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RESEARCH ARTICLE

Genetic diversity of two lake minnow, *Eupallasella percnurus* (Pall.), populations based on microsatellite DNA polymorphism

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Abstract. Two populations of lake minnow, *Eupallasella percnurus* (Pall.), from Bledzewo and Kowalicha were investigated. The Bledzewo population has existed for many decades, while Kowalicha was established between 2004 and 2006 by translocations of cultured juveniles. The genetic variation and genetic distance between these populations was evaluated. The size of the genetic bottleneck/founder effect that affected genetic variation was also investigated. In most of the examined microsatellite loci, only 2-3 alleles/population were detected. Heterozygosity across the investigated loci was low with ranges of *Ho*: 0.24-0.45 and *He*: 0.24-0.50. Both populations remained at Hardy-Weinberg equilibrium. The *M* value was low, and suggested a reduction in the genetic variation because of the founder/bottleneck effect. The genetic distance between populations was high (FST 0.23, $\delta\mu^2$ 2.96). High genetic differences existing between the investigated *E. percnurus* populations and possibly other ones suggest that the inter-population translocations of this species should be preceded by an evaluation of the genetic differences existing between source and destination populations.

Keywords: *Eupallasella percnurus*, genetic distance, lake minnow, microsatellite DNA

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Introduction

The lake minnow, *Eupallasella percnurus* (Pall.), is listed in the red book as an endangered species facing extinction in Poland. It is protected under the Natura 2000 Network (Wolnicki et al. 2008). The conservation of this species is promoted by applying several techniques such as the translocation of wild fish or the hatchery-produced juveniles (Wolnicki et al. 2008). These techniques are accompanied by efforts focused on the conservation of the biodiversity specific for *E. percnurus* populations present in Poland. The successful conservation and management of *E. percnurus* populations requires an assessment of the level of genetic variability specific for its populations and an assessment of the observed genetic differences between the populations.

Microsatellite DNA belongs to the class of short tandem repeats of DNA fragments (STR), and differences in these repeats can be applied in the detection and assessment of genetic differences within and between populations. The assessment of genetic variations based on the polymorphism of the microsatellites has been successfully applied in the management of resources of many endangered fish species (Zhao et al. 1996, O'Connell and Wright 1997). It has also proved useful in evaluating the results of enhancing natural populations with hatchery-produced juveniles (Wąs and Wenne 2002, 2003). The current studies are the first attempt to characterize the genetic properties of *E. percnurus*

populations as well as to evaluate the influence of population size changes on their genetic variation using this molecular marker. The aims of the present study were (1) to establish a set of microsatellite markers useful for assessing genetic variation in *E. percnurus* populations, and (2) to assess within-population and inter-population genetic variation in two *E. percnurus* populations, and to evaluate the size of bottleneck and founder effects that might have occurred in the populations' histories, and finally (3) to compare and evaluate genetic variation between a newly-established population (Kowalicha) and a long-standing population (Bledzewo).

Materials and methods

Fish samples

Material was derived from two small water bodies (Bledzewo and Kowalicha), with are inhabited by *E. percnurus* populations. The populations are about 110 km apart, which is sufficient to isolate them from each other. The Bledzewo population has existed for many years, the Kowalicha was established in 2004-2006, through translocations of cultivated juvenile individuals. The materials used for these studies were fragments of fin tissue (about 25 mm^2) taken from 50 individuals from the Kowalicha and 56 fish from the Bledzewo populations. The tissue was preserved by drying.

DNA extraction

Genomic DNA was extracted and purified from the fin tissues using a Sherlock AX DNA Extraction and Purification Kit. The extraction procedure was performed following the manufacturer's recommendations (A&A Biotechnology, Poland). DNA samples were stored at a temperature of 20°C. The integrity of the DNA samples was visually inspected after their electrophoresis in 1.5% agarose gel stained with etidium bromide. All agarose gels were photographed using a gel imaging system and the pictures were computer recorded. Samples of the DNA yields were quantified by spectrophotometric analysis, and only samples containing more than 30 pg μl^{-1} of double stranded DNA were qualified for the PCR stage.

