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RESEARCH PAPER

Polymorphism of the Lake, Migratory Populations and Reared Broodstocks of Whitefish (*Coregonus* spp. L.) in Northern Poland and its Importance in Maintaining Ecological Biodiversity

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Abstract

Whitefish of the genus Coregonus is a valuable component of ichtyofauna, whose area of occurrence has decreased in the recent years. While whitefish still occur in natural populations in north-western Poland, the diversity of Polish whitefish is poorly understood. In the present study we investigate migratory whitefish from the Oder River as coastal whitefish from eastern and western populations of the Polish Baltic Sea, whitefish from lakes of northern Poland and raised whitefish stock from hatcheries. The subject of the study was the application of mtDNA PCR-RFLP and sequencing techniques to evaluate the genetic potential of the whitefish populations. For this purpose *ND1*, *ND5/6* and a D-loop fragment were analysed. The most haplotypes were observed only in the migratory whitefish population from the Oder River. The greatest genetic distance of 0.029 was between the migratory populations from Lake Dąbie, Oder River, and the populations from Lake Morzycko, the Pomeranian Bay and the eastern migratory populations. The obtained results showed low variability between the analysed populations of the migratory, lake and whitefish broodstocks. The results of this study are a valuable molecular characterization of the populations and will provide data to conduct further genetic monitoring required for the ongoing protection and reintroduction activities.

Keywords: Whitefish, mtDNA, *ND1*, *ND5/6*, D-loop.

Introduction

The care for natural resources and species protection require the use of modern techniques in order to protect the natural populations and support the endangered ones. Whitefish of the genus *Coregonus* is a valuable component of water ecosystems, being also an important species for aquaculture. In Europe, the range of occurrence of whitefish has rapidly decreased in the recent decades (Kottelat & Freyhof, 2007; Winfield, Fletcher, & Winfield, 2002). It is considered that the Lower Oder River, Lake Dąbie and the coastal zone of the Baltic Sea are inhabited by *Coregonus nilssoni*, while Lake Morzycko, Lake Miedwie and the Radunia River are inhabited by *Coregonus marena* (Kottelat & Freyhof, 2007, FishBase 2013). The dramatic decline in the size of the whitefish population in the Polish coastal waters in the 1980s (Schulz *et al*., 1996) forced protective measures such as reintroduction, which contributed to the recovery of this indigenous population (Szczerbowski, 2000; Polewacz *et al*., 2015). However, due to the adverse anthropogenic changes in lakes and the high requirements of whitefish regarding water quality, the size of the lake populations is decreasing (Winfield *et al*., 2002; Witkowski, Kotusz, & Przybylski, 2009). Whitefish has been extirpated from many lakes, and the extant populations are small and rely on supplementary stocking (Wołos & Bnińska, 1998). Implementing protective and supportive measures for populations, including stocking, planned to last many years should be preceded by genetic analysis (Blohm *et al*., 2007; Jacobsen *et al*., 2012; Mysłowski, Panicz, Sadowski, & Hofsoe, 2011). The characteristics of the genetic pools can be used to analyse relatedness between populations, and to evaluate the degree of their diversity and the level of variability within the populations of *Coregoninae* (Bernatchez & Dodson, 1994; Bochkarev, Zuykova, Alexey, & Katokhin, 2011). In earlier studies of this group of fish, protein electrophoresis was used (Ferguson, Himberg, & Svardson, 1978; Vuorinen, Bodaly, Reist, & Luczynski, 1998). Currently, molecular methods are mainly used, including the analysis of selected mtDNA fragments using the PCR-RFLP technique (Bernatchez, Dodson, & Colombani, 1991; Hansen, Mensberg, & Berg, 1999; Gordeeva, Karmanova, &

Shitova, 2008; Borovikova, Artamonova, & Makhrov, 2012; Kirczuk, Rymaszewska, Czerniawski, Pilecka-Rapacz, & Domagała, 2015) and microsatellite analysis (Saisa *et al*., 2008; Fopp, 2010).

To this date, genetic studies in Poland included the analysis of the short nucleotide sequences D-loop (100 bp) (Brzuzan, 2000; Brzuzan & Ciesielski, 2002) and PCR-RFLP analysis of that region in the whitefish from Lake Maroz (Brzuzan, 1998), as well as *ND1* analysis in the fish caught in the lakes of Western and Central Pomerania (Kohlmann, Kempter, Kersten, & Sadowski, 2007; Kempter, Kohlmann, Panicz, Sadowski, & Keszka, 2010). In order to conduct genetic characterization, analyses of microsatellite DNA of the endemic whitefish from Lake Łebsko and several lakes of northern and north-eastern Poland were also conducted (Fopp-Bayat, 2010; Fopp-Bayat & Ciereszko, 2012). In the case of *Coregonidae*, there is also the problem of hybrydisation which results in the presence of fertile hybrids in the environment. For example in Poland, after the introduction of peled (*C. peled*) conducted at the end of the 1960s, the species crossbred with whitefish (Falkowski, Luczynski, & Vuorinen, 1988; Dembska-Zakes, & Mamcarz, 1992). This has forced actions that would help to maintain precious, genetically clean populations and obtain valuable material for reintroduction (Falkowski, 1992; Fopp-Bayat, 2010). Therefore, as the populations of migratory whitefish are recovering and the lake populations are decreasing, it is essential to support the fisheries economy based on reintroduction and catches with proper genetic monitoring.

In the present study, we have genetically surveyed four indigenous migratory populations of whitefish from the Polish coast of Baltic Sea, two non-migratory lake populations as well as two broodstocks kept under captivity. The aim of the study was to characterize each population for variability and genetic potential, as well as understand the potential effect of broodstocks used for stocking on wild populations. In this context, the comparison of the genetic resources of the Polish populations with those of other European populations is a contribution to the discussion that completes information about this species. It is particularly important due to the protection of whitefish by European law.

Materials and Methods

Study Sites and Sampling of Biological Material

The study was conducted using 6 whitefish populations from natural habitats and 2 spawning broodstocks, 30 individuals per site (Figure 1). The study material was obtained in 2012–2013. The study involved taking muscle or fin samples using a sterile technique, which were subsequently stored in Eppendorf-type tubes and frozen until analysis.

