Genetic variation in three paddlefish (*Polyodon spathula* Walbaum) stocks based on microsatellite DNA analysis

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ABSTRACT: There are two stocks of American paddlefish *Polyodon spathula* in Poland, one in Pogorze and the other in Wasosze. These stocks were established from small quantities of eggs imported from the USA in 1995. In this study, we examined genetic variation at seven microsatellite loci in adult fish from the two Polish farms and one stock farmed in Gorny Tykich in Ukraine. Our data were compared with those reported for one native population from the Mississippi River (USA). The polymorphism of examined loci varied in the Polish and Ukrainian stocks, showing 25–30 alleles across each stock (3.6–4.3 alleles per locus), though they were less polymorphic than those in the Mississippi (49 across the population, 7.0 per locus). The mean observed heterozygosity per locus estimated in Polish stocks (0.59–0.60) was comparable to that found in the Mississippi (0.68). The Garza-Williamson index and values of the heterozygosity excesses revealed a reduction of genetic variation in all the three European stocks (probably resulting from bottleneck or founder effect). Finally, genetic distance measurements confirmed a closer relationship between Pogorze and Wasosze (F_{ST} = 0.007) stocks than between these two Polish stocks and that from Ukraine (F_{ST} = 0.096 and F_{ST} = 0.054).

Keywords: broodstocks; aquaculture; founder effect; genetic distance; genetic variation

The paddlefish has been present in Europe since 1974 when a sample of eggs was imported from the USA to the Soviet Union (Vedrasco et al., 2001). Polish stocks were founded from part of a consignment of eggs imported in 1995 from the USA to Hungary, thus it is possible that all fish reared in Poland are the progeny of only a few spawners. Consequently, genetic variation in Polish stocks may be reduced due to the bottleneck effect.

Polish aquaculture of this species is still at the experimental level and is carried out at two centres: Pogorze and Wasosze. American paddlefish may become a popular additional fish species for Polish fish farms, especially because similar additions in neighbouring countries have been successful (FAO, 2007). The broodstocks, however, are small and the total number of fish is only two or three dozen. Some fish have reached their sexual maturity, others are close to maturity and are expected to become a broodstock for producing stocking material. Because both stocks originated from the same group of fish it is important to identify their genetic characteristics and to evaluate the differences between them. In this study we examined polymorphism at ten microsatellite loci in both of the Polish stocks and in the Ukrainian stock (Gorny Tykich) as a reference and potential source for genetic enrichment of Polish stocks. We have also evaluated genetic divergence between the examined stocks, as well as the extent of founder/bottleneck effects that might have affected genetic variation within those stocks.

If the aquaculture of this species is to be successfully developed there is an urgent need to apply these measures to other European stocks which might be regarded as potential sources of gene pool enrichment.

MATERIAL AND METHODS

Fish samples

Pelvic or pectoral fin clips (approximately 100 mm^2) were taken from 100 individuals (aged from 3 to 11 years). Samples were taken from all individuals of the Wasosze and Pogorze stocks (29 and 24 fish, respectively) and from 47 fish reared in Ukraine which were a part of the stock from Gorny Tykich. The fin clips were placed in Eppendorf tubes and kept in 97% ethanol at a temperature of 4°C until their examination.

DNA extraction

Genomic DNA was extracted and purified from the fin tissues using Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA), following the procedure described by Fopp-Bayat and Luczynski (2006) and Kaczmarczyk et al. (2007). DNA samples were stored at a temperature of –20°C. The integrity of the DNA samples was visually inspected after their electrophoretic separation in 1.5% agarose gel stained with ethidium bromide. All agarose gels were photographed using KODAK gel Logic 200 Imaging System (Kodak Japan Ltd., Tokyo, Japan). Samples of the DNA yields were quantified by spectrophotometric analysis. Low concentration of the template DNA often caused problems in amplifying the microsatellite loci, therefore only samples containing more than 30 µg/ml of double stranded DNA were qualified for the PCR stage; where this concentration was not achieved the procedure of DNA extraction was repeated.

