Application of nine species-specific microsatellite loci to characterize three pike-perch (*Sander lucioperca*) populations from the Aral Sea basin in Uzbekistan

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ABSTRACT

The pike-perch is one of the main commercial fish species in Uzbekistan. Historically, it inhabited the Aral Sea and deltas of inflowing rivers (Amu-Darya and Syr-Darya). At the beginning of the 1960s it was introduced into water bodies of the middle Amu-Darya and Syr-Darya from the Ural River. The present study was aimed to evaluate the current genetic status of wild pike-perch populations from different water bodies using nine recently isolated species-specific microsatellite markers. The three examined Uzbek pike-perch populations expressed significantly higher average numbers of alleles per locus (5.56 to 14.22) than three year-classes of a German wild population (3.11 to 3.78) included for comparison. On the other hand, average observed and expected heterozygosities were not significantly different in both regions (0.743 and 0.760 in Uzbek pike-perch; 0.576 and 0.471 in German pike-perch). After sequential Bonferroni corrections all Uzbek populations were found to be in Hardy-Weinberg equilibrium. Genetic differentiation between all populations as estimated by F_{ST} values was statistically significant. Pike-perch from Amu-Darya River delta and Tudakul reservoir were less differentiated from each other probably due to gene flow between them (both water bodies are connected) whereas the more distinct Syr-Darya River population showed indication for introgression with non-native individuals stocked in that region. Since stocking is widely practiced to satisfy the growing demand of pike-perch fisheries in Uzbekistan, fish seed for such activities should be obtained from local, genetically undisturbed populations such as the Amu-Darya River delta population.

INTRODUCTION

Pike-perch, *Sander lucioperca* (L.), is a widely distributed species, whose native distribution extends from the Elbe River to Lake Onega in the north and from the Maritsa River (Meriç/Évros) to the Aral Sea in the south. In the mid-20th century, numerous introductions expanded its geographical range to the east. Historically, pike-perch inhabited the Aral Sea and the Amu-Darya and Syr-Darya River deltas. A wide irrigation network in the Aral Sea basin allowed the pike-perch to expand its range. In parallel with that, in the beginning of the 1960s pike-perch from the Ural River was introduced into the region as a predator with a narrow throat to eliminate pest fishes when "fauna improving" and "biomelioration" were very popular. Such transplantations may have negatively affected the genetic integrity of local populations. At present, the pike-perch is widespread in plains throughout the basin.

The pike-perch is one of the main commercial species in Uzbekistan, where annual catches from the Aral Sea reached 12,000 tons in the 1970s, equivalent to 70% of total fish yield. Due to salinity tolerance (up to 13‰) pike-perch was one of the last commercial native fish species in the Aral Sea. Such a euryhaline species is very important for Uzbekistan, where most commercially fished water bodies are brackish-water due to saline soil and agriculture run-off. With the shrinking of the Aral Sea the fisheries importance of another large brackish water body, the manmade Aydar-Arnasai Lake System (AALS; Figure 1), has increased – with its pike-perch constituting up to 47% of the total fish yield in 1990s. However, since then exploitation has depleted most of the valuable fish stocks, foremost predators such as the pike-perch, leading to the necessity of stocking.

Knowledge on the genetic structure of wild populations is required for their successful conservation and supportive stocking but so far completely missing for pike-perch from Central Asia. Thus, the present study was aimed to characterize pike-perch populations from different water bodies of the Aral Sea basin using for the first time nine species-specific microsatellite markers recently published by Kohlmann and Kersten (2008).