PCR amplification

The primer sequences regarded as universal for cyprinid fish were tested for amplification of microsatellite loci from *E. percnurus* DNA (Dimsoski et al. 2000, Holmen et al. 2005). Out of twenty sets of primers tested, only six were found to be usable as markers of genetic variation of *E. percnurus*. These were the sequences designed for zebra fish, *Danio rerio*

Table 1
Names of analyzed microsatellite loci, sequences of their primer sets, and repeat motifs as well as annealing temperatures

Locus name	Repeat motif	Primers sequence	Annealing temperature (°C)
		F:AGCTACTGAAACCCTTTGGC	
Z10362	$(CA)_N$	R:AGCTACTGAAACCCTTTGGC	54
		F:ACATCCACACCGTCTGTCAA	
Z9878	$(CA)_n$	R:CACGTCATCAAGCAGAGGAA	55
		F:AGGTTTCAGAGCCCTCATCA	
Z13419	(TG)n, $(TC)_N$	R:TGGGCTTCAGAGTTCACATG	56
		F:GGACAGTGAGGGACGCAGAC	
Ca3	$(TAGA)_n$	R:TCTAGCCCCCAAATTTTACGG	56
		F:CGGTATCGGTGCATCCCTAAA	
Ca4	$(AC)_N$	R:AACAGCGCGAGCGTCATTC	56
		F:GTGAAGCATGGCATAGCACA	
Ca12	$(TAGA)_n$, $(CAGA)_n$	R:CAGGAAAGTGCCAGCATACAC	57

(Hamilton) (*Z9878, Z10362,* and *Z13419)* and central stoneroller, *Campostoma anomalum* (Raf.) microsatellites (*Ca3, Ca4,* and *Ca12).*

The primers sequences, repeat motifs, annealing temperatures, and amplified product size, are summarized in Table 1. The forward primer of each primer pair was 5' -end labelled with phosphoramidite dyes (6FAM, VIC, NED). The labelled primers and PCR products were protected against sunlight, following the method of Kaczmarczyk et al. (2007). The PCR product was verified by agarose gel electrophoresis and stained using ethidium bromide.

Genotyping

The lengths of amplified DNA fragments were determined using an Applied Biosystems 3130 Genetic Analyser against GS400LIZ size standards. Amplified fragments were arranged in two sets. Set No.1 consisted of amplicons *Z9878, Z10362* and *Z13419*; set No. 2 consisted of amplicons *Ca3, Ca4* and *Ca12*. Within these sets, the products of each individual microsatellite amplification were marked with different attached phosphoramide labels, thus enabling their separation in a multiplex mode. Fragment size and allele determination was performed using GeneMapper 3.0 software (Applied Biosystems), following the manufacturer's recommendations.

