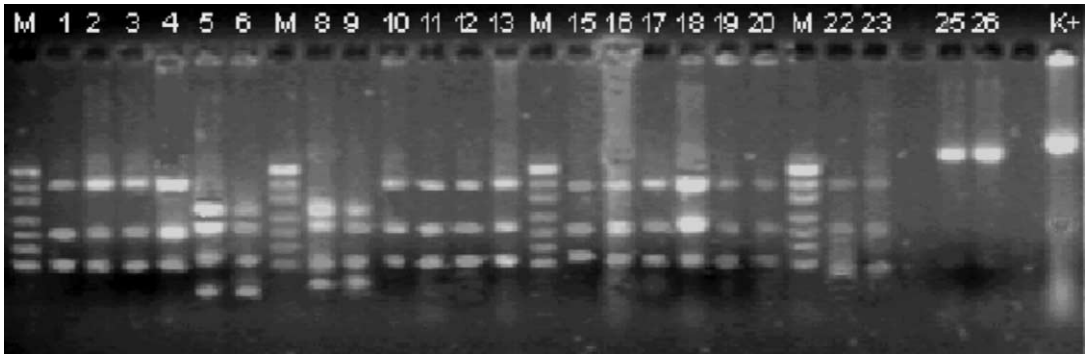

Fig. 2. Restriction ScrFI digestion of a mitochondrial *COI* gene fragment. Lane M, 501 Ladder (501, 404, 331, 242, 190, 147, 110 bp). Lanes 1 and 2, *Dreissena polymorpha* from Poland; lanes 3 and 4, *D. polymorpha* from Ukraine; lanes 5 and 6, *D. bugensis*; lanes 8 and 9, *Unio crassus*; lanes 10 and 11, *U. pictorum*; lanes 12 and 13, *U. tumidus*; lanes 15, 16 and 17, *A. cygnea*; lanes 18, 19 and 20, *A. woodiana*; lanes 22 and 23, *Pseudanodonta complanata*; lanes 25 and 26, *Anodonta anatina* and K^+, undigested fragment of the *COI*

the *COI* gene, which allowed the identification of five different genotypes (Table 1). In five species (*U. tumidus, A. anatina, A. cygnea, A. woodiana,* and *P. complanata*), digestion with endonuclease Csp6I resulted in two almost identical (about 350 bp) DNA fragments, whereby a single band was visible on the gel (Fig. 3). This restriction pattern was referred to as genotype E (Tables 1 and 2). *D. polymorpha* showed genotype A, characterised by two DNA fragments, one 480 and the other 240 bp long (Table 1; Fig. 3). A clear differentiation was observed between the three *Unio* species of genotypes C, D, and E as well as in the two *Dreissena* species with genotypes A and B (Tables 1 and 2; Fig. 3). The genotypes: A, B, C, and D, occurring in *D. polymorpha*, *D. bugensis, U. crassus,* and *U. pictorum,* respectively, were specific for those species only.

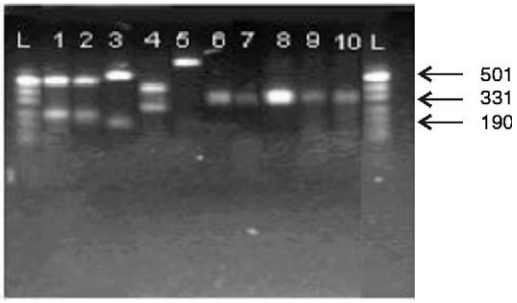

Fig. 3. Restriction Csp6I digestion of a mitochondrial *COI* gene fragment. Lanes L, 501 Ladder (501, 404, 331, 242, 190, 147, 110 bp). Lane 1, *Dreissena polymorpha* from Poland; lane 2, *D. polymorpha* from Ukraine; lane 3, *D. bugensis*; lane 4 *Unio crassus*; lane 5, *U. pictorum*; lane 6, *U. tumidus*; lane 7, *Anodonta anatina*; lane 8, *A. cygnea*; lane 9, *A. woodiana*; lane 10, *Pseudanodonta complanata*

Table 1. Approximate sizes (bp) of mitochondrial *COI* gene fragments generated by five restriction enzymes in nine bivalve species. Abbreviations: *Dp – Dreissena polymorpha; Db – D. bugensis*; *Uc – Unio crassus*; *Up – U. pictorum*; *Ut – U. tumidus*; *Aa – Anodonta anatina*; *Ac – A. cygnea*; *Aw – A. woodiana*; *Pc – Pseudanodonta complanata*