PCR amplification

The microsatellites examined were: *Ls 57*, *Ls 62*, *Psp 12, Psp 18, Psp 20, Psp 21, Psp 26, Psp 28, Psp 29,* and *Psp 32* (May et al., 1997; Heist et al., 2002). All forward primers were 5'-labeled using phosphoramidite dyes (D2-PA, D3-PA, and D4-PA). Fluorescent-labeled primers and PCR products were stored in black boxes to protect them against daylight. In order to reduce the exposure of chemicals to daylight during PCR setup, aluminium covers were put on tubes containing primers and PCR mixture. In the case of base dilutions the number of freezing-thawing cycles was reduced as far as possible. Amplification reactions and PCR product verification followed (Kaczmarczyk et al., 2007).

Genotyping

The lengths of amplified DNA fragments were determined using CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, USA) against 60–400 bp size standard. Amplified fragments were arranged in three sets. Set No. 1 consisted of amplicons *Psp 12*, *Psp 18*, and *Psp 26*; set No. 2 consisted of amplicons *Psp 20*, *Psp 21*, and *Psp 28*; and set No. 3 of *Psp 29, Psp 32,* and *Ls 57*. Within these sets each individual microsatellite was amplified using primers with different, attached, phosphoramide labels, thus enabling their separation in a multiplex mode. For fragment size and allele determination CEQ™ 8000 Software for Sequencing and Fragment Analysis (Beckman Coulter, Inc., Fullerton, USA) was applied following the manufacturer's recommendations.

Statistical analyses

The observed number of alleles per locus, allele frequency, number of private alleles, allelic range, and allelic richness were computed by MSA software (Dieringer and Schlötterer, 2003).

In order to accommodate the obtained results to the requirements of MSA and Arlequin software, a tetrasomic locus *Psp 29* was divided into two isoloci: *Psp 29A* and *Psp 29B*. An observed heterozygosity (H_o) was calculated for each locus applying an algorithm described by Nei (1987).

The Exact Hardy Weinberg (H-W) Test (Guo and Thompson, 1992) was used to test deviations from the H-W equilibrium. Each locus and each population was tested separately (Guo and Thompson, 1992). The number of steps in the Markov chain equaled to 1 000 000 and the number of dememorisation steps equaled to 100 000. Expected heterozygosity (H_e) and observed heterozygosity (H_o) were calculated using Arlequin 3.0 software (Excoffier et al., 2005).

Genetic divergence between stocks was analysed using two different parameters: fixation index (F_{ST}) (Wright, 1951) and variation of average allelic size ($\sigma\mu^2$) (Goldstein et al., 1995). F_{ST} values and their statistical significance were calculated by Arlequin

Table 1. Comparison of number of alleles and values of theGarza-Williamson index (*M* values) in the European stocks and Mississippi River population of *Polyodon spathula* (Heist et al., 2002)

Locus	Pogorze (24 fish)			Wasosze (29 fish)			Gorny Tykich (47 fish)			Mississippi (28 fish)		
	no. of alleles	allelic range (bp)	*M* value	no. of alleles	allelic range (bp)	*M* value	no. of alleles	allelic range (bp)	*M* value	no. of alleles	allelic range (bp)	*M* value
Psp 18	3	4	1.00	3	6	0.75	3	12	0.43	6	10	1.00
Psp 20	2	2	1.00	2	2	1.00	3	4	1.00	4	6	1.00
Psp 21	4	8	0.80	4	8	0.80	4	8	0.80	7	28	0.47
Psp 26	5	24	0.38	9	24	0.69	8	26	0.57	11	30	0.69
Psp 28	5	28	0.33	6	24	0.46	7	20	0.64	14	36	0.74
Psp 29	4	12	0.67	4	20	0.67	4	20	0.67	4	20	0.67
Psp 32	2	2	1.00	2	2	1.00	1	–	–	3	4	1.00
Average	3.6	10.0	0.74	4.3	9.8	0.77	4.3	10.3	0.68	7.0	19.1	0.80
SD	1.3	9.5	0.27	2.5	8.5	0.18	2.4	8.2	0.18	3.8	11.8	0.2
Number of private alleles	0	–	–	1	–	–	7	–	–	no data	–	–