Statistical analyses

Genetic diversity was measured using the average number of alleles, allelic frequency, observed heterozygosity (*Ho*), and expected heterozygosity (*He*) (Nei 1987). The number of alleles observed per locus, allele frequency, the number of private alleles, allelic range, and allelic richness were computed with MSA software (Dieringer and Schlötterer, 2003). The tetrasomic locus *Ca4* was divided into two isoloci: *Ca4A* and *Ca4B* in order to accommodate these genotypes to the requirements of MSA and Arlequin software. An observed heterozygosity (*Ho*) 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 H-W equilibrium. The test was performed separately for each locus in both populations (Guo and Thompson, 1992). The number of steps in the Markov chain equaled 1,000,000 and the number of dememorisation steps equaled 100,000. Expected heterozygosity (*He*) and observed heterozygosity (*Ho*) were calculated using Arlequin 3.0 software (Excoffier et al. 2005). The likely occurrence of bottleneck or the founder effect, and their influence on within-population genetic variability was based on the Garza-Williamson index (Garza and Williamson 2001) including Excoffier's adjustment (Excoffier et al. 2005). Genetic divergence between populations was analysed using two different methods: inbreeding coefficient (F_{ST}) (Wright 1951) and variation of average allelic size ($\delta\mu^2$) (Goldstein et al. 1995). F_{ST} values and their statistical significance were given by applying Arlequin 3.0 (Excoffier et al. 2005). The size of genetic distance based on F_{ST} values and their ranges were interpreted according to Wright (1978), and Baloux and Lugon-Moulin (2002). The estimation of genetic divergence was also performed using the sample-size independent method $\delta\mu^2$, and their values were given by applying MSA software (Dieringer and Schlötterer 2003). The genetic divergence between the investigated populations as well as differences observed at within--population level were also analysed using the assignment test. The log likelihood of occurrence of given fish genotypes in their source or alternative population was calculated using Arlequin 3.0 software. The obtained likelihood values were presented as points in a system of coordinates following Waser and Strobeck (1998). Genotypes (at occurrence probability) higher in Bledzewo than Kowalicha were located above the diagonal and genotypes (at occurrence probability) higher in Kowalicha than Bledzewo were located below diagonal.

Results

Out of six studied microsatellites, five (*Z9878, Z10362, Z13419, Ca3, Ca12*) were successfully amplified from all samples. DNA sequences of locus

Ca4 were not amplified from nine samples belonging to the Bledzewo group. Six investigated loci were polymorphic, but at five of them only two or three alleles were detected (Table 2). The Kowalicha and Bledzewo samples differed in number of alleles detected at a given locus as well as in the overall number of alleles identified across all investigated loci.

In both populations the investigated microsatellites differed in their values on the Garza-Williamson index. The lowest *M* was recorded at loci *Z9878*, *Z10362*, and *Z13419* in both populations. A low *M* value was also detected at locus *Ca12* in the Bledzewo population. The mean value of the Garza-Williamson index was similar for both populations (Table 3).

Table 2
Number of alleles and their size ranges detected within investigated loci

Locus	Allelic size range (bp)	Number of alleles	
		Bledzewo	Kowalicha
Z10362	109-129	2	3
Z9878	105-121	2	2
Z13419	192-206	2	2
Ca3	199-219	2	3
Ca4	78-90	3	3
Ca12	218-258	5	9
Number of alleles across populations		16	22
Mean number of alleles in locus		2.7	3.7

The observed heterozygosity (*Ho*) calculated as a mean for six loci was higher in Kowalicha than Bledzewo (Table 4). In both the investigated populations the mean observed heterozygosity (*Ho*) was close to the value expected under H-W equilibrium (*He*). Significant departures from H-W equilibrium were detected at two loci in the Bledzewo and one in the Kowalicha populations (Table 4).

Table 4
Comparison of Garza-Williamson index (M values) in the Bledzewo and Kowalicha populations

Locus	Bledzewo	Kowalicha	Mean
Z10362	0.33	0.27	0.30
Z9878	0.22	0.22	0.22
Z13419	0.22	0.22	0.22
Ca3	1.00	0.50	0.75
Ca4	0.50	0.47	0.48
Ca12	0.28	0.41	0.34
Average	0.43	0.35	0.39

Genetic divergence between the investigated populations, calculated as F_{ST} value, was 0.23 and was statistically significant at P<0.001. The size of genetic distances was confirmed using the $\delta\mu^2$ method, and its value was 2.96. Most analysed fish genotypes have a higher occurrence probability in their home population than in a foreign one. Only one genotype from Bledzewo was misassigned, with higher probability of occurrence in the Kowalicha population. Points showing the probability of

Table 3
Comparison of observed (H_o) and expected (H_e) heterozygosity as well as deviations from the Hardy-Weinberg equilibrium in the Bledzewo and Kowalicha populations