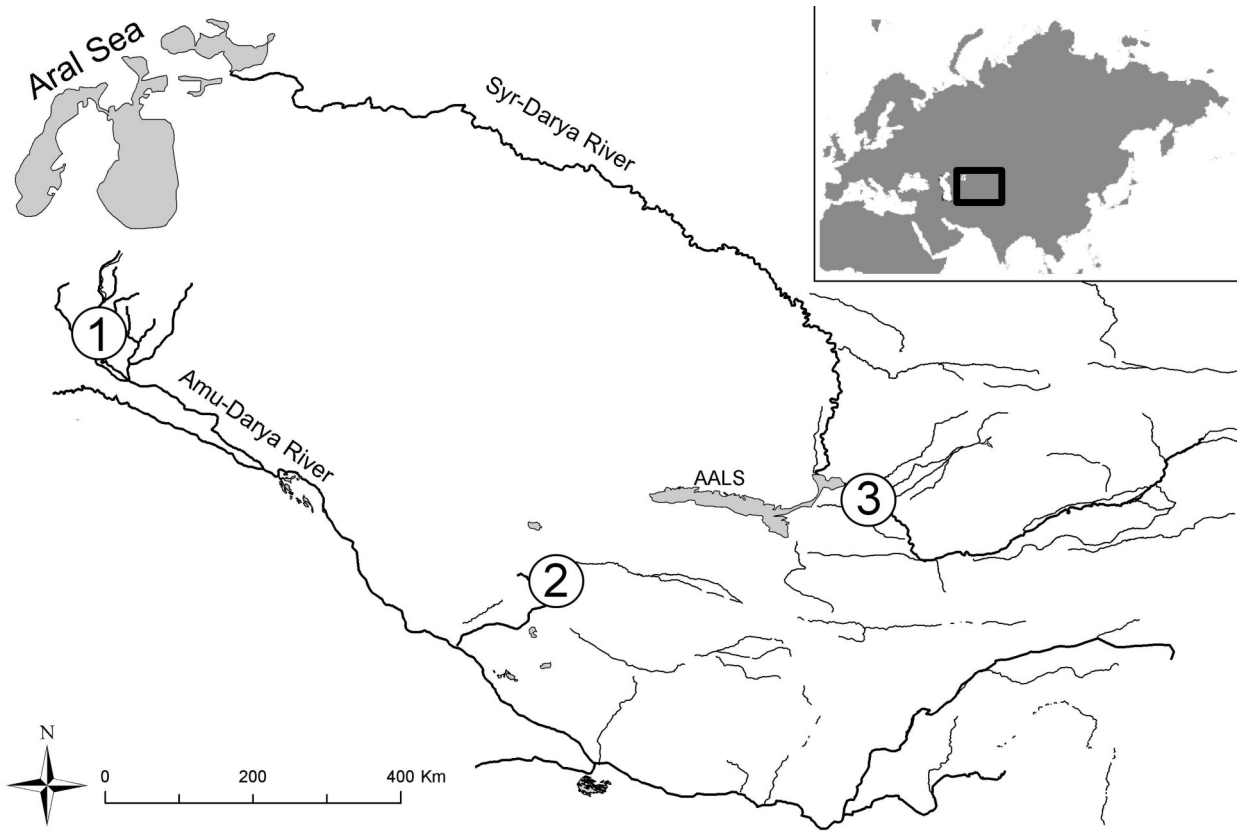

Figure 1. Map showing the origin of Uzbek pike-perch populations studied: 1 – Amu-Darya River delta (Amud), 2 – Tudakul reservoir (Tudk), 3 – Syr-Darya River (Syrd).

MATERIAL AND METHODS

Pike-perch fin clips were collected from three populations (Figure 1): Amu-Darya River delta (42°52' N, 59°18' E; sample code – Amud; 49 individuals), Tudakul reservoir (39°54' N, 64°51' E; sample code – Tudk; 50 individuals) and middle reaches of the Syr-Darya River (40°56' N, 68°40' E; sample code – Syrd; 47 individuals). For comparative reasons data on three year-classes (2005-2007; 50 individuals each; sample code – Ly05, Ly06, Ly07) of wild pike-perch from Germany were included. These fish hatched from eggs collected at natural spawning sites in lake Großer Lychensee (53°12' N, 13°17' E) and were raised in a closed recirculating system at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin until fin clip sampling at an age of approximately one year.