Molecular Survey

Total DNA was isolated from the sampled material using phenol-chloroform extraction, following Bernatchez, Savard, Dodson and Pallotta (1988), and kept at −70°C until analysis. DNA of all fish was analysed using the PCR-RFLP technique. Altogether, 3 mitochondrial DNA (mtDNA) fragments were analysed: *ND1* (NADH dehydrogenase, subunit 1 [complex I]) of approx. 1300 bp, *ND5/6* (NADH dehydrogenase, subunit 5–6 [complex I]) of approx. 2400 bp, and a noncoding control region (D-loop), approx. 1300 bp. The apropriate primers and restriction enzymes were used to analyse the DNA fragments of *Coregonus* sp.: *ND1* (Pamminger-Lahnsteiner, Weiss, Winkler, & Wanzenbock, 2009), *ND5/6* (Nielsen, Hansen, & Mensberg, 1998), D-loop (Bernatchez, Guyomard, & Bonhomme, 1992; Reed, Dorschener, & Phillips, 1998) as previously described. The PCR regime was adjusted to the requirements of GoTaq®Flexi DNA Polymerase, according to the manufacturer's recommendation. Final reagent concentrations were 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5mM $MgCl_2$ for *ND1*, 2,5mM $MgCl_2$ for *ND5/6* and 3,0mM $MgCl_2$ for region D-loop, 50 μM for each deoxynucleoside triphosphate, 10 pM for each primer and DNA. The results of PCR amplification were visualised by electrophoresis of 5 μl of each sample in 1.5% agarose gels with GPB Gold View Nucleic Acid Stain (GenoPlast, Biochemicals, Poland).

The amplicons were digested using 8 restriction enzymes: *Alu*I, *Ava*I, *Bsu*I, *Dde*I, *Msp*I, *Hha*I, *Hinf*I, *Rsa*I (Thermo Scientific, USA). The digestion was performed according to the manufacturer's recommendation. The digestion products were checked on 3.0% agarose gels with GPB Gold View Nucleic Acid Stain (GenoPlast, Biochemicals, Poland). The detailed list of restriction enzymes used for each gene and the numbers of haplotypes obtained using each enzyme are presented in Table 1. Digestion products under 100 bp not visible on the gel have been omitted.

For obtained haplotypes from each sampling site, sequence analysis was conducted. The sequencing was carried out by Macrogen Europe (the Netherlands) using the same primer sets as in the amplification. The results were analysed using the Finch TV, BLAST and MEGA7 software. All original sequences of the selected mtDNA fragments of *Coregonus* spp. were submitted to GenBank [*ND5*: KX230401, KX230402, KX230403, KX230404, KX230405, KX230406, KX230407, KX230408, KX230409, KX230410, KX230411, KX230412, KX230413; *ND1*: KX230415, KX230416, KX230417, KX230418, KX230419, KX230420, KX230421, KX230422, KX230423, KX230424, KX230425, KX230426; D-loop: KX230427, KX230428, KX230429, KX230430, KX230431, KX230432, KX230433, KX230434, KX230435].

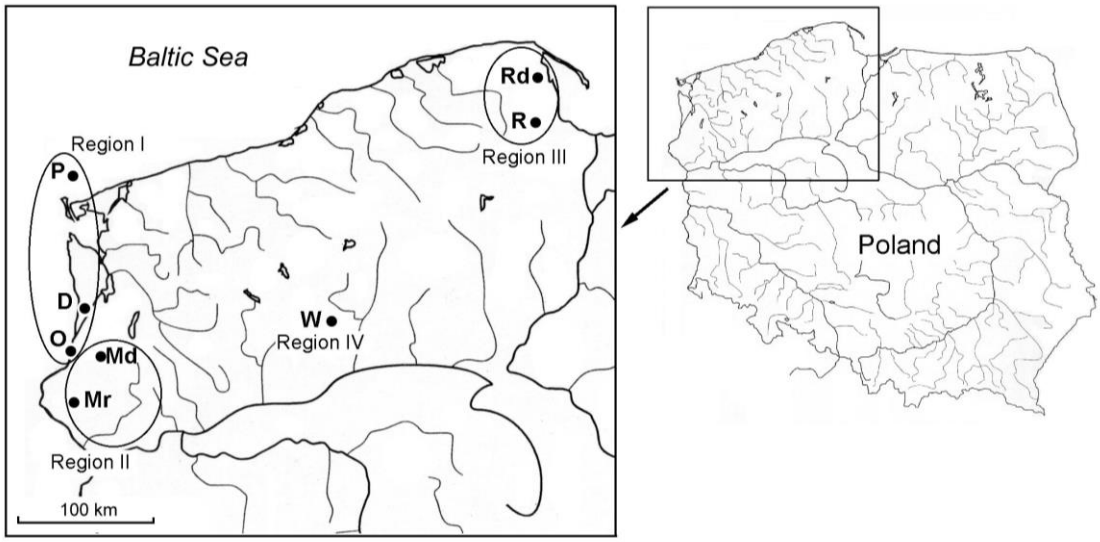

Figure 1. Map of the Poland selected sampling location. Research area: Western Pomerania: Dąbie Lake (D), Pomeranian Bay (P), Oder River (O) – Region I (migratory whitefish, *Coregonus nilssoni*); Miedwie Lake (Md), Morzycko Lake (Mr) – Region II (population of lake, *Coregonus maraena*); Eastern Pomerania: Reda River (Rd - migratory whitefish, *Coregonus maraena*), Rutki Hatchery (R - broodstock) – Region III; Wałcz Hatchery (W- broodstock) – Region IV.

Table 1. Pattern of restriction fragments for whitefish after digestion of the *ND1* gene with the used endonucleases *Alu*I, *Ava*II, *Bsu*RI, *Dde*I, *Hinf*I, and *Rsa*I

Restriction endonuklease	*Alu*I	*Ava*II			*Bsu*RI	*Dde*I	*Hinf*I		*Rsa*I	
No of haplotypes	1	1	2	3	1	1	1	2	1	2
Fragment sizes (bp)				1000				709		
					570					
		532	532							540
						468				
							416	416		
		380	380			390			390	390
	375									
			230				268			
	238								200	
		190							190	190
			158		172					
	145				121					

Data Analysis

Genetic similarity (GS) of investigated haplotypes defined by PCR-RFLP was calculated according to Nei and Li's (1979) coefficient, defined as;

$$GS = 2N_{AB}/(N_A+N_B),$$

where N_{AB} is the number of fragments shared by accessions A and B, N_A is the number of amplified fragments in sample A, and N_B is the number of amplified fragments in sample B. The haplotypes were grouped using the unweighted pair group method with arithmetic mean (UPGMA). Similarities among haplotypes were visualized with dendrogram.

