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Detection of two major cytochrome *b* lineages in pike-perch, *Sander lucioperca*, and first data on their distribution in European populations

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ABSTRACT

Despite of the growing interest in pike-perch for aquaculture and its economic importance in fisheries, knowledge on the population structure and phylogeography of the species is still limited. We report the discovery of two major cytochrome *b* lineages and describe a simple method for their detection based on PCR amplification followed by restriction digestion with Alw26I. Screening of 708 individuals showed that haplotype A was fixed or dominating in Central and East European countries, whereas haplotype B was mainly found in several French populations and Tunisian pike-perch introduced from Europe. Sequencing the complete cytochrome *b* cds of 17 representative individuals revealed that haplotypes A and B differed by five substitutions but also showed further differentiation of both haplotypes due to an additional substitution in a single haplotype A and B individual from France, respectively. Five partial pike-perch cytochrome *b* cds available from NCBI GenBank could also clearly be assigned to one or the other of the two major lineages. Therefore, this new mtDNA marker might be considered as suitable not only for studies on population structure and phylogeography of pike-perch but also to trace its introduction history and to assess the genetic composition of aquaculture brood stocks.

INTRODUCTION

The pike-perch, a freshwater fish native to the Caspian, Baltic, Black and Aral Sea basins but also widely introduced outside its natural distribution range (Kottelat and Freyhof 2007), is a valuable species in commercial as well as recreational fisheries. Due to locally and/or temporarily high fishing pressures many populations had been or are subjected to over-fishing. As a result, enhancement and supportive stocking programs for wild pike-perch became common practice in many countries. At the same time, pike-perch is also a promising candidate species for diversification of finfish aquaculture.

Despite of this growing interest in the species and its economic importance, relatively little is known on pike-perch population structure and phylogeography what might be at least partly due to a lack and/or deficiency of suitable genetic markers. For example, Björklund et al. (2007), Poulet et al. (2009) and Säisä et al. (2010) still used microsatellite loci originally isolated from the closely related North American species walleye, *Sander vitreus* (Borer et al. 1999; Wirth et al. 1999) and yellow perch, *Perca flavescens* (Leclerc et al. 2000) in their studies on the population structure and genetic differentiation of pike-perch in the Baltic Sea region and France, respectively. The first species-specific microsatellite loci were isolated by Kohlmann and Kersten (2008) and later on used by Khurshut and Kohlmann (2009) to characterize three wild pike-perch populations from the Aral Sea basin in Uzbekistan.

In the present paper, we report the discovery of two major pike-perch cytochrome *b* lineages, describe a simple and fast PCR-RFLP assay that allows to distinguish between them, and present first data on the distribution of both lineages in European wild and farmed populations. This study should only be considered as a demonstration of the potential of this new mtDNA marker for future applications in conservation and management of natural genetic resources as well as in aquaculture that would, of course, require a more systematic and hypothesis driven fish sampling approach.

MATERIAL AND METHODS

PCR-RFLP assay of the pike-perch cytochrome *b* gene

Pike-perch genomic DNA was isolated from muscle tissue or fin clips using the peqGOLD Tissue DNA Mini Kit (Peqlab Biotechnologie). The complete cytochrome *b* gene of 708 individuals was PCR amplified with primers taken from Song et al. (1998) that anneal to tRNA genes flanking the cytochrome *b* region of percid fishes:

F: 5´-GTGACTTGAAAAACCACCGTTG-3´,
R: 5´-CTCCATCTCCGGTTTACAAGAC-3´.

Each PCR reaction mix was composed of 2.5μL of 10xPCR buffer with $(NH_4)_2SO_4$ (MBI-Fermentas), 1.75μL of 2% BSA, 2.5μL of 25mM $MgCl_2$, 1.25μL of 1.25mM dNTPs, 0.5μL of each primer (10pmol·μL^{-1}), 5.0μL template DNA, 0.1μL of *Taq* DNA-polymerase (5units·μL^{-1}; MBI-Fermentas) and sterile water up to a final volume of 25.0μL. The hot start PCR program consisted of an initial denaturation at 95°C for 3min followed by 34 cycles of denaturation at 95°C for 1min, annealing at 51.5°C for 1min, extension at 72°C for 2min, and a final extension at 72°C for 7min.