Genomic DNA was isolated from the fin clips using the peqGOLD Tissue DNA Mini Kit (Peqlab Biotechnologie). The nine microsatellite loci *MSL-1* to *MSL-9* described by Kohlmann and Kersten (2008) were PCR-amplified on a Mastercycler gradient apparatus (Eppendorf). Primer sequences taken from the original publication can be found under GenBank accession numbers EF694018-EF694026 and in Table 1. Each reaction mix was composed of 1.5μl of 10xPCR buffer with $(NH_4)_2SO_4$ (MBI-Fermentas), 1.2μl of 25mM $MgCl_2$, 1.2μl of 1.25mM dNTPs, 0.3μl of each primer (10pmol·μl^{-1}), 3μl genomic DNA, 0.1μl of *Taq* DNA-polymerase (5units·μl^{-1}; MBI-Fermentas) and sterile water up to a final volume of 15μl. For cycling, a "touch down" thermal profile was used: initial denaturation at 94°C for 3min, 10 cycles of 40s at 94°C, 40s at 60 to 50°C (1°C decrease per cycle), 1min at 72°C and 25 cycles of 40s at 94°C, 40s at 50°C, 1min at 72°C and a final extension for 10min at 72°C. All nine loci were amplified separately, but to allow multiplex fragment analyses on a CEQ 8000 (Beckman Coulter) the forward primer of each pair was labeled with one of three WellRed fluorescence dyes (Sigma-Proligo).

Table 1. Primer sequences (F = forward; R = reverse), total number and number of private alleles, and size range of alleles observed at nine microsatellite loci in six pike-perch populations.

Locus	Primer sequences (5'>3')	Total number of alleles	Number of private alleles	Size range (bp) of alleles
MSL-1	F: TGTTTGTCAGCGTCAAGAGG R: TTCCGCTCCAACATATCACA	23	10	134-186
MSL-2	F: TTTTCACACCGTGCATGACT R: ACCCTCAGCCTCTGTGTACG	16	7	191-225
MSL-3	F: CCGGCATCCATACACCTTAC R: CACACCTGTGTCTGCCTAACA	12	3	234-268
MSL-4	F: TCAAGACCCCAGAACCAATC R: CAGACAGCTAAGAGAACAACAGG	11	3	180-204
MSL-5	F: CAATCGCTCTGAGGATGTCA R: AAGGGTGGGGAAATTATTCG	25	10	198-320
MSL-6	F: GTCGTCATCGTCAGCACAGT R: ACTACACGGGACGCTGGA	19	6	208-286
MSL-7	F: CACACAGCAGCATGTGACAA R: GGCACGGAGGTAGAATGGTA	5	0	254-274
MSL-8	F: AACACCTTCCTTCGTCCATC R: CGTGTTTGCCTCACACAAAG	12	3	207-231
MSL-9	F: GCATCACTTGCGTCACTTTC R: GCAGTCAGTGCTTGAAGTGG	23	9	210-264
Total		146	51	

