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Short Communication

Cross-species amplification of selected zebrafish, central stoneroller, and finescale dace microsatellites in lake minnow populations

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ABSTRACT. Fifteen sets of PCR primers designed for the amplification of microsatellite loci from *Danio rerio* and *Phoxinus neogaeus Campostoma* DNA that have been proven applicable for molecular studies of several species of cyprinids were tested for amplification of microsatellites from lake minnow DNA. The samples were taken from 298 fish inhabiting 6 distinct populations located in Poland, and DNA was extracted from dried fin clips. There were 6 sets of primer loci that were identified as useful for amplification of microsatellites from lake minnow DNA, which were polymorphic and could be applied in population genetics of this species. Three other sets of primers provided PCR products with a considerable number of stutter bands obscuring the identity of true microsatellite alleles. The amplification of lake minnow microsatellites using the 6 remaining primer sets was unsuccessful.

Key words: Cross-species amplification; Lake minnows; Microsatellites

INTRODUCTION

The lake minnow (*Eupallasella percnurus*) is a small cyprinid fish listed in the Red Book of endangered species, and is facing extinction in Poland. It is protected under the Natura 2000 program (Radtke et al., 2011). The conservation of this species is promoted by several methods (Wolnicki et al., 2011), including the transfer of fish or enhancement of existing populations with hatchery-produced juveniles. These techniques have been accompanied by efforts focused on conservation of the biodiversity specific for lake minnow populations present in Poland. The successful conservation and management of lake minnow populations requires an assessment of the level of genetic variability specific to lake minnow populations and an assessment of the observed genetic differences between the populations. The assessment of genetic variations can be based on polymorphism of microsatellite loci, also known as simple short repeats (SSRs). This technique has been successfully applied in the management of resources of many endangered fish species (Zhao et al., 1996; O'Connell and Wright, 1997; Spruell et al., 2003) and can be applied for lake minnow.

Microsatellites are known as markers specific for given species or groups of species, enabling their identification (Fopp-Bayat, 2002), but several studies have shown that flanking sequences of microsatellites might be conserved well enough through evolution to serve as primer-annealing sites for closely related species (Primmer et al., 1996; Tong et al., 2002), therefore enabling cross-species amplification. The taxonomy and genetics of the lake minnow are unclear and mostly unknown. In available publications there are no primer sequences designed for amplification of lake minnow microsatellites or reports describing the successful amplification through application of sets of primers taken from other cyprinid species. In this study, we attempted to identify the sets of primers that could be useful in the amplification of microsatellites from lake minnow DNA by testing a group of primer sets that are known to be common for several cyprinids.

MATERIAL AND METHODS

Material

Fin clips (approximately 25 mm^2 in size) were taken from 298 fish inhabiting 6 lake minnow populations in various regions of Poland [Bledzewo, Kowalicha, Siedliszcze, Sosniak, Mikolajki Pomorskie (Mikolajki Pom.), and Podpakule]. Approximately 50 fish from each population were investigated.

DNA extraction

Genomic DNA was extracted and purified from the fin tissues using a Sherlock AX DNA Extraction and Purification Kit and Genomic Mini AX Tissue SPIN DNA Extraction and Purification Kit (A&A Biotechnology, Poland). The extraction procedure was performed following manufacturer recommendations. DNA samples were stored at a temperature of -20°C. The integrity of the DNA samples was visually inspected after electrophoresis on 1.5% agarose gel stained with ethidium bromide. All gels were photographed using a gel imaging system and the pictures were digitally recorded. Samples of the DNA yields were quantified by

spectrophotometric analysis. Only samples containing more than 30 µg/mL double-stranded DNA were qualified for the PCR stage.

Selection of the primer sets

The primer sets *Z8356*, *Z9068*, *Z9523*, *Z9878*, *Z10363*, *Z11841*, *Z13419*, *Z14008* were designed for zebrafish (*Danio rerio*) (Shimoda et al., 1999) and their sequences were taken from http://www.ncbi.nlm.nih.gov. The primer sets *CA3*, *CA4*, *CA5*, *CA11*, and *CA12* were designed for central stoneroller (*Campostoma anomalum*) and their sequences were taken from Dimsoski et al. (2000). The primer set *Phox23* was designed for finescale dace (*Phoxinus neogaeus*) and its sequence was taken from GenBank (accession No. FJ807698). The sequence of primer *Phox30* was the same as in set *Phox23*, but both oligonucleotides were shortened at 3' at one nucleotide. From among the sets of primers tested by Holmen et al. (2005) in crosspiece amplification of the microsatellites from DNA of various cyprinid fish, we selected those which provided polymorphic, high-quality PCR product across a wide range of investigated species, including *Phoxinus phoxinus*, the other minnow species inhabiting streams in Poland. The investigated primer sets and their sequences are given in Table 1.

Table 1. List of primer sequences taken from zebra fish (*Z*), central stoneroller (*Ca*), and finescale dace (*Phox*) tested for amplification of microsatellites from lake minnow DNA.