SD = standard deviation, – = monomorphic locus, excluded from calculations

Table 2. Comparison of observed (H_o) and expected (H_e) heterozygosity in Polish and Ukrainian stocks to Mississippi River population (Heist et al., 2002), and departures from the Hardy-Weinberg equilibrium ($P \leq 0.05$) within investigated stocks

Locus	Wasosze			Pogorze			Gorny Tykich			Mississippi	
	H_o	H_e	*P* value	H_o	H_e	*P* value	H_o	H_e	*P* value	H_o	H_e
Psp 18	0.21	0.38	0.008	0.17	0.16	1.000	0.90	0.80	0.914	no data	no data
Psp 20	0.76	0.49	0.006	0.46	0.47	1.000	0.72	0.54	0.002	0.71	0.56
Psp 21	0.69	0.55	0.630	0.62	0.57	0.104	0.40	0.40	0.620	0.71	0.72
Psp 26	0.79	0.72	0.452	0.83	0.72	0.006	0.70	0.75	0.021	0.82	0.82
Psp 28	0.97	0.87	0.000	0.96	0.77	0.000	0.94	0.74	0.000	0.93	0.89
Psp 29A	0.62	0.44	0.027	0.71	0.47	0.020	0.66	0.50	0.037	no data	no data
Psp 29B	0.45	0.39	0.636	0.79	0.49	0.002	0.17	0.23	0.142		
Psp 32	0.21	0.19	1.000	0.29	0.25	1.000	–	–	–	0.21	0.20
Average	0.59	0.50		0.60	0.49		0.56	0.49		0.68	0.64
SD	0.26	0.20		0.26	0.20		0.32	0.26		0.24	0.25

SD = standard deviation, – = monomorphic locus, excluded from calculations; for the Mississippi River *P* values were not available;

3.0 software (Excoffier et al., 2005), the variation of average allelic size was calculated by MSA software (Dieringer and Schlötterer, 2003). Moreover, genetic differences among stocks were also evaluated using allelic differentiation tests in Genepop 4.0 software (Russet, 2008).

The estimations were based on heterozygosity excess in progeny generation of the Polish group (53 fish from Wasosze and Pogorze) and animals from Gorny Tykich. The estimations were based on excess heterozygosity in progeny generation according to Pudovkin et al. (1996). Estimates of the effective number of parents (N_b) and their confidence intervals (CI) were calculated from genotypic data at all investigated microsatellite loci by using Nb_HetEx software at 10 000 iterations (Zhdanova and Pudovkin, 2008) and rounded to an integer.

The likely effect of bottleneck on genetic variability within the investigated stocks was evaluated according to the Garza-Williamson index (Garza and Williamson, 2001) including Excoffier's adjustment (Excoffier et al., 2005). The monomorphic loci were excluded from these calculations. We also conducted a test for bottleneck assessment applying the BOTTLENECK software (Piry et al., 1999). This method is based on the assumption that in non-bottlenecked population (close to mutation-drift equilibrium) the value of H_e heterozygosity (expected heterozygosity according to the Hardy-Weinberg equlibrum) is equal to H_{eq} (heterozygosity expected in a mutation-drift equilibrium). The excess of H_e over H_{eq} is the evidence of a severe reduction in population effective size that may occur because of a bottleneck event (Cornuet and Luikart, 1996; Luikart and Cornuet, 1998). The differences between H_e and H_{eq} were statistically tested applying the Wilcoxon signed-rank test under the two-phase model (TPM) of microsatellite evolution (Di Rienzo et al., 1994). The fit to the mutation-drift equilibrium was tested applying Significance Test (BOTTLENECK software; Piry et al., 1999). The significance of differences in the number of alleles and their ranges between examined paddlefish samples were tested using the one-tailed Wilcoxon test for paired samples at a level of $P = 0.05$ (Statistica 8.0, Tulsa, USA).