Moreover, analysis of molecular variance analysis (AMOVA) was used to compute the distribution of genetic variability among and within sampling regions. AMOVA was performed using the program GenAlEx 6.5. Significance levels for variance component estimates were computed using 999 permutations.

Analysis of the nucleotide sequences was inferred using the Test Neighbor-Joining. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches (Kumar, Stecher, & Tamura 2016). The evolutionary distances

were computed using the Kimura 2-parameter method implemented in Mega 7 software (Kimura, 1980). The analysis was repeated using other tests (Maximum Likelihood – ML; Minimum Evolution – ME). For phylogenetic analysis, we used two own whitefish sequences, as well as other sequences from neighboring countries submitted to GenBank (Jacobsen *et al*., 2012).

Results

RFLP-PCR Analysis

The PCR-RFLP analysis of selected mtDNA fragments of whitefish revealed 40 restriction patterns using 8 different enzymes (Table 2). All haplotypes were grouped into compositions of haplotypes. The applied digestion technique of the *ND1* gene by *Alu*I, *Bsu*RI and *Dde*I (Table 1) as well as the *ND5/6* gene by *Dde*I (Table 3) revealed only one shared haplotype for each population/broodstock studied. The analysis of the broodstocks for haplotype prevalence demonstrated that 12 haplotypes were unique and present at only one site. Generally, only broodstock from Rutki hatchery was characterized by the presence of unique haplotypes in all individuals. In the case of the rest studied groups, the unique haplotypes were found only in single. The presence of one haplotype in all individuals of the migratory whitefish from the Reda River and all spawning individuals from the Rutki hatchery. In 80% analysed individuals from the Reda River, no variability of restriction sites was observed. This was distinctive compared to the other investigated populations and broodstock as particularly the D-loop fragment in the whitefish from other sites demonstrated variable restriction patterns. Half of the analysed haplotypes occurred in over 90% of all analysed fish, therefore the unique haplotypes occurred in a small number of individuals. The most genetic variants were obtained for the control region using two restriction enzymes, *Msp*I, 5 patterns, and *Rsa*I, 7 patterns (Table 4f).

Taking into account all restriction patterns of the two analysed genes and D-loop, 33 different compositions of haplotypes were obtained. The analysis of the combinations of gene patterns demonstrated that 10 of them occurred at more than one site (covering a total of 54% individuals), with the most frequent pattern 1 (4 sites), 2 and 6 (3 sites), as well as 4, 5, 7–10 (2 sites), Figure 2.

Based on the combination of haplotypes obtained in the restriction analysis, genetic distance between the combinations from each site was calculated (Table 5). The highest values were observed between the migratory whitefish from the Oder River and the whitefish from Lake Morzycko, the Pomeranian Bay and the eastern populations of the migratory whitefish (Rutki hatchery and Reda River), 0.029, as well as between the Oder River population and that of Lake Miedwie, 0.031, (Figure 3).

Sequence Analysis

For the analysed *ND1* fragment, 1011 bp were identified in all tested samples (974 bp within the gene and a 37-bp flanking region) and compared with each other. In total, 21 variable sites were identified, including 14 parsimony informative sites. All observed mutations were substitutions, including 10 transitions and 11 transversions. The most substitution was G↔C (38.1% substitutions), while

Table 2. List of restriction enzymes used for the digestion of mtDNA amplicons, including the number of haplotypes obtained and the percentage of their share in all analysed *Coregonus* spp. Restriction enzymes used and their cleavage sites: *Alu*I AG/CT, *Ava*II GG/CC, *BsuR*I GG/CC, *Dde*I C/TNAG, *Hinf*I G/ANTC, *Hha*I GCG/C, *Msp*I C/CGG, *Rsa*I GT/AC. Letters in parentheses mark the presence of the restriction pattern only in individuals from that site

Gen, part of mtDNA	Restriction enzyme	Number of haplotype						
		1	2	3	4	5	6	7
					%			
	*Alu*I	100						
	*Ava*II	88	4 (R)					
	*Bsu*RI	100						
ND1	*Dde*I	100						
	*Hinf*I	96	4 (O)					
	*Rsa*I	72	28					
	*Alu*I	98	2 (Md)					
	*Dde*I	100						
ND5/6	*Hinf*I	98	2(W)					
	*Rsa*I	25	7 (Mr)	60	7 (D,O)	1 (D)		
	*Ava*II	15	70	15 (D,W)				
	Hha	37	58	3 (O)	2 (Rd)			
D-loop	*Hinf*I	79	18	3 (O)				
	*Msp*I	79	4 (Mr,W)	11	6 (D,W)	1 (Rd)		
	*Rsa*I	50	20 (R,Rd)	3(D,O)	6 (Mr)	5 (D)	8 (W)	7

Table 3. Pattern of restriction fragments for whitefish after digestion of the *ND5/6* gene with the endonucleases *Alu*I, *Dde*I, *Hinf*I and *Rsa*I

Restriction endonuklease	*Alu*I	*Dde*I	*Hinf*I				*Rsa*I	
No of haplotypes	1	1	1	2	1	2	3	4
Fragment sizes (bp)				1425				
							1064	
					941	941	941	941
			724					
		643						
					607	607		607
	520	528						
	500					443		
	310		382	378				
					305	305	305	305
	279	283	270					
	237	244						
	184	215			206			
	164	156	172	172				
		100		100				

there was only one occurrence of the A↔T transversion. Seven substitutions led to amino acid changes in the protein, including those occurring twice at codon 322 (covering the mtDNA nucleotides at positions 3793–3795). As a result of substitution at the first or second position in the codon, substitution of three different amino acids, i.e. alanine↔threonine↔glycine, occurred in the protein, Table 6.

In the *ND5* gene, chromatograms allowed identification of 976 bp, which corresponded to 325 codons. The variability of DNA resulted from nucleotide substitution at 31 sites, 17 of which were parsimony informative sites (Table 7). Transversions were 59.4%, i.e. 19 mutations, and the most commonly observed substitution was C↔G (9 times). Among transitions, the most one was A↔G (9 times), while T↔C occurred only 3 times. At mtDNA position 12,041, a double substitution A↔G↔C occurred, resulting in a non-synonymous codon change, i.e. double glutamine↔lysine↔glutamic acid substitution in the protein. In total, 16 amino acid substitutions were observed.