Locus	Forward primer (5'-3')	Reverse primer (5'-3')
Z8356	AACAGGGGGGCAAATAATTC	GTTCCACACAATCGACATGTG
Z9068	TGCGCTTCATCCTCTACTAAA	GTGAGCTGCTGCCCTGTG
Z9523	AAACCTGTAATGGGACAGCC	ACTGCTTTGGCAGCTGTGC
Z9878	ACATCCACACCGTCTGTCAA	CACGTCATCAAGCAGAGGAA
Z10362	GGTGACCTCATGGAAGCATT	AGCTACTGAAACCCTTTGGC
Z11841	TTCTGTCACCTGCAGTTTGC	GGCACGTAAAAGCGGTTTAA
Z13419	AGGTTTCAGAGCCCTCATCA	CATGTGAACTCTGAAGCCCA
Z14008	CTTCAGCCGTGGAGAAAGAC	CTGAACATTCAAGCGTTCGA
Ca3	GGACAGTGAGGGACGCAGAC	TCTAGCCCCCAAATTTTACGG
Ca4	CGGTATCGGTGCATCCCTAAA	AACAGCGCGAGCGTCATTC
Ca5	TTGAGTGGATGGTGCTTGTA	GCATTGCCAAAAGTTACCTAA
Ca11	TCCCTCACTGTGCCCTACA	GGCGTAGCAATCATTATACCT
Ca12	GTGAAGCATGGCATAGCACA	CAGGAAAGTGCCAGCATACAC
Phox23	GGCTAGACAAGTGTTCCCGC	GGAAACAACGGATTTCACATCA
Phox30	GGCTAGACAAGTGTTCCCG	GAAACAACGGATTTCACATCA

PCR amplification

Samples of 20-100 ng genomic DNA extracted from 4 randomly selected fish belonging to Bledzewo populations and 4 from Kowalicha were used as templates in the PCR mixtures. Each PCR composition contained a 3 µL TAQ buffer, 430 µmol dNTP, 12 pmol of each primer, 0.3 U Run *Taq* polymerase (A&A Biotechnology), and was filled to 30 µL with nuclease-free water. A PCR protocol was started with an initial denaturation at 94°C for 3 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing for 30-45 s and elongation at 64°C for 30-45 s, and ended by a final elongation at 64°C for 35 min. The annealing temperature was determined empirically for each locus based on a series of tests carried out at temperatures between 50° and 67°C performed in Mastercycler Gradient thermal cyclers (Eppendorf, Germany) with a gradient of 1°C. The highest temperature that allowed ample PCR product while preventing stutter bands and other

unspecific fragments as much as possible was recorded as optimal for a given set of primers. The PCR product was verified by agarose gel electrophoresis and stained using ethidium bromide.

The primer sets providing successful amplification were reordered. In the new pairs of primers, the forward oligonucleotides were 5'-end labeled with phosphoramidite dyes (6-FAM, VIC, NED, or PET) to enable genotyping of the PCR product using the Applied Biosystems 3130 Genetic Analyzer. The amplifications performed during the previous optimization stage were repeated using labeled primer sets and the electrophoretic profiles of obtained fragments as well as possible stutter bands were inspected during automatic capillary electrophoresis and analyzed by the Genemapper 3.0 software. The primer sets providing the PCR product with easily identifiable alleles of microsatellites were selected for amplification of the microsatellites obtained from the remaining DNA samples and applied in population studies.

Amplification quality and genotyping

The lengths of amplified DNA fragments were determined using an Applied Biosystems 3130 Genetic Analyzer against GS500LIZ size standards. The quality of the obtained PCR product was assessed using the following scale: U, U_{ST}, ST, and NA. Only primers enabling clear amplification of microsatellites from DNA samples derived from all investigated fish were marked as useful (U). Samples with minor stutter bands surrounding microsatellite fragments were marked as (U_{ST}). Both U and U_{ST} amplification quality enabled clear identification of true microsatellite alleles among the obtained bands. Samples with considerable stutter and multiple bands hindering the identification of true microsatellite bands were marked as stutter (ST). Samples not amplifying or amplifying from few samples were marked as "no amplify" (NA). Due to the possible confusion between true microsatellite alleles and other fragments, bands having no trace of a weaker band 1 repeat below were included in the category NA, even when only 1 or 2 bands were observed. Due to possible confusion with primer/dimer fragments, the bands shorter than 70 bp were scored as NA even when 1 difference in their lengths between samples was detected and traces of a weaker band 1 repeat below were observed.

Statistical analysis

To successfully amplify microsatellites (amplification quality U and U_{ST}), the number of alleles observed per locus and in populations was computed by the MSA software (Dieringer and Schlötterer, 2003). Tetrasomic locus *Ca4* was divided into 2 independent inherited isoloci to accommodate it to the requirements of microsatellite alleles and the Arlequin software.

