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Genetic structure of the cisco (*Coregonus albula* L.) from lakes of glacial origin in northern Poland

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Abstract: European cisco (*Coregonus albula* L.) is one of the most precious species of the European lake ichtyofauna, however, due to progressive eutrophication of water, the range of its occurrence has decreased. Deteriorating ecological conditions are the main cause of this decline in population, and most of the existing populations in lakes are maintained thanks to reintroduction. Thus, it is important to determine the genetic structure of the European cisco. The study involved PCR-RFLP-based genetic analysis of *C. albula* caught in 15 lakes in northern Poland, including four lakes located in national parks. The analysis covered 3 genes located in the mitochondrial DNA: ND1, ND3/4 and cytochrome *b*, as well as a control region (D-loop). The PCR product was digested with 4 endonucleases (*Rsa*I, *Msp*I, *Bsu*I *and Hha*I) and the resulting haplotypes were grouped into combinations. Statistical analysis were then performed on these groups. Based on the genetic distance, a phenogram was constructed in which two groups could be distinguished. One group was represented by *C. albula* populations from most lakes in north-western Poland, including the three protected lakes. The other group consisted of the European cisco population from Lake Wigry (north-eastern Poland) and commercially exploited lakes from the southern part of the investigated north-western Poland area. The results of the study of *C. albula* from northern Poland present a valuable molecular characterization of the populations and can be a starting point for further genetic monitoring.

Keywords: Cisco, *Coregonus albula*, mtDNA, PCR-RFLP

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1 Introduction

The European cisco (*Coregonus albula* L.) is one of the most precious species of the Polish and European lake ichtyofauna due its role in the preservation of lake biodiversity and ecosystem balance, as well as its economic significance [1,2]. The species has high requirements with respect to the quality of waters it inhabits, however, due to the increasing eutrophication of lakes, the range of occurrence of its natural population has decreased [3-7]. In Poland, its conservation status is considered as vulnerable [8]. Deteriorating ecological conditions have lead to a decline in the population of the European cisco, and most of its populations in Polish lakes are maintained thanks to reintroduction [9]. The European cisco populations from natural spawning sites constitute a small fraction of the total population [10]. In north-western Poland, the European cisco is present in less than 3% lakes, and half of these lakes are monitored by professional fishermen [11]. Previous studies of the European cisco population from this area have focused on age structure, growth rate, condition [10,12] and fertility [13]. Most lakes from which the investigated fish originate are located in protected areas included in the Natura 2000 network. These are very valuable populations, which have not been genetically analysed to date. Conducting their genetic characterization is particularly important for the protection of the existing natural populations and sustainable management of the European cisco stocks [14,15].

For many years, the PCR-RFLP technique of analysing mtDNA has been successfully used to study populations of fish, allowing the differentiation of characteristic haplotypes [16]. The investigated species include *Clupea harengus* [17], *Salmo trutta* [18,19] *C. peled* [20] and other *Coregonus* sp.[21]. Most analyses conducted to date regarded the origin and migration destinations of *Coregonidae* [22,23], as well as interspecific differences [24,25,26,27] and phylogenetic links between the investigated populations [16,28,29,30]. Studies focused on the directions of introgression and the estimation of gene

migration rate and its impact on the genetic structure of the population require an exact genetic characterization.

The studies of the European cisco conducted to date in Poland included small sample size from few lakes and focused on selected aspects determined using phylogenetic analyses of mtDNA [31]. The studies were conducted primarily based on the sequence of D-loop control region and a fragment of the gene encoding NADH dehydrogenase subunit 3 and 4 (ND3/4) [25,32,33]. In other countries, studies of the genetic structure of the European cisco were carried out mainly by means of the RFLP or PCR-RFLP techniques.

The aim of this study is to perform a genetic characterization of the European cisco population from lakes located in two national parks and inhabited by natural populations of this species, as well as lakes in which the fish are introduced and commercially caught. The study identified 15 natural lakes of glacial origin in north-western Poland, including 3 lakes located in Drawa National Park (DNP), and Lake Wigry located in Wigry National Park (WNP) in north-eastern Poland. In the lakes of DNP, the local populations have not mixed with individuals from other lakes for several decades. Our analysis covered very valuable natural populations, as well as populations from lakes included in the Natura 2000 network. This is a particularly interesting area of analysis, as it will elucidate the genetic diversity of populations from lakes located in these protected areas. The obtained results may be helpful in the restoration of the genetic structure of the European cisco in lakes whose populations have decreased or have become extinct. The genetic analysis was performed based on methods commonly used by many research centres, thus allowing comparison of the obtained data.

