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

Klaus Kohlmann¹, Manel Louati², Petra Kersten¹, Lilia Bahri-Sfar², Nicolas Poulet³, Oum Kalthoum Ben Hassine²

¹Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Department of Ecophysiology and Aquaculture, Müggelseedamm 310, 12587 Berlin, Germany
²Unité de Recherche de Biologie Intégrative et Écologie Evolutive et Fonctionnelle des Milieux Aquatiques, Faculté des Sciences de Tunis, 2092 El Manar, Tunisie
³French National Agency for Water and Aquatic Environment (ONEMA), “Le Nadar” Hall C5, square Félix Nadar, 94300 Vincennes, France

Corresponding author: Klaus Kohlmann; Phone: 0049-30-64 181 634; Fax: 0049-30-64 181 663; E-mail: kohlmann@igb-berlin.de

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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

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 flavesens* (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:

\[
\begin{align*}
F: & \quad 5'\text{-GTGACCTTGGAAAACCACCGTTG-3'} \\
R: & \quad 5'\text{-CTCCATCTCCGGTTTACAAGAC-3'}
\end{align*}
\]

Each PCR reaction mix was composed of 2.5µL of 10xPCR buffer with (NH\(_4\))\(_2\)SO\(_4\) (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 (5 units·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 additional restriction sites in haplotype B, which cut the 392 bp long PCR product 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

**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):

\[
\begin{align*}
1F: & \quad 5'\text{-TAATGGCAAGCCTCCGAAA-3'} \\
1R: & \quad 5'\text{-GTTTAAGCCAAGGGGTTTG-3'} \\
2F: & \quad 5'\text{-CTGAGCTACTAATGCAGGATCA-3'} \\
2R: & \quad 5'\text{-CTIGGCCTACATTAGCAGGATCA-3'}
\end{align*}
\]

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, CEQUEase Investigator module (Beckman Coulter) and a complete, 1,141 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
Kohlmann et al. Pike-perch cytochrome b3 from Europe (according to a microsatellite study most probably from France [M. Louati, Unité de Recherche de Biologie Intégrative et Écologie Évolutive 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 “Retene 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.

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).

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

<table>
<thead>
<tr>
<th>Country</th>
<th>Population</th>
<th>Status</th>
<th>Sample size</th>
<th>Frequency (%)</th>
<th>Individuals sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Haplotype A</td>
<td>Haplotype B</td>
<td>Haplotype A</td>
</tr>
<tr>
<td>Germany</td>
<td>Lychensee 2005</td>
<td>wild</td>
<td>50</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Lychensee 2006</td>
<td>wild</td>
<td>50</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Lychensee 2007</td>
<td>wild</td>
<td>50</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Müggelsee</td>
<td>wild</td>
<td>50</td>
<td>96.0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>Hatchery</td>
<td>farmed</td>
<td>41</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>Poland</td>
<td>3 Lakes</td>
<td>wild</td>
<td>10</td>
<td>100.0</td>
<td>1</td>
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<tr>
<td>Czech Republic</td>
<td>Vodnany</td>
<td>farmed</td>
<td>23</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>France</td>
<td>Complexe d’Eguzon</td>
<td>wild</td>
<td>31</td>
<td>80.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Arders</td>
<td>wild</td>
<td>30</td>
<td>66.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Etang du Puits</td>
<td>wild</td>
<td>27</td>
<td>100.0</td>
<td>-</td>
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<tr>
<td></td>
<td>Lac de Pont</td>
<td>wild</td>
<td>22</td>
<td>50.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lac de Crescent</td>
<td>wild</td>
<td>38</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lac de Madine</td>
<td>wild</td>
<td>30</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Etang des Aulnes</td>
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<td>30</td>
<td>46.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Retene de Saint-Ferréol</td>
<td>wild</td>
<td>27</td>
<td>88.9</td>
<td>-</td>
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<tr>
<td></td>
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<td>30</td>
<td>100.0</td>
<td>-</td>
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<tr>
<td></td>
<td>Retene du Tordre</td>
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<td>30</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lac de Aydat</td>
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<td>16</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Retene de Hautefage</td>
<td>wild</td>
<td>19</td>
<td>94.7</td>
<td>-</td>
</tr>
<tr>
<td>Tunisia</td>
<td>Sidi Salem</td>
<td>wild</td>
<td>50</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nebhana</td>
<td>wild</td>
<td>54</td>
<td>100.0</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>708</td>
<td>78.4</td>
<td>21.6</td>
</tr>
</tbody>
</table>

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
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 Rímov 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 Rímov 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.
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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