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RESEARCH ARTICLE

Genetic variability in European populations of *Coregonus lavaretus* (L.): an assessment based on mitochondrial *ND-1* gene haplotypes

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Abstract. The genetic variability of whitefish, *Coregonus lavaretus* (L.), was studied based on 114 individuals from nine populations inhabiting Polish lakes, including the Szczecin Lagoon, and from one population each from lakes in Austria and Switzerland. Differences within and among populations were assessed with mitochondrial *ND-1* gene sequences that were PCR amplified and digested with ten restriction enzymes. The ten composite haplotypes obtained were sequenced and analyzed with MEGA4 software. The highest intrapopulation variability was noted in the whitefish populations inhabiting lakes Ińsko, Miedwie, Marianowo, Wisola, Śremskie, Morzycko, the Szczecin Lagoon, and Lake Lucerne, which possessed from two to five composite haplotypes. In contrast, populations inhabiting lakes Woświn, Czarne, and Traunsee were fixed for the most common haplotype H2.

Keywords: *Coregonus lavaretus,* PCR-RFLP, *ND-1*, haplotype variability, mtDNA

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Introduction

Due to the necessity of exploring the culture potential of fish species novel to aquaculture as well as attempts to implement the principles of responsible fisheries, including in inland fisheries, scientific interest focuses on fish species which are either of great economic importance or promise good market products. Coregonid species, particularly whitefish, *Coregonus lavaretus*, (L.), is one such species. Since responsible aquaculture and sustainable fisheries management both require knowledge of the genetic status of the species being managed, comparative genetic research makes it possible to set up a "gene bank" in the form of information on interpopulation differences and allows assessing the genetic purity of studied populations. The artificial reproduction of whitefish resulted in the emergence of whitefish x peled, *Coregonus peled* (Gmelin) and whitefish x vendace *Coregonus albula* (L.) hybrids. Therefore, the practical importance of studying the genomes of potential breeding stocks is of particular significance. In addition, the whitefish is known to have formed populations of great economic or historic value. One example is furnished by Lake Miedwie whitefish which for years has been a source of stocking material for numerous European lakes. Moreover, if the historical evidence is to be believed, Lake Miedwie whitefish

was an ancestor of whitefish that made their way to Japan (Toshikazu and Tetsuro 2004).

Coregonids, including whitefish, are particularly frequently subjects of studies on genetic variability. The intense interest in this aspect of the whitefish stems from its high interpopulation variability, and its ability to form numerous varieties and races differing in morphology and/or behavior. This raises the question of whether these differences are reflected in genotypic variability. Thus far, the literature dealing with whitefish has identified numerous varieties and sub-species differing in mouth structure, body shape, and the number of gill rakers, among others (Szczerbowski 1969). Numerous studies have also addressed whitefish genetic variability by examining mitochondrial DNA (Bernatchez et al. 1991, Bernatchez and Dodson 1994, Brzuzan 1998, 2000, Brzuzan et al. 1998) and microsatellite markers (Hansen et al. 1999, 2008, Winkler and Weiss 2008).

An earlier paper by Kohlmann et al. (2007) focused on the variability of the mitochondrial *ND-1* gene in whitefish caught in Poland, primarily in Western Pomerania. The present study broadens the scope of the previous study by extending the sample size from some Polish lakes and by including samples obtained from two European countries with whitefish populations that have been known for years. Analyses of their intrapopulation variability will make it possible to determine the degree of affinity between these populations and to estimate the genetic homogeneity of populations so in the future they can serve as a potential gene bank for aquaculture. For stocking operations, knowledge of the genetic status of individual stocks permits maintaining biodiversity resulting from the geographic isolation of individual populations. In extreme cases, heterogeneous populations will be identified, and it will be recommended that they are excluded from further exploitation.

Materials and Methods

Tissue or fin clip samples of 114 whitefish individuals were collected from eight Polish lakes (Ińsko, Woświn, Miedwie, Marianowo, Wisola, Czarne (Drawieński National Park), Śremskie, Morzycko), the Szczecin Lagoon, and one lake each in Austria (Traunsee) and Switzerland (Lucerne). The numbers in Figure 1 denote the number of samples collected in each country.