Microsatellite genotypes were recorded using the CEQ 8000 Genetic Analysis System, Fragment Analysis module (Beckman Coulter), and examined with MICROCHECKER v.2.2.3 (van Oosterhout et al. 2004). The GENEPOP v.1.2 software package (Raymond and Rousset 1995) was used to calculate allele numbers, observed and expected heterozygosities and to test for deviations from Hardy-Weinberg expectations (probability test: estimation of exact *P*-values by the Markov chain method). The number of private alleles was recorded at each locus and for each population. Hierarchical partition of microsatellite genetic diversity (two geographical regions with three populations/samples each) was examined by analysis of molecular variance (AMOVA; Excoffier et al. 1992) using Arlequin v. 3.0 (Excoffier et al. 2005). In order to include all individuals and loci in this analysis, the allowed level of missing data had to be set at 0.08. Genetic differentiation between populations was evaluated by calculating pairwise estimates of F_{ST} values and testing their significance by bootstrapping analysis (1,000 replicates) using the FSTAT software (Goudet 2002). FSTAT was also used to compare average values of heterozygosities and F_{ST} between the two geographical regions: Uzbekistan and Germany. In all cases with multiple tests, significance levels were adjusted using the sequential Bonferroni correction (Rice 1989). In order to examine the genetic relationships among populations a matrix of pairwise D_A distances (Nei et al. 1983) was calculated with bootstrapping (1,000 replicates) using the MSA v.4.0 program (Dieringer and Schlötterer 2003). The D_A distance measure was chosen because it is independent of the mutation models (Nei 1987) and superior to other distance measures in correct tree topology construction using microsatellites (Takezaki and Nei 1996). The resulting set of 1,000 distance matrices was then taken to run the NEIGHBOR and CONSENSE modules of the PHYLIP software package (Felsenstein 1993) in order to construct a consensus neighbor-joining tree. The publication-ready tree was plotted using the MEGA program (Kumar et al. 2004). Finally, the interpopulational dispersal of individuals was assessed by an assignment test (GeneClass software; Cornuet et al. 1999) using the Likelihood and Bayesian method and algorithm.

RESULTS

All of the nine pike-perch specific microsatellite loci could be amplified successfully and were found to be polymorphic. In total, 146 alleles were recorded across loci with 51 of them being private alleles (Table 1). Thus, the overall mean number of alleles per locus was 16.22. The number of distinct alleles showed large variation among loci ranging from five (locus *MSL-7*) to 25 (locus *MSL-5*). Evaluation of genotypes with the MICROCHECKER software indicated a possible presence of null allele(s) at loci *MSL-8* and *MSL-9* in the Amu-Darya River delta population.

Table 2. Genetic variability of six pike-perch populations at nine microsatellite loci (n = sample size; A = total number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; P_{HW} = Hardy-Weinberg probability test: * $P < 0.05$, ** $P < 0.01$, n.s. = nonsignificant).

Locus	Parameter	Ly05	Ly06	Ly07	Amud	Syrd	Tudk
MSL-1	n	46	50	50	32	47	50
	A	6	8	5	13	19	5
	H_E	0.713	0.686	0.678	0.717	0.922	0.528
	H_O	0.913	0.760	0.940	0.719	0.915	0.440
	P_{HW}	**	n.s.	**	n.s.	n.s.	n.s.
MSL-2	n	48	50	50	39	47	50
	A	6	3	4	9	13	5
	H_E	0.407	0.493	0.625	0.722	0.869	0.633
	H_O	0.354	0.480	0.960	0.641	0.915	0.680
	P_{HW}	**	n.s.	**	n.s.	n.s.	n.s.
MSL-3	n	47	50	50	41	47	50
	A	2	3	3	9	9	5
	H_E	0.362	0.672	0.366	0.811	0.817	0.725
	H_O	0.468	0.700	0.420	0.805	0.787	0.700
	P_{HW}	*	*	**	n.s.	n.s.	n.s.
MSL-4	N	46	50	50	49	47	50
	A	3	3	4	7	9	4
	H_E	0.652	0.598	0.534	0.636	0.802	0.704
	H_O	0.543	0.580	1.000	0.510	0.851	0.800
	P_{HW}	**	*	**	n.s.	n.s.	n.s.
MSL-5	N	45	50	50	47	47	50
	A	3	3	3	12	22	8
	H_E	0.401	0.097	0.403	0.875	0.941	0.822
	H_O	0.489	0.080	0.540	0.787	0.936	0.840
	P_{HW}	n.s.	*	*	n.s.	**	n.s.
MSL-6	N	46	50	50	47	47	50
	A	4	4	2	10	18	7
	H_E	0.462	0.341	0.368	0.766	0.890	0.669
	H_O	0.478	0.400	0.480	0.787	0.894	0.740
	P_{HW}	n.s.	n.s.	*	n.s.	n.s.	n.s.
MSL-7	N	45	50	50	46	47	50
	A	2	3	2	4	5	4
	H_E	0.502	0.543	0.505	0.689	0.608	0.509
	H_O	0.644	0.660	0.940	0.630	0.660	0.540
	P_{HW}	n.s.	n.s.	**	n.s.	n.s.	n.s.
MSL-8	N	47	50	50	48	47	50
	A	3	1	2	8	12	5
	H_E	0.415	-	0.020	0.815	0.827	0.752
	H_O	0.511	-	0.020	0.667	0.809	0.720
	P_{HW}	n.s.	-	n.s.	*	n.s.	**
MSL-9	N	48	50	50	48	47	50
	A	5	6	3	11	21	7
	H_E	0.775	0.762	0.403	0.793	0.936	0.788
	H_O	0.938	0.740	0.520	0.625	0.894	0.820
	P_{HW}	**	**	n.s.	*	n.s.	n.s.
Number of private alleles		0	1	1	7	41	1
Mean number of alleles per locus		3.78	3.78	3.11	9.22	14.22	5.56
P_{HW} population		**	**	**	*	n.s.	n.s.