Previous tests with several restriction enzymes identified Alw26I (MBI-Fermentas) to be capable of producing two different fragment patterns. The reaction mix for digestions of the amplified cytochrome *b* genes with Alw26I consisted of 10.0μL PCR product, 1.5μL enzyme specific buffer, 0.2μL restriction enzyme and 3.3μL sterile water. Resulting restriction fragments were electrophoretically separated and visualized on ethidium bromide stained 1.7% agarose gels. Exact fragment sizes were determined by sequencing of representative individuals and virtual digestions with Webcutter 2.0 (http://bio.lundberg.gu.se/cutter2/).

Cytochrome *b* sequencing of representative individuals

Due to technical restrictions of the available DNA sequencer the complete pike-perch cytochrome *b* gene had to be split into two overlapping segments. Using a preliminary sequence obtained with the Song et al. (1998) primers as template, new primers for PCR amplification of both segments were designed with the Primer3, v. 0.4.0 software (http://frodo.wi.mit.edu/primer3/):

1F: 5´-TAATGGCAAGCCTCCGAAA-3´,
1R: 5´-GTTTAAGCCAAGGGGGTTGT-3´,
2F: 5´-CTCGATTCTTTGCCTTCCAC-3´,
2R: 5´-CTGAGCTACTAATGCAGGATCA-3´.

Each PCR reaction mix was composed of 5.0μL of 10xPCR buffer (MBI-Fermentas), 4.0μL of 25mM $MgCl_2$, 4.0μL of 1.25mM dNTPs, 1.0μL of each primer (10pmol·μL^{-1}), 1.0μL template DNA, 0.1μL of *Taq* DNA-polymerase (5 units·μL^{-1}; MBI-Fermentas) and sterile water up to a final volume of 50.0μL. The hot start PCR program was as described above. PCR products were purified using the peqGOLD Cycle-Pure Kit (Peqlab Biotechnologie), and DNA concentrations were measured with a BioPhotometer (Eppendorf). Cycle sequencing was performed using the CEQ DTCS - Quick Start Kit (Beckman Coulter) according to manufacturer instructions. Forward and reverse sequences of both cytochrome *b* segments were recorded on a CEQ 8000, and aligned and edited manually using the Genetic Analysis System v.7.0, CEQuence Investigator module (Beckman Coulter) and a complete, 1141 bp long pike-perch cytochrome *b* cds (Matschiner et al. 2011; GenBank accession number: HM049965) as reference. The two resulting segments for each of 17 pike-perch individuals were assembled manually into complete cytochrome *b* cds using the MEGA v.3.1 software (Kumar et al. 2004).

Cytochrome *b* sequence analyses

In order to identify variable nucleotide positions manual alignments of the 17 complete cytochrome *b* cds were performed with MEGA v.3.1. All pike-perch cytochrome *b* haplotypes detected were used in Megablast searches for highly similar sequences in NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi; last accession date: 18 January 2013). In addition to the complete reference sequence five highly similar but only partial pike-perch cytochrome *b* sequences were found. They were used together with all haplotypes from the present study to construct a Neighbor-Joining (NJ) tree with MEGA v.3.1 (parameters: p-distance, 1,000 bootstrap replicates), based on the maximum number of shared nucleotides (1,106 bp) and including the *Gymnocephalus schraetser* cytochrome *b* (Matschiner et al. 2011; GenBank accession number: HM049946) as outgroup.

RESULTS AND DISCUSSION

The digestion of the PCR amplified cytochrome *b* gene from 708 individuals with the restriction enzyme Alw26I revealed two fragment patterns, designated as haplotypes A and B, which could clearly be distinguished on agarose gels (Figure 1). An additional restriction site in haplotype B cut the 392 bp long fragment of haplotype A into two smaller fragments of 119 and 273 bp, respectively. Among the pike-perch populations examined so far, haplotype A was fixed or dominating in Central and East European countries, haplotype B was mainly found in several French populations, and Tunisian pike-perch introduced