The exact Hardy-Weinberg (HW) test (Guo and Thompson, 1992) was used to test deviations from HW equilibrium. The test was performed separately for each locus in all populations (Guo and Thompson, 1992). The number of steps in the Markov chain equaled 1,000,000 and the number of dememorization steps equaled 100,000 and was computed by the Arlequin 3.0 software (Excoffier et al., 2005).

RESULTS

Of the total 15 primer pairs tested, 6 amplified successfully (amplification quality U and U_{ST}). The optimal annealing temperature for each of them is given in Table 2. The loci *Z9878*, *Z10363*, *Z13419*, *Ca4*, and *Ca12* were proven to be polymorphic in all populations, but locus

Ca3 was polymorphic in only 3 of 6 populations (Table 3). Deviations from the HW equilibrium were detected at locus *Z9878* in all populations and at *Ca12* in the Bledzewo population. The loci *Z9878*, *Z10363*, *Z13419*, *Ca3*, and *Ca12* were disomic, but *Ca4* was identified as tetrasomic. The amplification with 3 other primer sets, *Z14008*, *Phox23*, and *Phox30*, was not clear because of stutter bands. Locus *Z9523* was amplified successfully in fish from Bledzewo and Kowalicha, but it was scored as NA because the obtained PCR product was very short (between 60 and 75 bp), approximately that of primer/dimer fragments. The amplification of locus *Z8356* from Bledzewo and Kowalicha samples derived many random bands in the range of 112 and 241 bp and therefore this primer set was qualified as NA. Amplification of loci *Z9068*, *Z11841*, *Z11841*, *CA5*, and *CA11* failed NA because no PCR product was observed in the range of 70-400 bp.

Table 2. Suitability of 15 primer sets for amplifying the microsatellites from lake minnow DNA and optimal annealing temperature identified for amplification of the 6 microsatellites (amplification quality U and U_{ST}).

Locus	Optimal annealing temperature	Amplification quality
Z8356	-	NA
Z9068	-	NA
Z9523	-	NA
Z9878	55	U_{ST}
Z10362	54	U_{ST}
Z11841	-	NA
Z13419	56	U
Z14008	-	ST
Ca3	56	U
Ca4	56	U_{ST}
Ca5	-	NA
Ca11	-	NA
Ca12	57	U
Phox23	-	ST
Phox30	-	ST

U = useful; U_{ST} = useful with minor stutter; ST = major stutter/multiple bands; NA = no amplify.

Table 3. Number of alleles detected at loci *Z9878*, *Z10362*, *Z13149*, *Ca3*, *Ca4*, and *Ca12* in Bledzewo, Kowalicha, Siedliszcze, Sośniak, Mikołajki Pomorskie (Pom.), and Podpakule populations.

Locus	Bledzewo		Kowalicha		Siedliszcze		Sosniak		Mikolajki Pom.		Podpakule		No. of alleles across populations
	Size range	No. of alleles	Size range	No. of alleles	Size range	No. of alleles	Size range	No. of alleles	Size range	No. of alleles	Size range	No. of alleles	
Z9878	105-121	2*	105-121	2*	105-121	2*	105-121	2*	105-121	2*	105-121	2*	5
Z10362	109-119	2	109-129	3	103-109	2	109-119	3	111-119	2	103-119	3	5
Z13149	192-206	2	192-206	2	192-206	2	192-206	2	192-206	2	192-206	2	2
Ca3	199-203	2	199-219	3	199	1	199	1	199	1	191-199	2	3
Ca4	78-90	3	78-84	3	84-90	2	78-90	4	78-90	3	78-90	3	4
Ca12	218-254	5*	218-258	8	246-262	4	242-258	5	214-226	2	214-242	7	12
No. of alleles across loci		16		21		13		17		12		19	

*Deviation from Hardy-Weinberg equilibrium at $P < 0.0001$. Fragment size ranges are given in bp.

DISCUSSION

Of the 15 primer sets developed for 3 cyprinid species tested, only 5 sets of primers (*Z10363*, *Z13419*, *Ca3*, *Ca4*, and *Ca12*) can be established as useful in various studies on lake

minnow population genetics. These results confirmed the deviation from HW equilibrium at locus *Z9878* reported by Holmen et al. (2005) in various species of cyprinids. We found that this was common for all lake minnow populations investigated in this study, which could limit the application of this locus in population genetics. The loci *Z9878*, *Z10363*, *Z13419*, *Ca3*, *Ca4*, and *Ca12* proved to be amplifiable from DNA derived from various populations. The polymorphism of these microsatellites is relatively low, probably as a consequence of specific lake minnow biology, where considerable changes in population size are often observed.

Nine sets of primers were not suitable for amplification of the microsatellites from lake minnow DNA for the following reasons: stutter bands, randomly amplifying fragments, very short PCR product, or no amplification.

The genomes of cyprinid fish species differ in ploidy levels (Ohno et al., 1967). Some species are diploid while others are tetraploid and even hexaploid. The ploidy of lake minnows is unknown, but it seems that some tetrasomic or duplicated disomic regions including locus *Ca4* may be present.

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