The three pike-perch populations from the Aral Sea basin expressed significantly higher average numbers of alleles per locus ranging from 5.56 (Tudakul reservoir) to 14.22 (Syr-Darya River) than the three year-classes of the German population (3.11 – 3.78 alleles per locus) (Table 2). Although average observed and expected heterozygosities were larger in Uzbek pike-perch (0.743 and 0.760) than in German pike-perch (0.576 and 0.471) the differences between the two geographical regions were statistically not significant (two sided *P*-values for both tests: 0.099). At the population level, the number of private alleles ranged from zero (Lychensee, year-class 2005) to 41 (Syr-Darya River). If populations and year-classes were grouped according to country of origin then the number of private alleles amounted up to 103 (Uzbekistan) and eight (Germany), respectively. Most of the microsatellite variability could be attributed to variation within populations (70.9%), whereas 18.6% were due to variation among the two geographical regions and only 10.5% were caused by variation among populations/year-classes within regions.

Hardy-Weinberg equilibrium tests revealed highly significant deviations ($P<0.01$) in all three year-classes of the German pike-perch population and a significant deviation ($P<0.05$) in the Amu-Darya River delta population (Table 2). The latter observation might be related to the possible presence of null allele(s) at loci *MSL-8* and *MSL-9* in this population (see above). However, after sequential Bonferroni corrections (initial *P*-value of 0.05 divided by nine tests = adjusted *P*-value of 0.0055) this deviation became nonsignificant and only the three year-classes of German pike-perch still expressed highly significant deviations from Hardy-Weinberg equilibrium.

The evaluation of genetic differentiation between populations by F_{ST} values revealed significant differences between all pairs of populations and year-classes even after sequential Bonferroni corrections (initial *P*-value of 0.05 divided by 15 possible tests = adjusted *P*-value of 0.0033) (Table 3). F_{ST} values between the three Uzbek populations were lower (0.040 – 0.099) than between the three year-classes of the German population (0.117 – 0.277). However, the difference between average F_{ST} values of Uzbek (0.069) and German pike-perch (0.215) was still not significant (two sided *P*-value of 0.099).

Table 3. F_{ST} values (below diagonal) and D_A genetic distances (above diagonal) between pairs of six pike-perch populations. All F_{ST} values are statistically significant ($P < 0.05$) after sequential Bonferroni corrections.

	Ly05	Ly06	Ly07	Amud	Syrd	Tudk
Ly05		0.128	0.280	0.592	0.556	0.642
Ly06	0.117		0.224	0.547	0.547	0.570
Ly07	0.277	0.237		0.618	0.595	0.621
Amud	0.265	0.277	0.326		0.214	0.122
Syrd	0.236	0.259	0.295	0.060		0.309
Tudk	0.318	0.313	0.342	0.040	0.099	

Table 4. Results of the GeneClass assignment test (self-classification) of pike-perch individuals based on nine microsatellite loci.