The sequencing of the non-coding D-loop region yielded a fragment of 543 bp. Twelve substitutions and one deletion were found in the fragment. The number of transitions and transversions was the same, i.e. 6 substitutions of each type (Table 8). The T↔C transition occurred two times more frequently than A↔G, while the most transversion was A↔T (4 times). All samples from the fish caught in Lake Morzycko had a T deletion at mtDNA position 15,887–15,888.

The phylogeographic analysis employed sequences obtained by the authors and those obtained from GenBank, originating from whitefish caught in Denmark (lakes and fjords, JQ661442, JQ661434, JQ661475, JQ661462), Estonia (Baltic coast, JQ661389), Germany (lake, JQ661397) and Czech Republic (lake, NC_002646, according to Jacobsen *et al*., 2012).

Based on the nucleotide sequence analysis, genetic distance between the whitefish populations from the investigated regions was estimated at 0.1% (region III/Germany, and Estonian coast/Germany) to 1.0% (Czech Republic/region III and IV, and Denmark/region IV) (Table 9).

Based on the sequences of all analysed mtDNA fragments, a dendrogram was constructed in the MEGA7 software using three methods (Kimura 2-parameter, ME, ML) that yielded comparable results. The sequences obtained from the whitefish from Lake Morzycko were phylogeographically closer to those obtained from the fish caught at various sites in Denmark. Single sequences from the fish from region III (eastern migratory whitefish) grouped together with the sequences from the whitefish from the Baltic sea, Estonian coast and Lake Achterwasser in Germany (Jacobsen *et al*., 2012).

Based on the nucleotide sequences of the analysed mtDNA fragments (*ND1*, *ND5/6*, D-loop), mean genetic distance between the populations of the investigated regions was calculated (Table 7). The greatest distance was observed between the populations of region III (Lake Miedwie and Lake Morzycko) and that of region IV (whitefish hatchery in Wałcz), 0.008, and between the population of region IV and those of region I (migratory whitefish from Lake Dąbie, the Oder River and Pomeranian Bay), 0.007 (Figure 4).

Discussion

The first molecular analyses in fish were based

Table 4. Pattern of restriction fragments for whitefish after digestion of the D-loop gene with the endonucleases *Ava*II, *Hha*I, *Hinf*I, *Msp*I and *Rsa*I

Restriction endonuklease	*Ava*II			*Hha*I				*Hinf*I			*Msp*I					*Rsa*I						
No of haplotypes	1	2	3	1	2	3	4	1	2	3	1	2	3	4	5	1	2	3	4	5	6	7
Fragment sizes (bp)	507	591	1000	800	800	880	759	1150	1500	1249	580	688	580	800	580	783	1050	746	1050	746	1500	699
	345	345	300	320	500		410	150			500	400	400	500	500	419	240			554		300
							100				210				210							

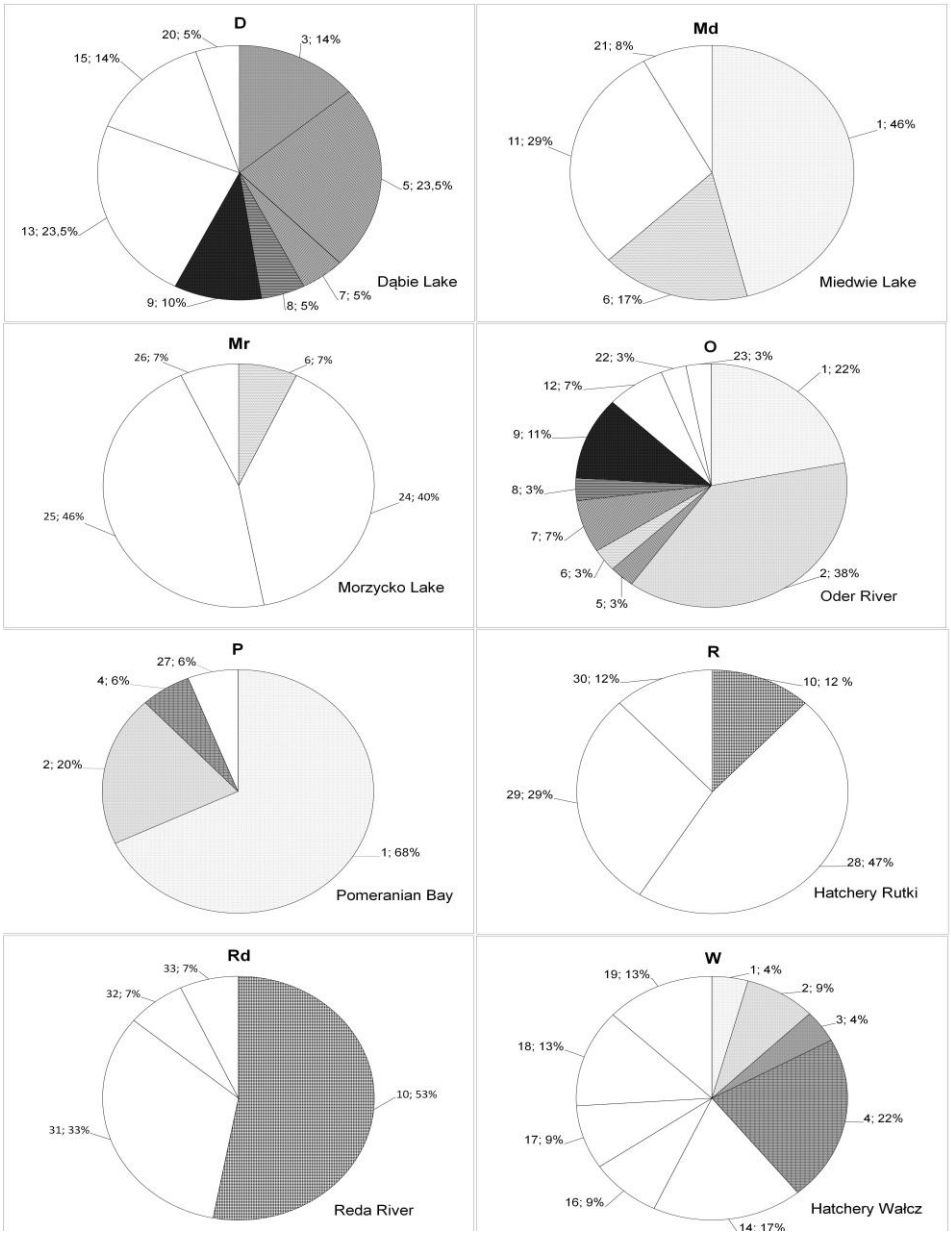

Figure 2. Haplotype combinations occurring in the investigated population of European whitefish (*Coregonus* spp.). Haplotype compositions 1–10 (with filling) were found at multiple sites; compositions 11–33 (without filling) were only found in individuals from single sites. Labelling: combination no., % individuals with the haplotype composition.