RESULTS

Out of ten studied microsatellites, seven (*Psp 18*, *Psp 20*, *Psp 21*, *Psp 26*, *Psp 28*, *Psp 29*, *Psp 32*) were successfully amplified from all samples. DNA sequences of locus *Ls 57* were also amplified, but only from a few samples. Amplification of locus *Ls 62* failed. Both, *Ls 57* and *Ls 62* were excluded from further analysis. Locus *Psp 12* was also excluded from further analysis due to numerous stutter bands present in almost 15% of the samples.

Table 1 shows that all loci (except *Psp 32* in the sample from Gorny Tykich) were polymorphic. At locus *Psp 18,* most fish from the Polish samples were homozygous. In stock from Pogorze, 25 alleles were identified whereas 30 were detected in

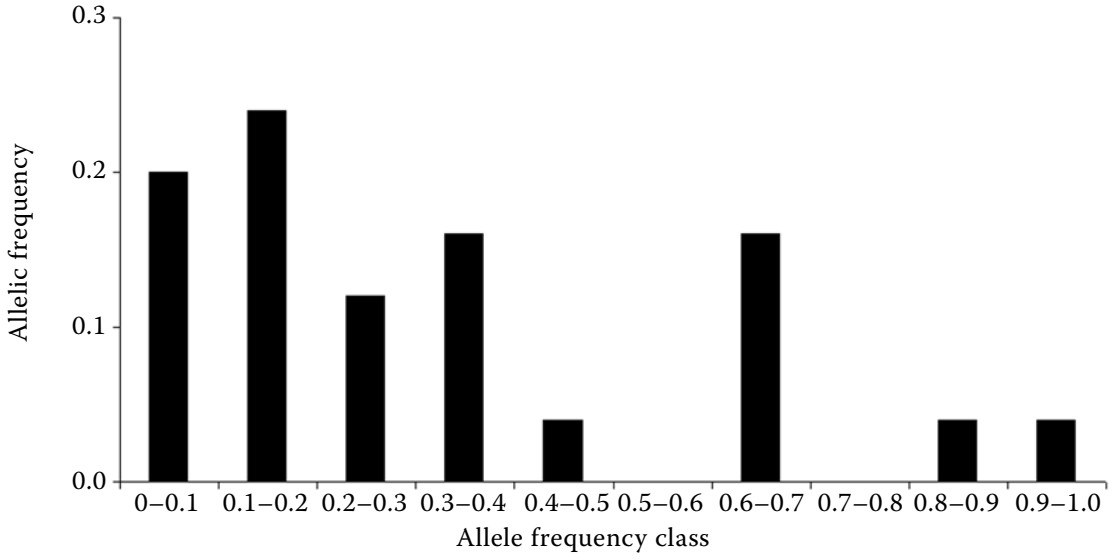

Figure 1. Distribution of allele frequencies in Pogorze stock

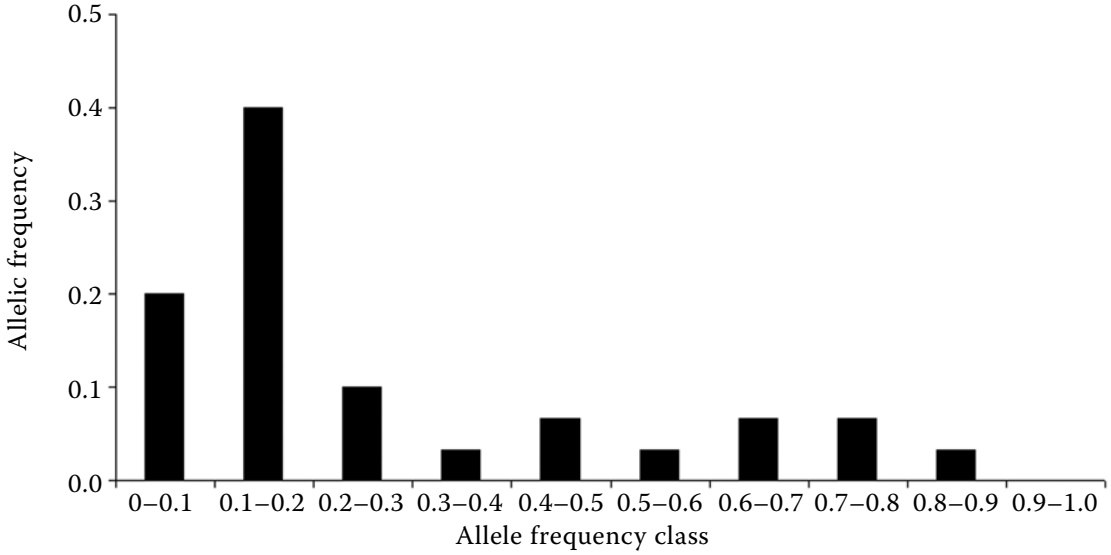

Figure 2. Distribution of allele frequencies in Wasosze stock

stock from Wasosze. Total number of alleles detected in stock from Gorny Tykich was also 30. Neither numbers of alleles, nor their allelic ranges differed significantly in the examined stocks ($P > 0.05$; Wilcoxon one-tailed test). We also identified private alleles that were specific for a given stock or group of stocks. Three alleles (230, 246 at locus *Psp 28* and 179 at locus *Psp 32*) were common in both Polish stocks, but allele 256 at locus *Psp 28* was observed only in Wasosze stock. The stock from Gorny Tykich differed from both of the Polish stocks by having 7 private alleles. These were: alleles 166, 176, and 178 at locus *Psp 18,* allele 212 at *Psp 20*, as well as alleles 154, 162 at locus *Psp 26* and 248 at locus *Psp 28*.

All investigated loci in this work were found to be different in values of the Garza-Williamson index (*M*) (Garza and Williamson, 2001). In all samples *M* values were the lowest for the most polymorphic loci (such as *Psp 26* and *Psp 28*) and the highest for the least polymorphic loci (*Psp 20* and *Psp 32*) (Table 1).

The mean observed heterozygosity (H_o) in the investigated stocks ranged from 0.56 to 0.60 (Table 2). In each of the investigated stocks heterozygosity observed at locus *Psp 28* significantly exceeded