Original population	Total number of fish	Number of fish classified as:						Correctly classified (%)
		Ly05	Ly06	Ly07	Amud	Syrd	Tudk	
Ly05	50	49	1					98.0
Ly06	50		50					100.0
Ly07	50			50				100.0
Amud	49				37	1	11	75.5
Syrd	47					47		100.0
Tudk	50				2		48	96.0

The significant genetic differentiation at the population level was also reflected by a high accuracy of the assignment test: 94.93% or 281 individuals out of 296 examined could be correctly classified according to the population of their origin (Table 4). At the population level the percentage of correctly classified individuals ranged from as low as 75.5% (Amu-Darya River delta) to as high as 100% (Syr-Darya River, and year-classes 2006 and 2007 of the German population). Remarkable misclassification was only observed among the less differentiated populations of the Amu-Darya River delta and Tudakul reservoir, which gets water from the Amu-Darya River delta.

The D_A genetic distances displayed large variation among pairs of populations/year-classes ranging from 0.122 to 0.642 (Table 3). The distance values between the Uzbek populations (0.122 – 0.309) were similar to the values observed between the three year-classes of German pike-perch (0.128 – 0.280), whereas remarkably larger distances were found between the two geographical regions (0.547 – 0.642).

The neighbor-joining tree based on D_A genetic distances showed two major clades supported by a bootstrap value of 100% and consisting of the three Uzbek populations and the three year-classes of the German population, respectively (Figure 2). Within the Uzbek clade the populations of the Amu-Darya River delta and Tudakul reservoir formed a sub-cluster with 99.3% bootstrap support.

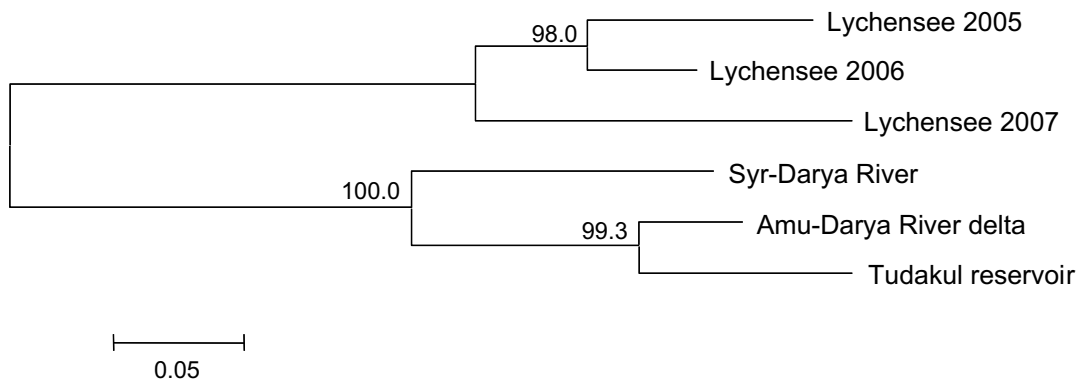

Figure 2. Neighbor-joining tree of six pike-perch populations/year-classes based on D_A genetic distances (Nei et al. 1983) and 1,000 bootstrap replicates.

DISCUSSION

The present study is the first one examining the recently isolated pike-perch specific microsatellite loci in a larger number of individuals originating from different populations and geographical regions. These nine new microsatellite loci demonstrated their suitability for population genetic studies.

The general microsatellite variability of pike-perch as found in the present study is comparable to that of anadromous fish species: DeWoody and Avise (2000) conducted a literature search in May 1999 covering 524 microsatellite loci examined in nearly 40,000 individuals of 78 fish species and non-piscine animals, and reported mean heterozygosities of $h = 0.68 \pm 0.22$ at the population level and $H = 0.68 \pm 0.12$ at the species level, and mean numbers of alleles per locus of $a = 11.3 \pm 10.1$ at the population level and $A = 10.8 \pm 7.2$ at the species level, respectively. The corresponding values for marine fish species were higher: $h = 0.79 \pm 0.26$, $H = 0.77 \pm 0.19$, $a = 20.6 \pm 11.8$ and $A = 19.9 \pm 6.6$ and those for freshwater fish species were lower: $h = 0.46 \pm 0.34$, $H = 0.54 \pm 0.25$, $a = 7.5 \pm 8.1$ and $A = 9.1 \pm 6.1$ (mean values ± standard deviations).