Dp	*Db*	*Uc*	*Up*	*Ut*	*Aa*	*Ac*	*Aw*	*Pc*
				ScrFI				
Genotype A	B	C	A	A	D	A	A	E
400	300	300	400	400	620	400	400	400
220	220	220	220	220		220	220	220
130	130	120	130	130		130	130	100
	50	80						
Total 750	700	720	750	750	620	750	750	720
				Csp6I				
Genotype A	B	C	D	E	E	E	E	E
480	530	400	735	350	350	350	350	350
240	200	300		350	350	350	350	350
Total 720	730	700	735	700	700	700	700	700
				BsiZI				
Genotype A	B	C	C	C	C	C	C	C
400	350	735	735	735	735	735	735	735
200	200							
120	100							
Total 720	650	735	735	735	735	735	735	735
				EcoRI				
Genotype A	A	B	C	A	D	C	C	B
735	735	520	520	735	620	520	520	520
		200	220		100	220	220	200
Total 735	735	720	740	735	720	740	740	720
				AluI				
Genotype A	B	C	D	E	F	G	H	I
250	350	420	250	250	300	200	350	250
150	250	150	150	180	270	150	150	230
120	100	80	130	100	130	100	130	150
120			130	100		100	50	80
80			50	80		100		
Total 720	700	650	710	710	700	650	680	710

Table. 2. Multigenotypes of nine bivalve species. Unique genotypes indicated in bold

Restriction enzyme	ScrFI	Csp6I	BsiZI	EcoRI	AluI	Number of multi-genotype
D. polymorpha	A	**A**	**A**	A	**A**	1
D. bugensis	**B**	**B**	**B**	A	**B**	2
U. crassus	**C**	**C**	C	B	**C**	3
U. pictorum	A	**D**	C	C	**D**	4
U. tumidus	A	E	C	A	**E**	5
A. anatina	**D**	E	C	**D**	**F**	6
A. cygnea	A	E	C	C	**G**	7
A. woodiana	A	E	C	C	**H**	8
P. complanata	**E**	E	C	B	**I**	9
Total genotypes	5	5	3	4	9	9

Restriction endonuclease BsiZI recognised two restriction sites only in the *Dreissena COI* sequence (Table 1). The enzyme revealed different restriction patterns in the two species; they are referred to as genotype A for *D. polymorpha* and genotype B for *D. bugensis*. In the remaining seven species, restrictase BsiZI recognised no *COI* gene restriction sites with the G/GNCC sequence; all those species showed the presence of an identical genotype C (Table 2).

Restrictase EcoRI resulted in the digestion of the *COI* gene of a single site in each of the six species of freshwater bivalves. The sequence it recognised (G/AATTC) was absent in the *COI* gene of the two *Dreissena* species and *U. tumidus* which all showed an identical genotype A (Table 1). The restriction patterns in *Unio pictorum* as well as in *A. cygnea* and *A. woodiana* were identical for the enzyme and contained two fragments of 520 and 220 bp, identified as genotype C. A very similar genotype B was present in *U. crassus* and *P. complanata*, the slight differences observed involving about 20 base pairs in the smaller DNA fragment. A clearly different genotype D, showing 620 and 100 base pairs, was present in *A. anatina*.

Restriction endonuclease AluI recognised from two to four restriction sites in all the bivalve species analysed (Table 1). The enzyme revealed nine different restriction patterns and genotypes within the nine bivalve species studied (Tables 1 and 2).

The *COI* gene fragment from all species analysed lacked the restriction site (735 bp) for BamHI.

RFLP involving six restriction enzymes did not reveal any variability within any of the nine bivalve species analysed. Each species showed an enzyme-specific restriction pattern. The *D. polymorpha* individuals from Poland and Ukraine, always showed identical endonuclease-specific restriction patterns.

The overall genotype analysis with respect to the five restriction enzymes revealed nine different genotypes in the nine bivalve species studied (Table 2). PCR-RFLP applied to the *COI* gene of *A. cygnea* and *A. woodiana* showed identical genotypes for four endonucleases and only AluI allowed the molecular identification of the two species (genotype 7 and 8). In contrast, the remaining species differed from one another in three to five restriction patterns.

DISCUSSION

Amplification of the *COI* mitochondrial gene and its restriction analyses involving six restriction enzymes showed the absence of individual variability within each species at the level of DNA sequences recognised by the endonucleases. The genetic variability of the *COI* gene involved differences at the species, genus, and family level, depending on the restriction enzyme used. No genetic differences between the Polish and Ukrainian populations of *D. polymorpha* were revealed.