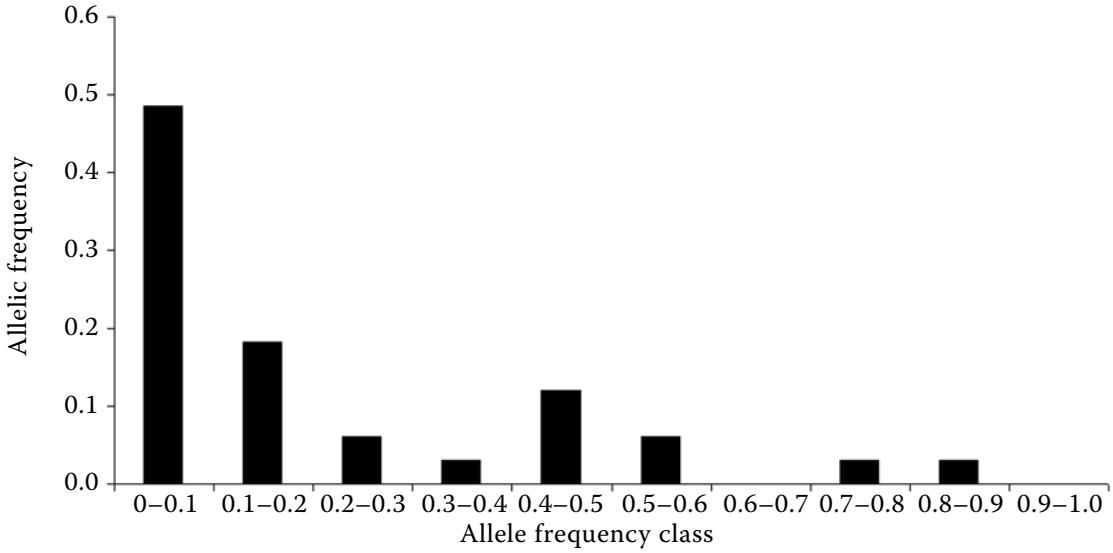

Figure 3. Distribution of allele frequencies in Gorny Tykich stock

expected heterozygosity calculated from the H-W equation.

The effective number of parents (N_b) for the pooled sample of Polish paddlefish was found to be 8 (95% CI from 5 to 39) fish whereas in Ukrainian stock N_b was 10 (95% CI from 5 to 68).

In all 3 stocks, under the TPM model, heterozygosity excesses were detected in most of the analysed loci (Pogorze – 6 out of 8 loci (P = 0.04633), Wasosze – 7 out of 8 loci (P = 0.17623), Gorny Tykich – 6 out of 7 loci (P = 0.09918)), however observed $H_e > H_{eq}$ differences were significant only in Pogorze stock. The hypothesis of mutation drift equilibrium in those populations was confirmed under the Wilcoxon test in Wasosze and Gorny Tykich stocks ($P < 0.05$) and rejected in Pogorze stock (P = 0.097).

An analysis of allele frequency distribution (Figures 1–3) revealed L-shape distribution for Gorny Tykich and Wasosze stocks, and mode-shift shape for Pogorze stock.

Genetic divergence between Polish and Ukrainian stocks, calculated as F_{ST} values, was 0.096 (Pogorze and Gorny Tykich) and 0.054 (Wasosze and Gorny Tykich), whereas that observed between the two Polish stocks was 0.007. All distances were statistically significant. Measures of genetic distances based on F_{ST} values were confirmed by using $\sigma\mu^2$ method; genetic distance between stocks from Pogorze and Gorny Tykich was 1.200, between Wasosze and Gorny Tykich 0.700, while that between the Polish stocks (Wasosze and Pogorze) was 0.107. The allelic differences between the three stocks were significant under the Fisher's test in most of the analysed microsatellites ($P < 0.05$) except loci: *Psp 29A* (P = 0.119), *Psp 26* (P = 0.110), and *Psp 20* (P = 0.088).

DISCUSSION

Evaluation of genetic variation in domesticated fish stocks requires their comparison with values observed in natural populations. The results of the present study were compared with the respective values observed in the natural paddlefish population inhabiting the Mississippi River (Illinois, USA) as described by Heist et al. (2002) (Tables 1 and 2).

The numbers of fish examined in the Polish stocks were similar to those from the Mississippi, but the Mississippi and European stocks exhibited different levels of polymorphism (computed across the seven microsatellites). The numbers of alleles within each locus as well as their allelic ranges in the natural Mississippi population (Heist et al., 2002) were significantly higher than in the Polish and Ukrainian stocks. The comparison of the overall number of alleles (25–30 alleles across the population) within European stocks to the Mississippi population (49 alleles across the population) and different allelic ranges in European stocks and the population from the Mississippi shows the presence of alleles in the American paddlefish which were undetected in those from investigated stocks. This finding may suggest a reduced genetic variation within Polish and Ukrainian stocks.

Case studies on various species of finfish, reptiles, and mammals presented by Garza and Williamson (2001) indicated that M value of the Garza-Williamson index being 0.8 or higher is specific for populations that have not suffered a reduction in their size. Values about 0.7 or less suggest that a population has gone through a recent reduction in size. Low M values ranging from 0.29 to 0.43 across a population are specific for remnant populations (for example the Galapagos iguana) and indicate significant reduction of the population size in the past (Tzika et al., 2008).