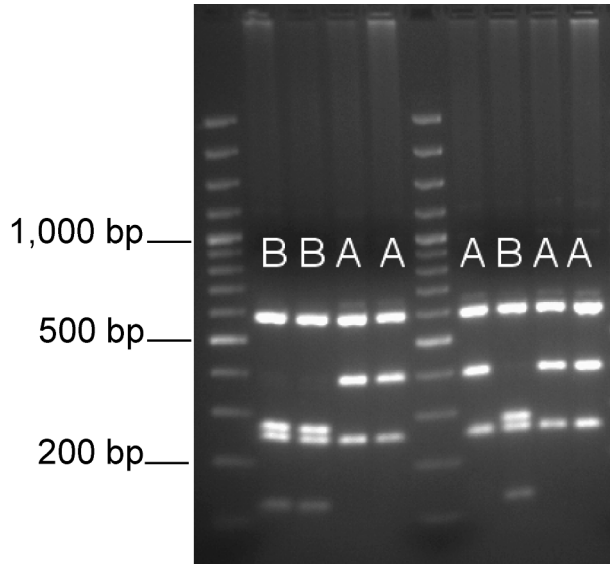

Figure 1. Fragment patterns of PCR amplified pike-perch cytochrome *b* genes after digestion with the restriction enzyme Alw26I. Fragment sizes (bp) are based on complete cds: haplotype A – 533, 392, 216; haplotype B – 533, 273, 216, 119. Size marker: GeneRuler™ 100 bp Plus DNA Ladder (MBI-Fermentas).

from Europe (according to a microsatellite study most probably from France [M. Louati, Unité de Recherche de Biologie Intégrative et Écologie Evolutive et Fonctionnelle des Milieux Aquatiques, Faculté des Sciences de Tunis, 2092 El Manar, Tunisie]) were fixed for haplotype B (Table 1).

Sequencing the complete cytochrome *b* cds (1141 bp) of nine individuals with haplotype A and eight individuals with haplotype B confirmed the fragment patterns observed on agarose gels. Haplotype A was fully identical with the cytochrome *b* reference sequence reported by Matschiner et al. (2011). Haplotypes A and B differed by five substitutions (Table 2), and the variable site (A/G) at nucleotide position 333 caused the difference in their fragment patterns. Additionally, one haplotype A individual of the French population "Retenue de Hautefage" possessed a further substitution (C/A) at nucleotide position 1098. Similarly, one haplotype B individual of the French population "Etang des Aulnes" showed an additional substitution (C/T) at nucleotide position 297. Since both substitutions had no effect on the corresponding fragment patterns, they were considered as variants of their basal haplotypes and designated as haplotypes A1 and B1, respectively (Table 2). Sequences of all four haplotypes were deposited in GenBank under accession numbers JX025362-JX025365.

Table 1. Pike-perch cytochrome *b* gene: distribution of PCR-RFLP derived haplotypes in European populations.

Country	Population	Status	Sample size	Frequency (%)		Individuals sequenced	
				Haplotype A	Haplotype B	Haplotype A	Haplotype B
Germany	Lychensee 2005	wild	50	100.0	-	1	-
	Lychensee 2006	wild	50	100.0	-	1	-
	Lychensee 2007	wild	50	100.0	-	1	-
	Müggelsee	wild	50	96.0	4.0	1	1
Netherlands	Hatchery	farmed	41	100.0	-	1	-
Poland	3 Lakes	wild	10	100.0	-	1	-
Czech Republic	Vodnany	farmed	23	100.0	-	1	-
France	Complexe d´Eguzon	wild	31	80.6	19.4	-	-
	Ardres	wild	30	66.7	33.3	-	-
	Etang du Puits	wild	27	100.0	-	-	-
	Lac de Pont	wild	22	50.0	50.0	-	1
	Lac de Crescent	wild	38	100.0	-	-	-
	Lac de Madine	wild	30	100.0	-	-	-
	Etang des Aulnes	wild	30	46.7	53.3	-	1[a]
	Retenue de Saint–Ferréol	wild	27	88.9	11.1	-	-
	Retenue de Bage	wild	30	100.0	-	-	-
	Retenue du Tordre	wild	30	100.0	-	1	-
	Lac dę Aydat	wild	16	100.0	-	-	-
	Retenue de Hautefage	wild	19	94.7	5.3	1[b]	-
Tunisia	Sidi Salem	wild	50	-	100.0	-	2
	Nebhana	wild	54	-	100.0	-	3
Total			708	78.4	21.6	9	8

[a] due to an additional substitution designated as haplotype B1 later on
[b] due to an additional substitution designated as haplotype A1 later on
- stands for 0

Table 2. Variable sites of the pike-perch cytochrome *b* complete cds.