2 Material and Methods

2.1 The studied localities

The study was conducted using 450 European cisco individuals, 30 from each lake. The fish were obtained from the following lakes: Western Pomerania: Miedwie (M), Morzycko (R); Drawa Lake District: Bytyń (B), Drawsko (D), Lubie (L), Pile (P), Siecino (S), Żerdno (Z); strict protection zone of Drawa National Park (DNP): Marta (E), Ostrowieckie (T), Płociowe (C); area around DNP: Szczuczarz (A); at DNP border: Tuczno (U), Liptowskie (I); and north-eastern Poland, Masurian Lake District (Wigry National Park, WNP): Wigry (W) (Figure 1). The catches of the European cisco from the lakes of DNP were performed with permission of the Ministry of the Environment, no. DLPpn-4102-229/17717/13/M. As for other lakes, the fish were bycatch in commercial fishing. The study involved taking muscle samples using a sterile technique, which were subsequently stored in Eppendorf-type tubes and frozen until analysis.

Most of the investigated lakes, in which the European cisco was caught, are of glacial origin. The lakes in DNP are dimictic, thus the water in the deepest areas mixes only in the spring and autumn. These lakes are characterized by a low fertility, water transparency of 2–4 m and a high oxygen content, even during the summer months. Water at depths are at 5°C favouring the European cisco, which is a naturally occurring species in these lakes. In the period 2004–2008 in the lakes of DNP reintroduction using juvenile individuals, originating from fish spawning in these lakes, was performed. The aim was to preserve the populations of the European cisco from the lakes in the strict protection zone of DNP. Water in other lakes where the European cisco was caught is of 1st class (A, L, S, Z), 2nd class (B, I, P, R, U) and 3rd class (D, M) purity. These lakes are, in part, deep (approx. 40 m) and have a sand and gravel bottom, which favours their inhabitance by the European cisco. Lake Wigry, located in WNP and listed by the International Union for Conservation of Nature (IUCN) among the world's most valuable waterbodies (Project Aqua). In this waterbody, the population of the European cisco has decreased since the 1990s, which was influenced by an increase in the fertility of the lake and the management of fisheries. Currently, due to the implementation of protection plans and reintroduction, the population of the European cisco in this waterbody remains constant and has been a source for species reintroduction into many nearby lakes [9]. According to the information obtained from fishermen, European cisco used for reintroduction in lakes outside of the strict protection zone of DNP and WNP, originated from different lakes, depending on the requirements. The process was continued for several decades and was rather arbitrary. Fish used for reintroduction in Lake Miedwie (M) and Lake Morzycko (R originated from the spawning fish from the same lake.

2.2 MtDNA RFLP anlysis

Total DNA was isolated using phenol-chloroform extraction, following Bernatchez *et al.* (1988), and kept at -70°C until analysis. The analysis of the genetic structure of the European cisco population from lakes in northern Poland was based on the PCR-RFLP technique.

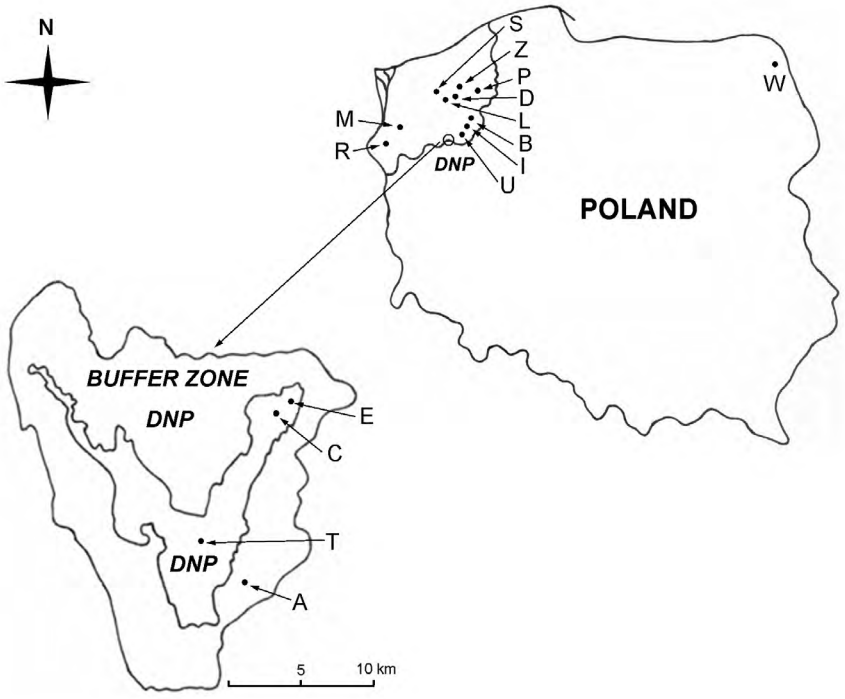

Figure 1. Map of Poland marking the selected sampling location. Research area: lakes with strict protection zone Drawieński National Park (DPN)[1]: Marta (E), Ostrowieckie (T), Płociowe (C); lake in the buffer zone[2] DPN: Szczuczarz (A); lake at the border of DPN: Tuczno (U), Liptowskie (I); other lakes Lakeland Drawieński: Bytyń (B), Drawsko (D), Lubie (L), Pile (P), Siecino (S), Żerdno (Z); other lakes north - west Poland: Miedwie (M), Morzycko (R); the lake from the north - eastern Polish (Wigierski National Park)[3]: Wigry (W).