The *COI* gene digestion with ScrFI resulted in restriction patterns that could serve as molecular markers with which to differentiate between bivalves of the genus *Dreissena* and *U. crassus, A. anatina* and *P. complanata*. On the other hand, the endonuclease in question did not differentiate between the two *Anodonta* species and the two *Unio* species which showed identical restrictive patterns (genotype A). It did, however, differentiate between *U. crassus* and the remaining members of the genus and the 400 and 220 bp fragments of the *COI* gene were typical of the unionid bivalves (except for *U. crassus* and *A. anatina*) (Table 1, Fig. 2).

The restriction patterns obtained using PCR-RFLP and the endonuclease ScrFI in *D. polymorpha* (from Poland and Ukraine; genotype A) and *D. bugensis* (from Ukraine; genotype B) are similar to the results

reported for these species from the North American Great Lakes (CLAXTON et al. 1997, CLAXTON & BOULDING 1998). However, certain differences in the length of the restriction fragments were apparent. In adult and juvenile *D. polymorpha* from Lake Erie, CLAXTON et al. (1997) arrived at an identical restriction pattern of 450, 200, and 100 base pairs. On the other hand, CLAXTON & BOULDING (1998) reported a restriction pattern of 400, 150 and 50 base pairs in *D. polymorpha* adults and veligers from lakes Erie, Ontario, and Simcoe. In the present study, an identical restriction pattern of 400, 200, and 130 base pairs was revealed in *D. polymorpha* from Poland and Ukraine.

In American and Ukrainian individuals of *D. bugensis,* endonuclease ScrFI always recognised three restriction sites, the discrepancies in restriction fragment size never exceeding 50 bp. The Canadian workers mentioned reported obtaining 250, 200, 175–150, and 50 bp DNA fragments (CLAXTON et al. 1997, CLAXTON & BOULDING 1998), while the lengths determined in this study were 300, 220, 130, and 50 base pairs. The differences between restriction patterns of the two *Dreissena* species did not exceed 50 base pairs and most probably resulted from differences in primers used in the *COI* gene fragment amplification and/or from the precision of gel image analysis. It does not seem likely that the discrepancies observed were a result of differences, at the DNA level, between conspecific individuals from different sites, even located very far apart. Both we and CLAXTON et al. (1997) used FOLMER's primers (FOLMER et al. 1994) which amplify about 710 bp *COI* gene fragments, while CLAXTON & BOULDING (1998) used the dreissenid A and dreissenid B primers and obtained an about 608 bp *COI* gene fragment.

Because of the noticeable differences in the restriction patterns obtained, restrictase Csp6I can be used as a marker to differentiate between all the species within the genera *Dreissena* and *Unio*. On the other hand, the restrictase produced identical patterns for the *Anodonta* species (two a. 350 bp long DNA fragments constituting genotype E).

Endonuclease Csp6I was earlier used to differentiate between *D. polymorpha* and *D. bugensis* from Lake Erie in North America (CLAXTON et al. 1997). Results reported by those authors are comparable with those obtained in this study. In both species, restrictase Csp6I recognised a single restriction site. In *D. polymorpha,* CLAXTON et al. (1997) obtained 450 and 250 bp fragments, corresponding to 480 and 240 bp fragments yielded by the present study. In Lake Erie *D. bugensis,* CLAXTON et al. (1997) obtained 550 and 150 bp restriction fragments, comparable to those produced for individuals from the Ukrainian River Dnieper used in this study (Table 1). CLAXTON et al. (1997) reported identical results for adult and juvenile individuals, both in *D. polymorpha* and *D. bugensis*.

Restriction endonuclease BsiZI (an isoschisozyme of enzyme Sau96I) was found to perfectly differentiate between the *Dreissena* bivalves and the unionids in which it did not recognise any restriction site (Table 1). Earlier, when searching for molecular markers of *D. polymorpha* and *D. bugensis,* CLAXTON et al. (1997) applied restrictase Sau96I. Their results were comparable; the differences observed in the lengths of the restriction fragments obtained involved 30 base pairs in single DNA fragments in *D. polymorpha* only. In this study, 400, 200 and 120 bp fragments were obtained for the species, while CLAXTON et al. (1997) reported 400, 200 and 150 bp fragments. For *D. bugensis*, both this study and that of CLAXTON et al. (1997) on the North American populations, resulted in identical 350, 200 and 100 bp fragments. The results reported were obtained when amplifying the *COI* gene with identical primers (FOLMER et al. 1994) and using a restriction analysis that involved enzymes recognising identical sequences (GGNCC). For this reason the results are almost identical. This demonstrates the reliability of the technique and its potential for wide applications. The differences in fragment lengths were small and never exceeded 50 base pairs, which could have resulted from the precision of the gel visualisations and/or from the amount of the product subjected to amplification and restriction.