Haplotype	Nucleotide position						
	297	333*	468*	546*	753*	897*	1098
A	C	A	A	C	G	T	C
A1	C	A	A	C	G	T	A
B	C	G	G	T	A	C	C
B1	T	G	G	T	A	C	C

* parsimony informative sites

The five highly similar, partial pike-perch cytochrome *b* cds originated from wild individuals caught in the Kokemäenjoki delta, western Finland (Kahilainen et al. 2011; GenBank accession number: GU936790), the Římov reservoir, Czech Republic (Kalous et al. 2010; GenBank accession numbers: FJ788394 and FJ788397), the River Danube, Slovakia (Sloss et al. 2004; GenBank accession number: AY374291) and the River Ouse, England (Sloss et al. 2004; GenBank accession number: AF546122). The NJ tree clustered haplotypes A and A1 as well as B and B1 into two groups with high bootstrap support (Figure 2). The haplotype of the Finnish pike-perch was identical with our haplotype A and the reference sequence. In contrast, the two identical haplotypes reported by Sloss et al. (2004) from Slovakia and England could be considered as varieties of our haplotypes B and B1. Interestingly, the two differing sequences of pike-perch from the Czech Římov reservoir did not belong to the same cytochrome *b* lineage: one clustered with our haplotypes A and A1, the other one with haplotypes B and B1.

Based on the still very preliminary data on the haplotype distribution within Europe we can only roughly speculate at the moment that the cytochrome *b* lineage consisting of haplotypes A, A1 and their varieties might be prevailing in North-Eastern Europe whilst the lineage consisting of haplotypes B, B1 and their varieties might be confined to South-Eastern Europe. A natural contact zone of both lineages might exist in Central Europe (Germany and Czech Republic), and the occurrence of both lineages in France might be due to mixed introductions. It is already evident, however, that the phylogeography of pike-perch in Europe is worth to be studied in more detail.

In conclusion, the present results demonstrate that the described simple and fast PCR-RFLP assay is able to distinguish two major cytochrome *b* lineages in pike-perch: all sequence variation observed so far could clearly be assigned to one of them. Therefore, this new mtDNA marker for pike-perch can be considered as suitable for future studies on population structure and phylogeography

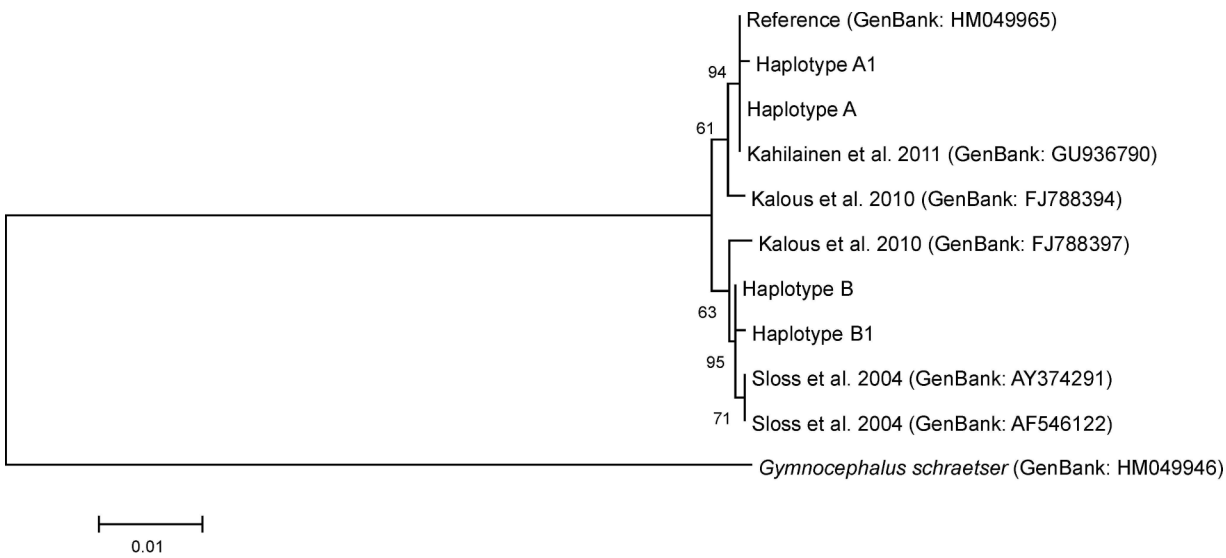

Figure 2. NJ tree of partial, 1106 bp long pike-perch cytochrome *b* haplotypes constructed with MEGA v.3.1 based on p-distances and 1,000 bootstrap replications.

but also to trace the introduction history and to assess the genetic composition of aquaculture brood stocks – in particular if used in combination with the species-specific microsatellite loci and those from walleye and yellow perch that are known to cross-amplify in the species.

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