Figure 1. Origin of samples used in this study and the number of samples collected in each country.

DNA from these samples was isolated using the E.Z.N.A. Tissue DNA Mini Kit (Peqlab Biotechnologie, Germany). The isolated DNA was subsequently used as a template for the amplification of the mitochondrial *ND-1* gene region (2012 bp) using a pair of primers described by Nielsen et al. (1998):

Forward: 5' GCC TCG CCT GTT TAC CAA AAA CAT 3'

Reverse: 5' GGT ATG GGC CCG AAA GCT TA 3'

The PCR procedure applied to each sample involved 10 µl genomic DNA, 0.2 µM of each primer, 1 x PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.5 mM $MgCl_2$, 80 µg BSA, 0.1 mM dNTPs mix, and 0.5 units *Taq* DNA-polymerase (Fermentas, Germany) in a total volume of 50 µl. The PCRs were performed in a thermal cycler (Eppendorf, Germany) programmed for initial denaturation for 3 minutes at 95°C followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2.5 minutes. A final extension at 72°C lasted for 10 minutes. The PCR

products obtained were digested with ten restriction enzymes (Fermentas, Germany). They were selected using Webcutter software 2.0 (available at http://rna.lundberg.gu.se/cutter2/) based on a reference mtDNA sequence for *Coregonus lavaretus* obtained from GenBank (accession number AB034824). Unless indicated otherwise, the enzymes were four-base cutters: *Xba*I (six-base cutter), *Eco*47I (five-base cutter), *Hin*fI (five-base cutter), *Bsu*RI, *Rsa*I, *Alu*I, *Mbo*I, *Hpa*I, *Hin*6I, and *Taq*I. Digestion proceeded in reaction mixtures of 15 µl total volume consisting of 10 µl of the PCR product, 1.5 µl buffer adjusting the reaction conditions to the optimum, 0.2 µl of the restriction enzyme, and 3.3 µl sterile water. The resulting fragments were separated by electrophoresis on 2% agarose gel with the TBE buffer system, stained with ethidium bromide (EtBr), and compared to peqGOLD 100 bp DNA-Ladder Plus (Peqlab Biotechnologie, Germany) size standards using BIO-1D Analysis Software for Electrophoresis Images (Vilber Lourmat, France). The fragment patterns observed were marked with capital letters starting from A, which was given to the expected pattern from the "virtual" digestion of the reference sequence. Letters from B on were used to mark the patterns deviating from expected pattern A. Subsequently, composite haplotypes were designated based on combinations of restriction fragments resulting from the different restriction enzymes.

Representative individuals of all observed composite haplotypes were sequenced on a CEQ 8000 capillary sequencer (Beckman Coulter, USA). The preparation of internal primers, sequencing parameters, and the assemblage of the accumulated sequence fragments was performed according to Kohlmann et al. (2007). Sequences representing individual haplotypes were also virtually digested to confirm the restriction sites obtained by the ten enzymes. In addition, to reconstruct phylogenetic relationships between the haplotypes, a neighbor joining tree based on Jukes-Cantor distances (Jukes and Cantor 1969) was constructed using MEGA4 software (Tamura et al. 2007).

Results

All digested samples were easily distinguishable on agarose gels. Compared to the previous coregonid study and applying the same ten restriction enzymes, only one additional haplotype could be detected in the *ND-1* gene region. It was very similar to haplotype H2, and it differed in the fragment pattern of only one restriction enzyme (*Eco*47I: C instead of A). This new haplotype HN (N – like new) was observed in a single Lucerne sample (Table 1, and L. Lucerne7 in Fig. 2).