Altogether, 3 mitochondrial DNA (mtDNA) genes were analysed: ND1 (NADH dehydrogenase, subunit 1 [complex I]), ND3/4 (NADH dehydrogenase, subunit 3–4 [complex I]) and cytochrome *b*, as well as a noncoding control region (D-loop). Primers used in the PCR and annealing temperatures for each pair are given in Table 1. For the PCR, the GoTaq Flexi® DNA Polymerase (Promega, USA) was used. The final reagent concentrations were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ for *cytochrome b* and ND1, 2.4 mM $MgCl_2$ for the D-loop region and 3.0 mM $MgCl_2$ for ND3–4, 200 µM of each deoxynucleoside triphosphate, 10 pM of each primer and DNA template. The PCR regime was adjusted to the requirements of the GoTaq®Flexi DNA Polymerase, according to the manufacturer's recommendation. The results of PCR amplification were visualised using electrophoresis of 5 µl of each sample on 1.5% agarose gels with GPB Gold View Nucleic Acid Stain (GenoPlast, Biochemicals, Poland).

The amplicons were digested using 4 restriction enzymes: *Rsa*I, *Bsu*I, *Msp*I, *Hha*I (Thermo Scientific, USA). Digestion was performed according to the manufacturer's recommendation. Cleavage products were checked on a 3.0% agarose gels with GPB Gold View Nucleic Acid Stain (GenoPlast, Biochemicals, Poland). For a detailed list of enzymes used for the digestion of individual genes, as well as the obtained number of haplotypes, see Table 2. In order to compare the European cisco populations from each lake, the obtained haplotypes were grouped into combinations.

2.3 Statistical treatments

The results were stored on a BioRad gel documentation system and analysed using the Quantity-One® software (BioRad, USA). The genetic similarity (GS) of the investigated haplotypes was calculated according to Nei and Li's [34] 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). The similarities among the haplotypes were visualized with a dendrogram.

Moreover, analysis of molecular variance (AMOVA) was used to compute the distribution of genetic variability among and within the populations. AMOVA was performed

Table 1. Primers and restriction enzymes used to amplify DNA of European cisco

mtDNA region/ gen	Primer sequence	Anneling temperature	Length of PCR product (approximate values)	Reference	Restriction enzymes
ND1	Tt-ND1-F1: 5'-GTA ATT GCG AAA GGC CTA AG-3' Tt-ND1-R1: 5'-CCC CTA TTA GCC ACG CTA TC-3'	52°C	whole NADH-dehydrogenase 1 gene 1300 bp	[15]	*Rsa*I, *Bsu*I, *Msp*I
ND3/4	ND3-4A: 5'- TTAATACGTATAAGTGACTTCCAA-3' ND3-4B: 5'-TTTTGGTTCCTAAGACCAATGGAT-3'	54°C	2 200 pz	[42]	*Rsa*I, *Bsu*I, *Msp*I
D-loop	L19: 5' CCACTAGCKCCAAACTA 3' H17: 5' ACTITCTAGGGTCCATC 3'	54°C	whole control region –D-loop 1300	[50] [44]	*Rsa*I, *Bsu*I, *Msp*I, *Hha*I
Cytochrome *b*	L14724B: 5'-CGAGATCTGAAAAACCATCGTTG-3 H15915: 5'- ACTGCAGTCATCTCCGGTTTACAAGAC-3'	52°C	1 000 pz	[51]	*Rsa*I, *Bsu*I, *Msp*I

Table 2. List of restriction enzymes used for the digestion of mtDNA amplicons, including the number of haplotypes obtained (nc* = not cut)

mtDNA region/gen	*Rsa*I *GT/AC*	*Bsu*I *GG/CC*	*Msp*I *C/CGG*	*Hha*I *GCG/C*
ND1	2	2	1	nc*
ND3/4	3	5	1	nc*
D-loop	7	1	7	7
Cytochrome *b*	1	1	1	nc*

using the GenAlEx 6.5 program. The significance levels for variance component estimates were computed using 1000 permutations.