Table 5. Pairwise Population Linearized PhiPT Values. Analysis of molecular variance analysis (AMOVA) was used to compute the distribution of genetic variability among whitefish populations

Locality	W	D	Md	O	Mr	P	R	Rd
W	0.000							
D	0.000	0.000						
Md	0.025	0.020	0.000					
O	0.000	0.000	0.031	0.000				
Mr	0.023	0.017	0.000	0.029	0.000			
P	0.023	0.017	0.000	0.029	0.000	0.000		
R	0.023	0.018	0.000	0.029	0.000	0.000	0.000	
Rd	0.023	0.017	0.000	0.029	0.000	0.000	0.000	0.000

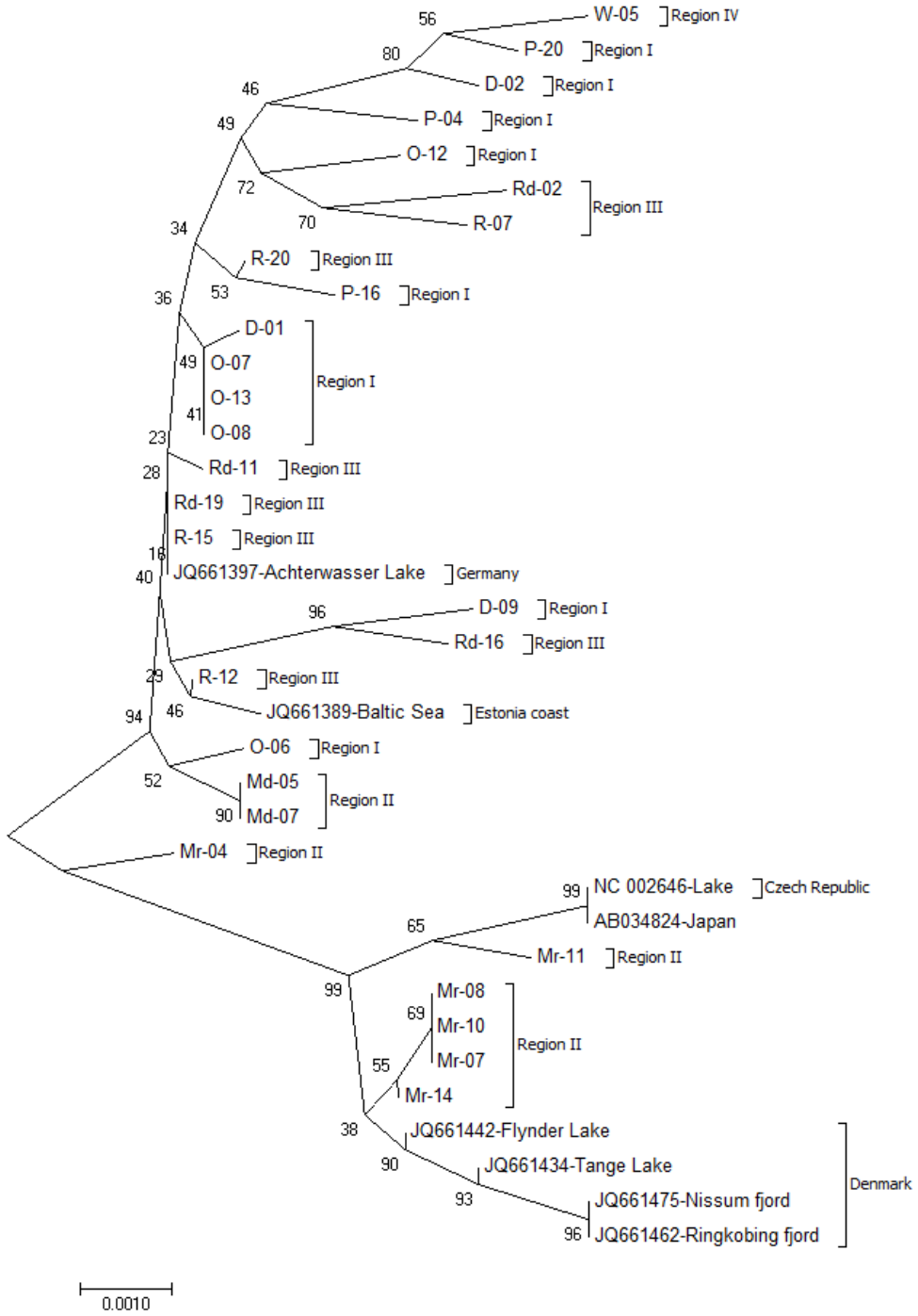

Figure 3. A dendrogram showing the genetic distances (Nei, & Li's 1979) between the whitefish populations studied (UPGMA).

on the PCR-RFLP technique that is successfully used to date. Characteristics of the species belonging to *Coregoninae* were presented by, e.g., Bernatchez *et al*. (1991), Bernatchez and Danzmann (1993), Hansen *et al*. (1999), Sukhanova, Smirnov, Smirnova-Zalumi, Griffiths, & Belikov (2002), Næsje, Vuorinen, & Sandlund (2004), Borovikova *et al*., (2012), and the described fish populations originated from different parts of the world, e.g., North America, Europe, Siberia. Regarding *C. lavaretus*, origin and relationship between populations from different regions were analysed (Bernatchez & Dodson, 1994; Hansen *et al*. 1999; Østbye, Næsje, Bernatchez, Sandlund, & Hindar, 2005).