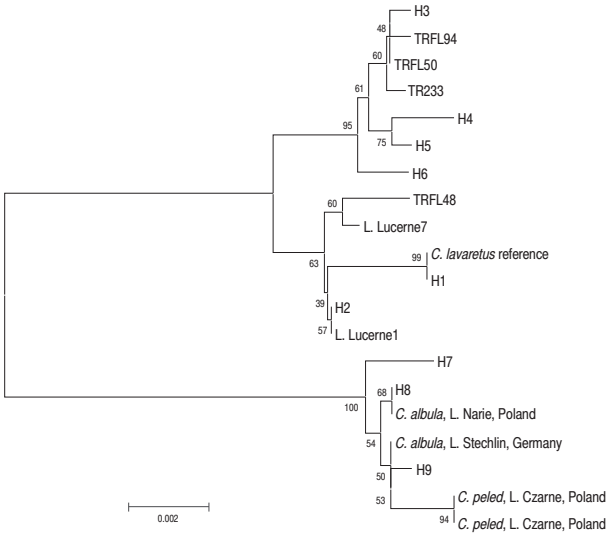

Figure 2. Neighbor-joining tree based on Jukes-Cantor distances (upper branch group number one, lower branch group number two).

Haplotype H2 was expressed with the highest frequency (68.42%); it was present in samples collected from all the lakes studied (Table 2). The rarest haplotypes were H6, H7, and HN, for each of which there was only one individual per haplotype (1.19%). As confirmed by the sequence analysis, haplotype H1 was expressed in as few as two specimens only caught in the Lake Śremskie. In the sequence alignment for 1929 nucleotides, 62 variable sites were detected. As shown by Jukes-Cantor distances (Fig. 2), the relative sequence differences in the first group were rather similar and ranged from 0.000 to 0.008 (mean 0.004). In the second group, the between-sequence distances ranged from 0.000 to

Table 1
Composite *ND-1* haplotypes revealed by digestion with ten restriction enzymes

Haplotype	*Eco*47I	*Bsu*RI	*Hinf*I	*Rsa*I	*Alu*I	*Mbo*I	*Hpa*I	*Hin*6I	*Taq*I	*Xba*I	Haplotype frequency (%)
H1	A	A	A	A	A	A	A	A	A	A	1.75
H2	A	A	A	A	A	A	A	B	A	A	68.42
H3	A	A	B	A	A	A	A	B	A	A	4.76
H4	A	A	A	B	A	A	A	B	A	A	10.71
H5	A	A	A	B	A	B	A	B	A	A	8.33
H6	B	B	A	A	A	A	A	B	B	A	1.19
"HN"	C	A	A	A	A	A	A	B	A	A	1.19
H7	C	C	C	C	B	A	B	B	A	A	1.19
H8	C	C	C	C	C	A	B	B	A	A	5.95
H9	C	C	C	D	C	A	B	B	A	A	2.38

Table 2
Distribution of ten composite haplotypes observed in whitefish populations from Poland, Switzerland, and Austria. Numbers in brackets are from Kohlmann et al. (2007)

Population	H1	H2	H3	H4	H5	H6	H7	H8	H9	HN	Total
Ińsko		(26)			(4)						30
Woświn		5 (4)									5
Miedwie		6	2(1)	1	3(1)						12
Marianowo		(2)				(1)	(1)	(1)			5
Wisola		2			(2)						4
Czarne (DNM)		8									8
Śremskie	(2)	(1)			(1)			(4)	(2)		10
Morzycko		(2)	(1)	(6)	(1)						10
Traunsee		13									13
Szczecin Lagoon		4	1	2							7
Lucerne		9								1	10
Total	2	76	4	9	11	1	1	5	2	1	114

0.004 (mean 0.002). The difference between the two groups was 2% (mean distance between groups 0.02).

Samples from the three lakes of Woświn, Czarne, and Traunsee, revealed the expression of only one type of haplotype, which might be strong evidence that the whitefish population in each of these lakes is rather uniform according to the *ND-1* gene region (Table 2). A sample from Lake Lucerne (10% of this population sample) exhibited a novel haplotype which did not appear in other samples analyzed in this study. Most probably, the question of whether this haplotype is more frequent or whether it appeared just once, could be resolved if further research were conducted. The highest number of haplotypes was observed in the Śremskie materials because of the number of stocking programs carried out there and probably from the high rate of hybridization between whitefish, peled, and vendace.