Enzyme EcoRI proved to be a restrictase that made it possible to differentiate between the genus *Dreissena* and *U. tumidus* (possessing 735 base-pair genotype A) and the remaining *Unio* and *Anodonta* species, as well as *P. complanata* (Table 1). The restriction pattern of *A. anatina* (genotype D) is specific for this species, while the other genotypes B and C (520 and 220 bp or 200 bp) are very similar and common for five of the freshwater bivalve species.

The highest number of genotypes (9) was recognised by restrictase AluI, five genotypes being observed for ScrFI and Csp6I, 4 for EcoRI, 3 for BsiZI, and 1 for BamHI. Individual enzymes recognised from one (EcoRI) to nine (AluI) unique genotypes (i.e., those present in a single species each), and can be regarded as species-specific markers. At the same time, from one (ScrFI, Csp6I and BsiZI) to three (EcoRI) genotypes were observed which were repeated in two or more species (Table 2).

Three restriction enzymes (ScrFI, Csp6I, and Sau96I/BsiZI), applied earlier to differentiate between *D. polymorpha* and *D. bugensis* (CLAXTON et al. 1997, CLAXTON & BOULDING 1998) proved useful in identifying European populations of the bivalves and may be used to identify the three *Unio* species (enzyme Csp6I only), *A. anatina* and *P. complanata* (ScrFI only). However, they did not differentiate between any of the *Anodonta* species, except *A. anatina*. Only enzyme AluI that was applied for the first time can make molecular identification of all species analysed possible.

Application of five restrictases produced identical restriction patterns in *A. cygnea* and *A. woodiana* (only AluI can differentiate this two species), which is indicative of their substantial homology within the *COI* gene fragment analysed (Table 2). It is interesting that the native *A. cygnea* shows a higher genetic affinity to *A. woodiana* (introduced from the Far East and present in Poland for 10 years only; SOROKA 2000) than to the remaining native unionids. However, a comparative analysis of their sequences in the *COI* gene, obtained from GenBank (accession numbers: AF461419 for *A. cygnea* and AF468683 for *A. woodiana*) showed certain heterogeneity, amounting to 14.9% within 630 base pairs. Comparisons between sequences of *A. cygnea* and *A. anatina* (AF462071), *P. complanata* (AF494102), and *U. crassus* (AF514296) revealed heterogeneity levels of 33.1%, 15.0%, and 16.6%, respectively (within 612–637 base pairs), the heterogeneity being higher than that observed in comparisons of *A. cygnea* with the Chinese bivalve *A. woodiana*. This demonstrates that restriction analyses of the mitochondrial *COI* gene reflect interspecific genetic similarities and differences at the DNA sequence level.

No bivalve species the individuals of which were collected from different distant localities in Poland (except for *A. woodiana* occurring at a single site only) showed intraspecific polymorphism in the restriction fragment of their *COI* gene fragments analysed. Moreover, no genetic variability was observed between *D. polymorpha* individuals from the Polish and Ukrainian populations. Identical restriction patterns also were present in the European and North American populations of *D. polymorpha* and *D. bugensis* (CLAXTON et al. 1997, CLAXTON & BOULDING 1998).

The absence of species-level heterogeneity demonstrates the utility of RFLP, as applied to the *COI* gene fragment in differentiating between and identifying bivalve species not only in Poland, but worldwide as well. The technique can be successfully used with adults, juveniles, and larval stages of the bivalves (CLAXTON et al. 1997, CLAXTON & BOULDING 1998), and it thus suitable for population studies and for monitoring changes in distribution ranges. An additional asset of the technique is its repeatability across various laboratories.

Results obtained when PCR-RFLP was applied to the mitochondrial *COI* gene are comparable to those produced by the more expensive and time-consuming gene sequencing. Theoretical restriction analyses of sequences registered in GenBank for different bivalve species have always resulted in identical numbers of DNA fragments obtained; the differences observed concerned fragment lengths only and were related to differences in the number of sequenced bases of the gene.

REFERENCES

BALDWIN B. S., BLACK M., SANJUR O., GUSTAFSON R., LUTZ R. A., VRIJENHOEK R. C. 1996. A diagnostic molecular marker for zebra mussels (*Dreissena polymorpha*) and potentially co-occuring bivalves: mitochondrial *COI*. Mol. Mar. Bio. Biotechnol. 5: 9–14.

BROWN W. M. 1985. The mitochondrial genome of animals. In: Molecular Evolutionary Genetics (MACINTYRE R. J., ed.), pp. 95–130, Plenum Press, New York.