To our knowledge in only two other studies (Björklund et al. 2007; Poulet et al. 2009) microsatellite loci were used so far to examine the genetic variability and structure of pike-perch populations. Even if the same six loci analysed in both of these studies were originally isolated from the related North American species walleye, *Stizostedion vitreum* (Borer et al. 1999; Wirth et al. 1999) and yellow perch, *Perca flavescens* (Leclerc et al. 2000) a comparison of results might be useful. Our present data on genetic variability measured as average number of alleles per locus and heterozygosity correspond very well with the data reported by Poulet et al. (2009) on a small pike-perch population introduced into the Rhône delta, southern France: expected heterozygosities ranged from 0.647 to 0.744 and allelic richness from four to six alleles per locus. On the other hand, our variability measures are a little higher than those observed by Björklund et al. (2007) in 18 pike-perch populations from the

Fennoscandian region. They reported an average allelic richness of 3.38 for ten native populations sampled in the southern Fennoscandian region and 4.62 for eight populations sampled in the northern Fennoscandian region. Gene diversities (i.e. expected heterozygosities) were 0.55 in the north and 0.49 in the south, respectively.

All three year-classes of the German pike-perch population showed a highly significant deviation from Hardy-Weinberg expectations. This observation might be explained by non-random sampling of related individuals (egg samples originated from only a few spawning nests collected by a diver). The possibility of mixing/introgression can be excluded, since the pike-perch from Großer Lychensee represent a single, closed population. Non-random sampling is also supported by the fact that all three year-classes displayed significant differentiation from each other as estimated by pairwise F_{ST} values.

The strong divergence between Uzbek and German pike-perch may reflect different phylogeographic lineages. This assumption is also supported by the high number of private alleles (103) recorded in Uzbek pike-perch. To clarify this problem additional, more comprehensive studies are needed which should cover more pike-perch populations from Central Asia and Europe and include other genetic markers such as mitochondrial DNA.

The closer relationship between pike-perch populations from the Amu-Darya River delta and the Tudakul reservoir might be caused by gene flow between them. The Tudakul reservoir belongs to the Zeravshan River basin. Since 1960s, after implementation of the Amu-Bukhara irrigation channel, it is filled with water from the Amu-Darya River. Moreover, in 1970s circa 1,000 individuals of pike-perch were introduced into the Tudakul reservoir (Isaev and Karpova 1989). The origin of that stocking material, however, is unknown.

The high genetic variability (average number of alleles per locus = 14.22) as well as the relatively large distinctness (41 private alleles) of the Syr-Darya River pike-perch from the other Uzbek populations might be a result of the extensive stocking activities with non-native fish in this area: in 1960s pike-perch was introduced in reservoirs of the middle reaches of the Syr-Darya River (Amanov 1990). In 1963 it was introduced into the Qayraqqum Reservoir, Tajikistan (origin is unknown). In 1965 pike-perch from the Ural River delta and Biylikol' Lake was introduced into the Shardara Reservoir, Kazakhstan. The Syrd sample of the present study was collected from the Syr-Darya River between the Qayraqqum and Shardara reservoirs.

The pike-perch is the only exported fish in Uzbekistan – a small amount of it is exported to Turkey and Israel. Breeding and stocking can satisfy the growing demand, which has already brought about the depletion of pike-perch stocks. Thus, the main practical conclusion from the present study is that fish seed for such activities should be obtained from local populations of the Amu-Darya River delta since the gene pool of the Syr-Darya River pike-perch is obviously already introgressed by non-native fish.

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