More detailed information was supplied by sequencing all samples for the *ND-1* gene region that was represented in several haplotypes. It is highly

probable that some digestion sites were located close to the ends of the DNA template and their cutting and short fragments were overlooked. In fact, all four sequenced Austrian whitefish samples from Lake Traunsee (TR 233) and the tributary River Traun (TRFL48, TRFL50, TRFL94) that expressed haplotype H2 according to the PCR-RFLP analyses revealed sequence differences with three samples being closer to H3 than to H2 (Fig. 2). On the other hand, haplotype H2 was confirmed for the Swiss L. Lucerne1 sample as was the close affinity of the new haplotype HN (expressed by sample L. Lucerne7) to haplotype H2.

The Neighbour-Joining dendrogram consisted of two clearly distinguishable clades. The first consisted of the samples with seven haplotypes (from H1 to H6 and new haplotype HN) being expressed, and the second comprised samples with haplotypes from H7 to H9. In addition, to check for possible hybrids or introgressed specimens, the dendrogram was also supported by analyses of vendace and peled samples. The second cluster supported by the bootstrap value of 100% was highly similar among sequences, which means that the last three haplotypes were highly similar to those of peled and vendace (Fig. 2). Samples of *C. albula* (Lake Narie, Poland; Lake Stechlin, Germany) and *C. peled* (Lake Czarne, Poland) showed a high similarity with three whitefish haplotypes: H7, H8, and H9. The most frequent haplotype of the three was H8 (N = 5), followed by H9 (N=2), and H7 (N=1).

Discussion

Studies on the systematic position of coregonids, including that of whitefish, are as frequent as research on whitefish biology primarily because of the complexity of the problem. In the case of whitefish, the lack of clearly defined taxonomy is noteworthy since individual taxonomic systems are very often chaotic and show regional differences (Heese 1986, 1990). Some authors, such as Regan (1908), classified coregonids based on the location of the mouth, Berg (1948) supplemented this by including the extension of the maxilla toward the eye, while others (e.g., Hubbs 1947 or Gąsowska 1960) used some bones as the basis for classification. Analysis of the gill raker count has been and remains important in distinguishing between ecological forms of whitefish (Lampert 1925, Gąsowska 1960), but classification based on this is also prone to error. Since the whitefish potential for hybridization is great, the mean gill raker count, which is the basis of classification, can vary around the mean gill raker counts typical of hybrid populations (Fabricius in Szczerbowski 1969b). Moreover, the gill raker count can change with age (Pęczalska 1962). Literature data on karyotype polymorphism of whitefish forms from different European locations indicate they have similar or identical diploid karyotypes consisting of 80 or 81 chromosomes (Jankun et al. 1995).

Generally, the fish of the subfamily Coregoninae pose a number of interesting problems for those studying evolution, zoogeography, and taxonomy as well as for those engaged in fisheries. The haplotypes obtained in this study, grouped on two branches of the cladogram (Fig. 2) seem to confirm the data referred to above. The numerous haplotypes (H1, H8 and H9) in the materials collected from Lake Śremskie are particularly distinct. The similarity with sequences obtained for peled and vendace is noteworthy, and might result from hybridization or introgression. The probability of this is enhanced by the fact that the Szwaderki farm was, in the 1960s and 1970s, the site of experimental introductions of alien coregonids and their hybrids, as mentioned by Bernatowicz (1964). The haplotype H7 represented in the sample collected from Lake Marianowo (Western Pomerania) could have also been a result of former biomanipulations. Numerous European populations of whitefish reveal traces of hybridization with other coregonids, particularly with peled (Vuorinen 1988). Instances of karyotype variability increasing because of hybridization have been reported for whitefish populations in Armenian and Italian lakes (Ruhkjan and Arakeljan 1980, Sola et al. 1989). On the other hand, the whitefish sample

collected in Lake Czarne contained only the haplotype H2.