CLAXTON W. T., MARTEL A., DERMOTT R. M., BOULDING E. G. 1997. Discrimination of field-collected juveniles of two introduced dreissenids (*Dreissena polymorpha* and *Dreissena bugensis*) using mitochondrial DNA and shell morphology. Can. J. Fish. Aquat. Sci. 54: 1280–1288.

CLAXTON W. T., BOULDING E. G. 1998. A new molecular technique for identifying field collections of zebra mussel (*Dreissena polymorpha*) and quagga mussel (*Dreissena bugensis*) veliger larvae applied to eastern Lake Erie, Lake Ontario, and Lake Simcoe. Can. J. Zool. 76: 194–198.

DOMAGAŁA J., MIGDALSKA B., ŁABĘCKA A. M., PILECKA-RAPACZ M. 2003. *Anodonta woodiana* (Lea, 1834) in the Western Pomerania. Zeszyty Naukowe Uniwersytetu Szczecińskiego, Acta Biol. 10: 199–202.

FALNIOWSKI A., WILKE T. 2001. The genus *Marstoniopsis* (Gastropoda: Rissooidea): intra- and intergeneric phylogenetic relationships. J. Moll. Stud. 67: 483–488.

FOLMER O., BLACK M., HOEH W., LUTZ R., VRIJENHOEK R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Bio. Biotechnol. 3: 294–299.

KISS A. 1995. The propagation, growth and biomass of the Chinese huge mussel (*Anodonta woodiana woodiana* Lea, 1834) in Hungary. University of Agriculture Science in Gödöllö, Tropical and Subtropical Department, Private Edition, Second ed.: 1–33.

KRASZEWSKI A., ZDANOWSKI B. 2001. Distribution and abundance of the Chinese mussel *Anodonta woodiana* (Lea, 1834) in the heated Konin lakes. Arch. Pol. Fish. 9: 253–265.

NOWAK E. 1974. Zwierzęta w ekspansji. WP, Warszawa.

PIECHOCKI A., RIEDEL A. 1997. Mollusca. In: Checklist of Animals of Poland (RAZOWSKI J., ed.), pp. 104–121, Polish Academy of Sciences, Cracow.

PROTASOV A. A., AFANASJEV S. A., SINICYNA O. O., ZDANOWSKI B. 1994. Composition and functioning of benthic communities. Arch. Pol. Fish. 2: 257–284.

RYBSKA E., PACAK A., SZWEYKOWSKA-KULIŃSKA Z., LESICKI A. 2000. Taxonomy of European Lymnaeidae (Gastropoda: Pulmonata) in studies with the use of molecular biology techniques. I. Preliminary view on the subgenus *Stagnicola* Leach, 1830 on the basis of RAPD analysis. Folia Malacol. 8: 277–284.

SOROKA M. 2000. Age structure and sex ratio of *Anodonta woodiana* (Lea, 1834) (Bivalvia: Unionidae) from Konin reservoirs (C Poland). Folia Malacol. 8: 239–244.

SOROKA M., ZDANOWSKI B. 2001. Morphological and genetic variability of the population of *Anodonta woodiana* (Lea, 1834) occurring in the heated Konin lakes system. Arch. Pol. Fish. 9: 239–252.

STAŃCZYKOWSKA A. 1977. Ecology of *Dreissena polymorpha* (Pall.) (Bivalvia) in lakes. Pol. Arch. Hydrobiol. 24: 461–530.

STAŃCZYKOWSKA A. 1997. Review of studies on *Dreissena polymorpha* (Pall). Pol. Arch. Hydrobiol. 44: 401–415.

VOSS M. C., JENNECKENS I., MEYER J.-N., HOPPE H.-H. 1999. Species differentiation of slugs (Mollusca, Pulmonata) by using DNA-fingerprinting techniques (SSR-a PCR; RAPD). Zeitschr. Pflanzenkrankheiten und Pflanzenschutz 106: 387–391.

WIKTOR J. 1969. Biology of *Dreissena polymorpha* and its ecological role in Szczecin Lagoon (in Polish). Studia i Materiały Morskiego Instytutu Rybackiego 5: 1–88.

WOOD A. R., BEAUMONT A. R., SKIBIŃSKI D. O. F., TURNER G. 2003. Analysis of nuclear-DNA marker for species identification of adults and larvae in the *Mytilus edulis* complex. J. Moll. Stud. 69: 61–66.

ZDANOWSKI B. 1994. Characteristics of heated Konin lakes, pollution sources, main results and conclusions. Arch. Pol. Fish. 2: 139–160.

Received: June 15th, 2005
Accepted: September 20th, 2005