In the studies of *Coregoninae*, *ND1* analysis is often used to assess the degree of hybridization, especially of the species that are very variable from the morphological point of view (Kohlmann *et al*., 2007; Gordeeva *et al*., 2008; Borovikova *et al*., 2012). As noted by Politov, Gordon, Afanasiev, Altukhov

Table 6. Polymorphic sites within the *ND1* gene. Positions are referred to the mtDNA sequence JQ661475[1]. Asterisks mark the positions of mismatch mutations

Position / Localities	2 025	2 967	3 049	3 078	3 142	3 207	3 237	3 333	3 387	3 534	3 567	3 598	3 615	3 712	3 759	3 788	3 790	3 792	3 793	3 794	3 822
	C	A	G*	C*	G*	T*	A*	G*	T*	G*	G*	C	C*	C	C*	C	C	G*	G*	C*	C
D-01	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
D-02	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
D-09	.	.	.	.	.	C	.	.	A	C	.	.	T	.	.	.	.	.	.	.	.
Md-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Md-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mr-04	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	A	.	.
Mr-07	.	.	.	T	.	.	G	C	.	.	.	.	.	.	T	.	.	A	A	.	.
Mr-08	.	.	.	T	.	.	G	C	.	.	.	.	.	.	T	.	.	A	A	.	.
Mr-10	.	.	.	T	.	.	G	C	.	.	.	.	.	.	T	.	.	A	A	.	.
Mr-11	A	.	.	T	.	.	G	C	.	.	.	G	.	.	T	.	.	A	A	.	.
Mr-14	.	.	.	T	.	.	G	C	.	.	.	.	.	.	T	.	.	A	.	.	.
O-06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
O-07	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-08	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-13	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P-04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P-16	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
P-20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
R-12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R-15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R-20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-02	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-11	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-16	.	.	.	.	.	C	.	.	A	C	.	.	T	.	.	G	A	.	.	.	G
Rd-19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
W-05	.	.	.	T	.	.	G	C	.	.	C	.	.	.	.	.	.	.	.	.	.
AA					V/ I							H/ D		L/ F			A/ G	L/ M	A/ T/ G		

AA: A – alanina, D – aspartic acid, F – fenyloalanina, G – glicyna, H – histydyna, I – izoleucyna, L – leucyna, M – metionina, V-walina, T – treonina; [1] Jacobsen et al. 2012

Table 7. Polymorphic sites within the *ND5* gene. Positions are referred to the mtDNA sequence JQ661475[1]. Asterisks mark the positions of mismatch mutations

Position	11 965	12 000	12 023	12 031	12 040	12 041	12 050	12 051	12 059	12 085	12 139	12 183	12 292	12 310	12 360	12 402	12 439	12 546	12 596	12 604	12 723	12 724	12 745	12 784	12 799	12 811	12 841	12 842	12 875	12 876	12 923
Localities	A	C*	A	T	C	C	G*	G*	G	C	G	A*	C*	C*	T	C*	A*	A*	A*	A*	T*	G*	G*	G	C	T*	C*	G*	G	C	C
D-01	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D-02	.	.	.	.	.	.	T	T	.	.	.	.	.	G	.	.	.	G	G	.	.	.	.	.	.	A	.	.	.	.	.
D-09	.	T	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Md-05	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
Md-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
Mr-04	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mr-07	.	T	.	.	.	.	.	.	.	.	.	G	T	.	.	T	G	.	.	.	.	A	A	.	.	.	.	.	.	.	.
Mr-08	.	T	.	.	.	.	.	.	.	.	.	G	T	.	.	T	G	.	.	.	.	A	A	.	.	.	.	.	.	.	.
Mr-10	.	T	.	.	.	.	.	.	.	.	.	G	T	.	.	T	G	.	.	.	.	A	A	.	.	.	.	.	.	.	.
Mr-11	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	T	G	.	.	.	.	A	A	A	.	.	.	.	.	.	.
Mr-14	.	T	.	.	.	.	.	.	.	.	.	G	T	.	.	T	G	.	.	.	.	A	A	.	.	.	.	.	.	.	.
O-06	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
O-07	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-08	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-12	C	.	.	.	.	A	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
O-13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P-04	.	.	.	.	.	.	T	T	.	G	T	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P-16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	.	.	.
P-20	.	.	.	.	.	.	T	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
R-07	.	.	.	A	G	G	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.
R-12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R-15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R-20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-02	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	G	C	C	G	A
Rd-11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
Rd-19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
W-05	.	.	.	.	.	.	T	T	.	.	.	.	.	G	.	.	.	G	G	.	.	.	.	.	.	A	.	.	.	.	.
AA	A/V	T/A			N/K	Q/K/E	G/L		A/P					R/G	V/G			N/S	T/A		M/K				C/W		C/W	A/P	A/R		L/I

AA: A – alanina, C – cysteina, G – glicyna, I – izoleucyna, K – lizyna, L – leucyna, M – metionina, N – asparagina, Q – glutamina, P – prolina, R – arginina, V-walina, T – treonina; [1] Jacobsen et al. 2012

Table 8. Polymorphic sites within the (D-loop) gene. Positions are referred to the mtDNA sequence JQ661475[1]. Asterisks mark the positions of mismatch mutations

Position / Localities	15 683	15 684	15 716	15 771	15 819	15 831	15 833	15 847	15 887	15 969	15 982	15 996	16 149
	A	T	A	T*	A	C*	T*	T	T*	T	G	C*	T*
D-01	.	.	.	.	.	.	.	.	.	.	.	.	.
D-02	.	.	.	.	.	.	A	.	.	.	.	.	.
D-09	.	.	.	.	.	.	A	.	.	.	.	.	.
Md-05	.	.	.	.	G	.	A	.	.	.	.	.	.
Md-07	.	.	.	.	G	.	A	.	.	.	.	.	.
Mr-04	.	.	.	.	.	T	.	–	C	.	.	T	.
Mr-07	.	.	.	.	.	T	.	–	C	.	.	T	.
Mr-08		.	.	.	.	T	.	–	C	.	.	T	.
Mr-10	.	.	.	.	.	T	.	–	C	.	.	T	.
Mr-11	.	.	.	.	.	T	.	–	C	.	.	T	C
Mr-14	.	.	.	.	.	T	.	–	C	.	.	T	.
O-06	.	.	.	.	.	.	.	.	.	.	.	.	.
O-07	.	.	.	.	.	.	.	.	.	.	.	.	.
O-08	.	.	.	.	.	.	.	.	.	.	.	.	.
O-12	.	.	.	.	.	.	.	.	.	G	C	.	.
O-13	.	.	.	.	.	.	.	.	.	.	.	.	.
P-04	.	.	.	.	.	.	.	.	.	.	.	.	.
P-16	.	.	.	A	.	.	.	.	.	.	.	.	C
P-20	.	.	G	.	.	.	A	.	.	.	.	.	.
R-07	.	.	.	.	.	.	.	.	.	.	.	.	.
R-12	.	.	.	.	.	.	.	.	.	.	.	.	.
R-15	.	.	.	.	.	.	.	.	.	.	.	.	.
R-20	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-02	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-11	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-16	.	.	.	.	.	.	.	.	.	.	.	.	.
Rd-19	.	.	.	.	.	.	.	.	.	.	.	.	.
W-05	T	A	.	.	.	.	.	.	.	.	.	.	.