Analyses based on comparing rDNA sequences identified peled as a sibling species of whitefish, but further comparisons in the ITS region demonstrated the presence of a single 65-67 bp repetition in the North American Coregoninae. The repetition has two copies in *C. peled*, *C. lavaretus*, and *Coregonus clupeaformis* (*Mitchill*) from the Bering Sea, while the North American *C. clupeaformis* population has three copies. This evidence indicates that the North American *C. clupeaformis* is a form of *C. lavaretus* (Sajdak and Philips 1997).

The most frequent haplotype in this study was H2; it occurred in populations from Poland, Austria, and Switzerland. H2 was the only haplotype revealed in the lakes Traunsee, Czarne, and Woświn samples. Of the 30 whitefish individuals from Lake Ińsko, 26 showed the presence of H2 as well. Whereas there is no information on stocking operations in the Austrian or Swiss lakes that supplied materials for this study, fishers operating in lakes Woświn and Ińsko reported that these lakes had been stocked with indigenous materials, and that some allochthonous materials were also used in a few instances in Lake Ińsko (J. Łojko unpublished data). In addition, the whitefish population in Lake Woświn is on the brink of disappearance. It seems then that the homogeneity of the Woświn sample resulted from selection mechanisms underpinned by adverse environmental changes in the lake (Czerniejewski et al. 2008) and, to some extent, by the lake's morphology that is facilitating the potential isolation of whitefish stocks within it.

The highest number of haplotypes (5 in Śremskie) was revealed in the populations inhabiting lakes Miedwie, Marianowo, and Morzycko. The most probable cause of such high variability is the intense management of these lakes, including stocking with diverse stocking materials. According to historical evidence, Lake Miedwie used to support a native form of whitefish, the Miedwie whitefish (*C. lavaretus maraena*, Bloch), characterized by dense gill rakers. Because of its high growth rate and large size, it was used in stocking operations in other lakes in Poland, the Czech Republic, and in the Alps (Pietrucha 1999). As reported by Pietrucha (1999), Lake Miedwie also supports another dense gill raker form, *C. lavaretus generosus* (Peters), originating from stocking operations carried out in 1997. The presence of the migratory whitefish (*C. lavaretus lavaretus* L.) is also possible. When studying the morphology of whitefish inhabiting lakes near Międzychód (the region of Wielkopolska, Poland), Kaj (1955) found a similar situation. The relict *C. lavaretus generosus* was accompanied by Miedwie whitefish. Without direct evidence, he intuited that the coexistence of genotypically pure forms is not plausible. However, he referred to the small size of the lakes he studied, the lack of isolated coves in them, and other features which could have facilitated the hybridization of different whitefish forms in the Międzychód lakes; the hybridization contention was also supported by the location of spawning grounds.

Elucidation of the full scale of variability in whitefish is of importance for taxonomy and has practical implications. It is crucial that a phenome be found which is best adapted to its habitat and which has advantageous growth parameters, and such a phenome should be protected and supported in cultures.

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Streszczenie

Zróżnicowanie genetyczne europejskiej populacji *Coregonus lavaretus* (L.): ocena na podstawie mitochondrialnego genu *ND-1*

Zróżnicowanie genetyczne 9 populacji siei pospolitej (*Coregonus lavaretus,* L.) określono w grupie 114 osobników pozyskanych ze zbiorników wodnych Polski, Szwajcarii oraz Austrii. Analizę przeprowadzono w oparciu o technikę PCR-RFLP zastosowaną względem mitochondrialnego genu *ND-1,* którego sekwencję poddano trawieniu 10 enzymom restrykcyjnym. Na tej podstawie otrzymano dziesięć haplotypów, których sekwencje reprezentatywne zostały poddane sekwencjonowaniu oraz analizie z wykorzystaniem programu MEGA4. Najwyższe zróżnicowanie międzypopulacyjne określono dla populacji siei zamieszkujących jeziora Ińsko, Miedwie, Marianowo, Wisola, Śremskie, Morzycko, Zalew Szczeciński oraz szwajcarskie jezioro Lucerne. Zawierały się ona w obrębie haplotypów od 2 do 5. Dla porównania osobniki z jezior Woświn, Czarne (DPN) oraz Traunsee (Austria) były zaklasyfikowane do pojedynczego haplotypu H2.