M values of the Garza-Williamson index, ranging from 0.68 to 0.77, were lower in Polish and Ukrainian stocks than that estimated for the Mississippi population (0.80). This may suggest reduction in genetic variation within Polish and Ukrainian stocks, particularly at the most polymorphic loci found in this study: *Psp 26* and *Psp 28*. The least polymorphic loci showed lower reduction in genetic variation with higher M values. Loci with two or three alleles were more resistant to changes of their allele frequency and to reduction of heterozygosity caused by the bottleneck effect.

Departure from the TPM mutation-drift equilibrium was found only in Pogorze stock, and the allele frequency distribution indicates that in this stock the loss of allelic diversity may have occurred probably because of bottleneck effect. Although the L-shape allele frequency distribution detected within other two stocks does not show significant reduction of allelic diversity, it seems that each of the investigated stocks suffered from bottleneck. In the case of fish from Wasosze (that share an ancestor with fish from Pogorze) the founder effect might not be clearly detected because an insufficient number of fish generations had been established since the foundation of this stock 16 years ago, and thus the analyses may not have reached their maximal value

(Cristescu et al., 2010). We do not know the initial number of founder fish for Wasosze stock but if we assume it was equal to the estimated effective number of spawners calculated for parental generation of fish from the Polish stocks, and the paddlefish reaches maturity after 11 years, then the maximal value of heterozygosity excess and power of this test according to the method of Cristescu et al. (2010) is anticipated about 88 years after the creation of the stock.

In Gorny Tykich the founder effect was probably limited due to the foundation of this stock from groups of diverse individuals taken from several parental stocks or populations. Indeed, Vedrasco et al. (2001) showed that there were more than two imports of paddlefish to the former USSR republics.

The effective number of spawners that were parents of the Polish group was close to the number of fish that would have effectively contributed to Gorny Tykich, but in both cases the CI interval was high. Although there are other effective methods of estimating the effective number of spawners, for example using temporal change in allelic frequencies between investigated fish and their parents (Waples, 1991), we could not apply them because we did not have data from parental generations.

The proportion of the H_e to H_{eq} values might be a useful estimate in the assessment of recent population changes, as the heterozygosity excesses ($H_e > H_{eq}$) may indicate a historical bottleneck event (Cornuet and Luikart, 1997). Despite the standardised differences test was not performed here because less than 20 loci were investigated, two other tests confirmed the significance of this parameter. In domesticated strains the observed heterozygosity (H_o) sometimes exceeds expected heterozygosity (H_e). This tendency was observed in Chinook salmon stocks (Kim et al., 2004) and was also detected in the three European paddlefish stocks examined, especially in that from Gorny Tykich. This excess may be the consequence of the use of a non-random subset of the broodstock as males would also tend to produce different allele frequencies in the male and female parents each generation, a condition that could account for the observed slight excess of H_o compared with H_e in the domesticated strains (Luikart and Cornuet, 1999). In general, both observed and expected heterozygosity of European stocks were similar to those in the Mississippi population (Heist et al., 2002) (Table 2), suggesting that future Polish aquaculture of paddlefish could be based on individuals reared in Pogorze and Wasosze.

The genetic differences among the three stocks cover differences between Polish and Ukrainian stocks as well as within the Polish groups. The high genetic similarity of Polish stocks confirmed their common origin. The genetic distance at level of 0.007 detected between them was small (Balloux and Lugon-Moulin, 2002; Knapen et al., 2003). The larger genetic distances detected between Ukrainian and Polish stocks can be accounted to different origin of parental populations and/or a consequence of genetic drift that may have occurred during breeding practices. Studies performed by others (Knapen et al., 2003) suggest that the size of genetic distance between populations is proportional to their geographic isolation. Consequently, if in the future an enrichment of the genetic pool of Polish stocks is necessary, then the stock from Gorny Tykich could be seen as a valuable source of paddlefish eggs or sperm for this procedure.

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