[1] Jacobsen et al. 2012

Table 9. Genetic distance between populations from different regions Polish and neighboring countries

	Region I	Region IV	Region II	Region III	Denmark	Czech Republic	Germany
Region I							
Region IV	0.005						
Region II	0.007	0.008					
Region III	0.003	0.006	0.006				
Denmark[1]	0.009	0.010	0.005	0.009			
Czech Republic[1]	0.010	0.010	0.006	0.009	0.004		
Germany[1]	0.002	0.005	0.005	0.001	0.007	0.008	
Estonia coast[1]	0.003	0.006	0.006	0.002	0.008	0.008	0.001

[1] Jacobsen et al. 2012

and Bickham (2000), RFLP analysis of the *ND1* gene using the restriction enzyme *Rsa*I is useful as the obtained restriction patterns are used for the discrimination between the species of *Coregoninae*. In our study, we observed a low degree of variability (2 patterns) in the *Rsa*I restriction sites. In the whitefish populations from north-eastern Poland, Kempter *et al*. (2010) found a higher degree of variability (4 haplotypes).

The use of *Ava*II, *Dde*I and *Hinf*I revealed the presence of 3, 1 and 2 haplotypes, respectively. Similar results were obtained by Hansen *et al*. (1999)

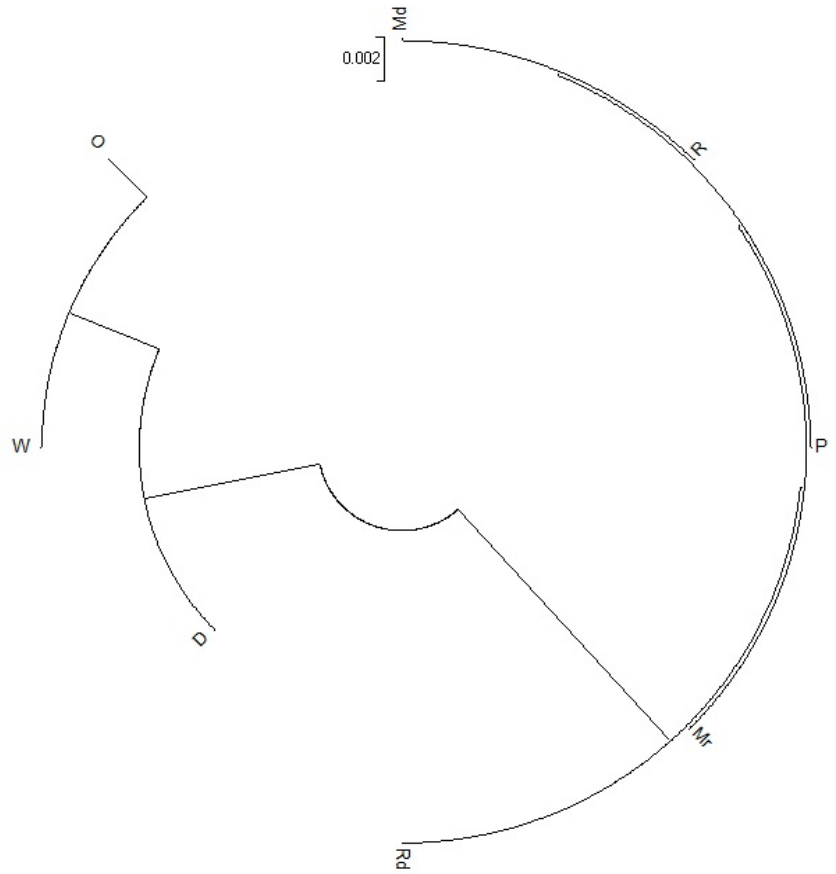

Figure 4. Analysis of the similarity of the Polish *Coregonus* spp. populations from natural and rearing reservoirs and the populations from neighbouring countries based on the sequences of mtDNA fragments (*ND1*, *ND5* and D-loop).

who investigated *C. lavaretus* from Denmark, however, three haplotypes were identified after digestion with *Dde*I. Single restriction patterns for each enzyme were spotted for the Polish and Danish populations. Gordeeva et. al. (2008) who analysed the population of *C. lavaretus pidschian* from the Tuva Republic, Russia, used *Bsu*I, *Dde*I, *Rsa*I that yielded 2, 2 and 3 haplotypes, respectively, for the *ND1* gene. The only patterns in with the Polish populations were those obtained using *Rsa*I (1 haplotype).

PCR-RFLP analysis of the mtDNA *ND5/6* gene of the Danish whitefish populations (Hansen *et al*., 1999) using *Dde*I and *Rsa*I yielded 2 haplotypes each, while in our study (approx. 2400 bp)—1 and 4, respectively. One haplotype (3) obtained with *Rsa*I present in the majority of the Polish whitefish populations was present in the fish investigated by Hansen *et al*. (1999).

Analysis of the noncoding mtDNA control region, D-loop, is often used in population studies due to the high intra- and interspecies variability (Schulz *et al*. 2006; Oleinik & Skurikhina, 2008). Therefore, as predicted, the restriction analysis of D-loop revealed more restriction patterns than that of *ND1* and *ND5/6*, and numerous unique haplotypes were obtained for the whitefish from each site. Our analysis of D-loop haplotypes (approx. 1300 bp) of the fish from the investigated sites revealed the presence of 3 different haplotypes per each of the two enzymes: *Ava*II and *Hinf*I. Comparable results were obtained by Brzuzan (1998) in the whitefish from Lake Maroz (Masuria, north-eastern Poland) (1300 bp) and Lake Baikal (Russia) (1130 bp), in which 3 and 2 haplotypes, respectively, corresponded to the size of restriction fragments obtained in this study.