Locus	Bledzewo			Kowalicha		
	H_o	H_e	P value	H_o	H_e	P value
Z10362	0.05	0.05	1.0000	0.54	0.51	0.8816
Z9878	0.66	0.45	0.0001*	0.82	0.49	0.0000*
Z13419	0.05	0.05	1.0000	0.02	0.02	1.0000
Ca3	0.04	0.08	0.0643	0.72	0.67	0.5704
Ca4A	0.04	0.04	1.0000	0.32	0.45	0.0560
Ca4B	0.36	0.44	0.2176	0.18	0.20	0.4586
Ca12	0.46	0.56	0.0001*	0.90	0.84	0.6646
Average	0.24	0.24		0.50	0.45	

Deviations statistically significant at P< 0.001

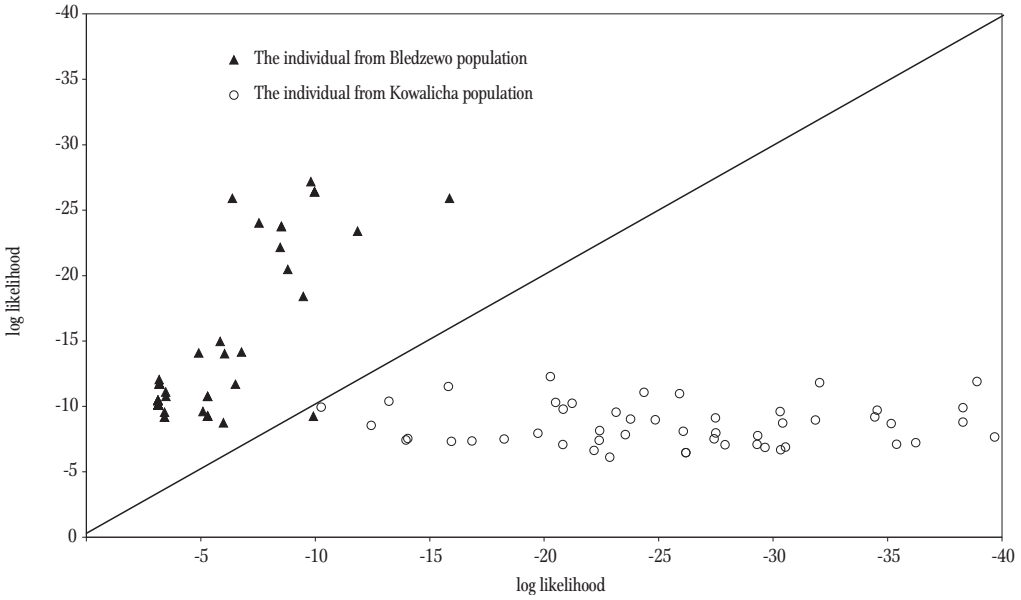

Figure 1. Result of the genetic assignment test performed between the Bledzewo and Kowalicha populations.

occurrence of fish genotypes were concentrated mainly in two groups that were distanced from the diagonal (Fig. 1).

Discussion

A tool to characterize the genetic properties of *E. percnurus* populations was developed by establishing, for this purpose, a set of six microsatellite markers. This set of six markers was taken from species other than *E. percnurus*. A further four microsatellite markers within *E. percnurus* genome that were identified in this project could be added to the tool in future work. It was determined that the Bledzewo and Kowalicha populations are in H-W equilibrium. The genetic differences existing within Bledzewo and Kowalicha were small when described as the number of alleles detected within and across the investigated populations and including the populations' heterozygosity. The values of *Ho* as well as *He* at loci *Ca3*, *Ca4*, and *Ca12* specific for the Bledzewo and Kowalicha populations were lower than those reported for a *C. anomalum* population (*Ho* 0.24-0.90; *He* 0.80-0.90) (Dimsoski et al. 2000). Although genetic variation in both investigated populations was low, they differed from the values of genetic variation indicators. Although the level of genetic variation within both populations was low, the newly established Kowalicha population showed a heterozygosity level and a number of alleles that was higher than those detected within the older Bledzewo population. It is likely that the detected higher level of genetic variation within the Kowalicha population might be a consequence of using numerous groups of genetically-diverse fish for establishing this population, which portends well for its future.