As shown by the results of the PCR-RFLP analysis of three mtDNA fragments, haplotype patterns obtained by digesting material from different whitefish populations with the given enzyme differ in the presence or absence of additional products compared to the whitefish from other (Hansen *et al*., 1999; Gordeeva *et al*., 2008). The variability of restriction sites may be of adaptive nature. The phylogeographic analysis conducted on the basis of the authors' nucleotide sequences for *ND1*, *ND5/6* and the control region, as well as the sequences of those mtDNA fragments from fish caught in Denmark, Germany or Estonia (Jacobsen *et al*., 2012) obtained from GenBank indicates their history. The results are in accordance with the hypothesis by Østbye *et al*. (2005) that the Polish and other Baltic whitefish populations belong to the northern Europe clad.

Whitefish as a species has undergone a rapid postglacial speciation through diversification and

colonization of post-glacial lakes with different ecological conditions (Østbye *et al*., 2006). Moreover, easy gene exchange was observed between the various populations (Politov *et al*., 2000). Due to the feeding habits of this species, ecological selection has contributed to the occurrence of various morphological forms and subspecies via adaptive radiation (Østbye *et al*., 2006). Also in lakes in which sympatric forms occur, parallel speciation may be observed (Landry, Vincent, & Bernatchez, 2007).

According to recent studies, *C. lavaretus maraena* (Mysłowski *et al*., 2011) previously identified in Polish studies is actually *C. maraena*, while *C. lavaretus generosus* (Szczerbowski, 2000; Polewacz *et al*., 2015) is actually *C. nilssoni* (Kottelat & Freyhof, 2007).

In this study, the whitefish from Lake Miedwie had 1 unique haplotype and few haplotype combinations which may result from the stability of the population thanks to the annual reintroduction of spawning individuals in this lake [PZW 2014 (Polish Fishing Association)]. This is also practised in the second investigated lake with whitefish, Lake Morzycko. The whitefish populations in both lakes are separate and have a high number of unique haplotype combinations, which may indicate adaptation to the environment. Phylogeographically, the population from Lake Morzycko is close to the Danish populations (as indicated by nucleotide sequence analysis), which is a feature particularly distinguishing that population from other investigated populations.

The populations of migratory whitefish from Lake Dąbie, the Oder River and Pomeranian Bay were small in the 1980s, but the later reintroduction in the Szczecin Lagoon and the neighbouring areas under the Polish-German cooperation in 1995–2002, 2005–2009 [MIR (Sea Fisheries Institute), 2015] and currently (data according to PZW) contributed to the recovery of these populations (Czerniejewski & Rybczyk, 2010). The species currently reproduces naturally and is caught commercially (MIR, 2015). The populations of migratory whitefish from the Oder River and Lake Dąbie are characterized by the highest variability among the fish from all investigated sites. A large number of haplotypes and their combinations was reported there, with part of them being common for both sites. Lake Dąbie is connected with the Oder River by channels, therefore free crossbreeding between the individuals from the two populations occurs. Lake Dąbie is a large lake (54.08 km²) with a well-developed coastline which allows formation of numerous microenvironments enabling differentiation of sympatric populations and maintaining their sizes at a constantly high level. Similarly, the lower Oder River is varied from the ecological point of view. The region covers the area of Międzyodrze with a network of natural and artificial channels, oxbow lakes and marshes, and belongs to the Landscape Park of the Lower Oder River Valley. Such conditions promote genetic variability in species living in such area. The presence of numerous haplotype combinations in the whitefish from these sites may be due to the fact that the material for reintroduction was obtained from different sources every time (spoken statement from the employees of MIR 2015).

The migratory whitefish caught in the Reda River enter to the Bay of Puck in which the reintroduction is conducted using material originating from a broodstock the Rutki hatchery maintained since early 1990s (spoken statements from the employees of PZW, Gdańsk). Thus conducted reintroduction may be the cause of the low intrapopulation variability of the migratory whitefish from the Reda river and the high frequency of the shared haplotypes with that of the fish from the Rutki hatchery, occurring only at these sites. The share of the haplotype combinations characteristic for reared fish among the individuals of whitefish caught in Reda is very clear, more than 50%. The other combinations are unique. The presence of unique haplotypes in the whitefish from Rutki and the Reda River, and one unique haplotype common for both groups may indicate the adaptation process. In contrast to the western migratory whitefish, the populations from the Reda River are characterized by a lower genetic variability. Furthermore, their size increases, which demonstrates the success of the multiannual stocking activities and the good adaptation of the species to the environmental conditions (Pelczarski, 2004). Such processes were noted earlier in whitefish by Brzuzan (1998) and Bernatchez and Danzman (1993). According to Gordeeva *et al*. (2008), the genetic appearance of the population is a result of interaction between the homogeneous baseline material and the influence of the environmental conditions, and this effect is the strongest at the earliest stages of introduction.

High variability is also demonstrated in the reared broodstock of whitefish from Wałcz and those caught from the nearby lakes (spoken statements of fishermen). Regarding the number of haplotype combinations, it is comparable with the populations from Lake Dąbie or the Oder River, but in this case, unique combinations have a high share in the overall variability. In the Wałcz hatchery, there are as many as 5 different unique combinations, constituting a total of 61%. The variability observed in this case results mainly from the use of spawners originating from multiply sites as well.

Monitoring of the genetic pool of individuals occurring in natural waters and reared individuals seems to be necessary. Reintroduction of closely related material, originating from a limited number of spawning individuals, is a threat to the population. The impoverishment of the gene pool and the possibility of inbred are some of the hazards of constant introduction of foreign material in the environment, particularly in the case of closed, small lakes with uncomplicated coastline, which does not

help the formation of specific microenvironments (Leberg & Firmin, 2008).

In the study, high variability of the western populations and lower variability of the eastern and lake populations of whitefish was demonstrated. It was also shown how important it is to implement a rational fishery policy based on reared fish, including genetic monitoring. Conservative aquaculture that uses only its own juvenile fish for stocking favors conserving the same gene combinations. This is associated with adaptation of the fish to the given environment, however, in closed populations kept in small basins that are poor in flora and fauna such policy can lead to a reduction of the genetic pool. Therefore, characterizing the European (including Polish) populations provides much information about this valuable species and helps to preserve biodiversity.

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