The values of the Garza-Williamson Index (*M* value) can be compared with the results of studies on various species of finfish, reptiles, and mammals. The *M* values specific for Bledzewo and Kowalicha populations covered a range of 0.29 to 0.43 for this indicator, which is specific for remnant populations (for example the Galapagos iguana) (Garza and Williamson 2001, Tzika et al. 2008), and this indicates that both investigated populations suffered from bottleneck (Bledzewo) or founder effects (Kowalicha). These studies showed that the Bledzewo and Kowalicha populations are genetically different. The results of the genetic assignment test showed that the vast majority of detected fish genotypes were population specific and the likelihood of their occurrence in

an appropriate parental population was much higher than an alternative one. The size of genetic distances calculated using the $\delta\mu^2$ method were comparable to the results described by King et al. (2001), which were specific for geographically distanced or isolated populations (Paetkau et al. 1997, Rowe et al. 1998, Bjørnstad et al. 2003). Moreover, the F_{ST} value calculated between Bledzewo and Kowalicha is not only confirmation of the results of the genetic distance assessment using the $\delta\mu^2$ method, but also suggests that this high genetic differentiation was a consequence of their larger genetic drift, probably increased by founder and bottleneck effects as well as possible inbreeding events (Balloux and Lugon-Moulin 2002).

It is likely that sustainable genetic differences may also exist between other *E. percnurus* populations occurred in Poland. These, and possibly other genetic differences between given *E. percnurus* populations, should be taken into consideration in conservation programs for this species, especially those that involve fish translocations, or enhancement with hatchery-produced juveniles. If such work is planned, it should be preceded with an assessment of the genetic distance between fish inhabiting the source and target locations.

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Streszczenie

Ocena polimorfizmu genetycznego dwóch populacji strzebli błotnej *Eupallasella percnurus* (Pall.) na podstawie sekwencji mikrosatelitarnego DNA

Przebadano populacje strzebli błotnej zamieszkujące zbiorniki Bledzewo i Kowalicha. Populacja z Bledzewa istnieje od wielu dziesięcioleci, zaś populacja z Kowalichy została utworzona pomiędzy 2004 i 2006 rokiem przez translokację młodocianych osobników z warunków hodowlanych. Oszacowano zmienność genetyczną w obrębie badanych populacji oraz dystans genetyczny istniejący pomiędzy nimi. Przeprowadzono szacowanie skali efektu genetycznego wąskiego gardła, jak i efektu założyciela, jako czynnika oddziaływującego na poziom zróżnicowania genetycznego badanych populacji. W obrębie większości przebadanych loci mikrosatelitarnych wykryto zaledwie 2-3 allele w każdej populacji. Średnia heterozygotyczność, określona dla przebadanych loci, była niska i zawierała się w przedziale (*Ho* 0,24-0,45 i *He* 0,24-0,50). W obu populacjach nie wykryto odstępstw od równowagi Hardy'ego-Weingerga. Wartości wskaźnika *M* były niskie i wskazywały na redukcję zmienności genetycznej obu populacji jako konsekwencje efektu genetycznego wąskiego gardła lub założyciela. Dystans genetyczny pomiędzy badanymi populacjami był znaczny (F_{ST} 0,23, $\delta\mu^2$ 2,96). Znaczne różnice genetyczne istniejące pomiędzy badanymi i prawdopodobnie innymi populacjami strzebli błotnej sugerują, że międzypopulacyjne translokacje tego gatunku powinny być poprzedzane określeniem różnic genetycznych pomiędzy populacją źródłową i populacją docelową.