3 Results

For all isolates of DNA from *C. albula*, the obtained amplification products were of the expected length (Table 1). The PCR-RFLP analysis of four mtDNA regions revealed 37 different restriction patterns, but since 1 haplotype per enzyme was obtained with the cytochrome b gene in all populations, only the haplotypes obtained with ND1, ND3–4 and D-loop were considered in further considerations (a total of 34 restriction patterns). For ND1, digestion with *Msp*I produced 1 haplotype (haplotype 1), while that with *Rsa*I (Figure 2a) and *Bsu*I (Figure 2b) produced 2 haplotypes per enzyme, Table 3. For the mtDNA fragment covering ND3 and ND4, 1 restriction pattern was obtained with *Msp*I (haplotype 6), 3 with *Rsa*I (Fiure. 3a) and as many as 5 with *Bsu*I (Figure 3b). The most genetic variants were obtained for the noncoding control region (D-loop): 7 patterns were obtained with *Msp*I (Fig. 4a) and *Hha*I (Figure 4b), 5 with *Rsa*I (Figure 4C) and only 1 with *Bsu*I (haplotype 27), Table 4.

Taking into account all restriction patterns of the 3 analysed genes and D-loop, 80 different compositions of haplotypes were obtained. Grouping all the obtained haplotype patterns into combinations allowed the comparison of each composition, including all the analysed mtDNA fragments. The analysis of the combinations of gene patterns demonstrated that 16 of them occurred at more than one site, with the most common patterns being pattern 8 (9 sites) and pattern 6 (5 sites), Table 5. Other patterns occurred two or three times. As many as 64 patterns were unique and were found only at one site (Figure 5a–w). In Lake Szczuczarz (A), located in the area around DNP, the least combinations (2) were observed, including one unique and one occurring at a number of sites (pattern 8) (Table 6, Figure 5). In Lake Marta (E), Lake Płociowe (C) and Lake Ostrowieckie (T), located within the strict protection zone of DNP, 5 haplotypes were distinguished in the former two lakes and 7 haplotypes were distinguished in the latter lake (Figure 5a-c). In these lakes, a total of 6 unique OF haplotype compositions were observed, while 3 (6, 11, 15) were common for two sites in various combinations. Variant 11 of haplotype compositions also occurred in Lake Szczuczarz (A) (Figure 5d) of the area around DNP, located near Lake Ostrowieckie (T) of DNP, and constituted 66% of haplotype compositions identified in that lake. The most combinations, i.e. 11 different patterns, were found in Lake Bytyń (B), (Figure 5g), Lake Miedwie (M) (Figure 5m)

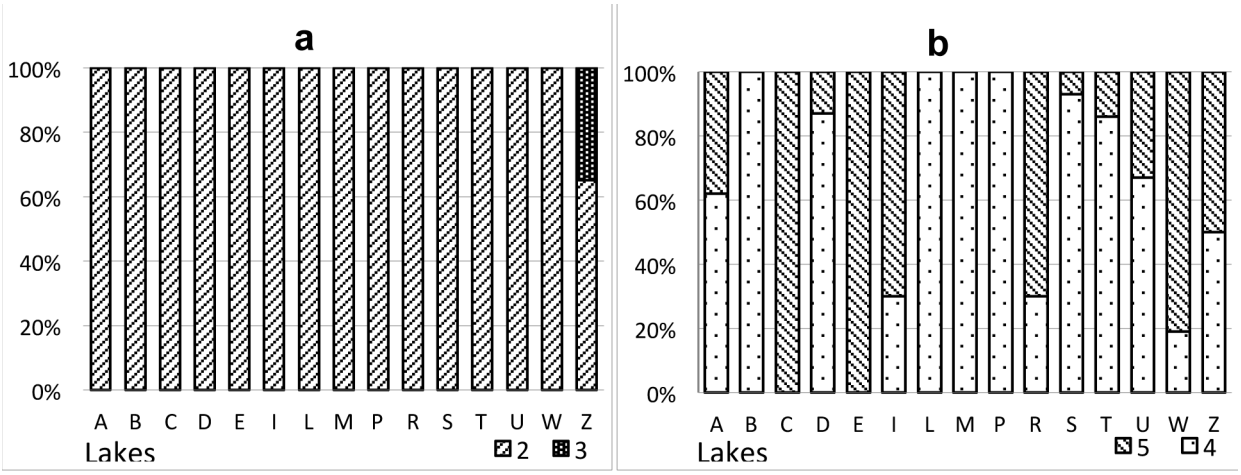

Figure 2. Distribution of haplotypes for the ND1 gene digested with *Rsa*I (a) and *Bsu*I (b) in the European cisco populations from the investigated lakes; legend: haplotype no. Site letter code as in Figure 1.

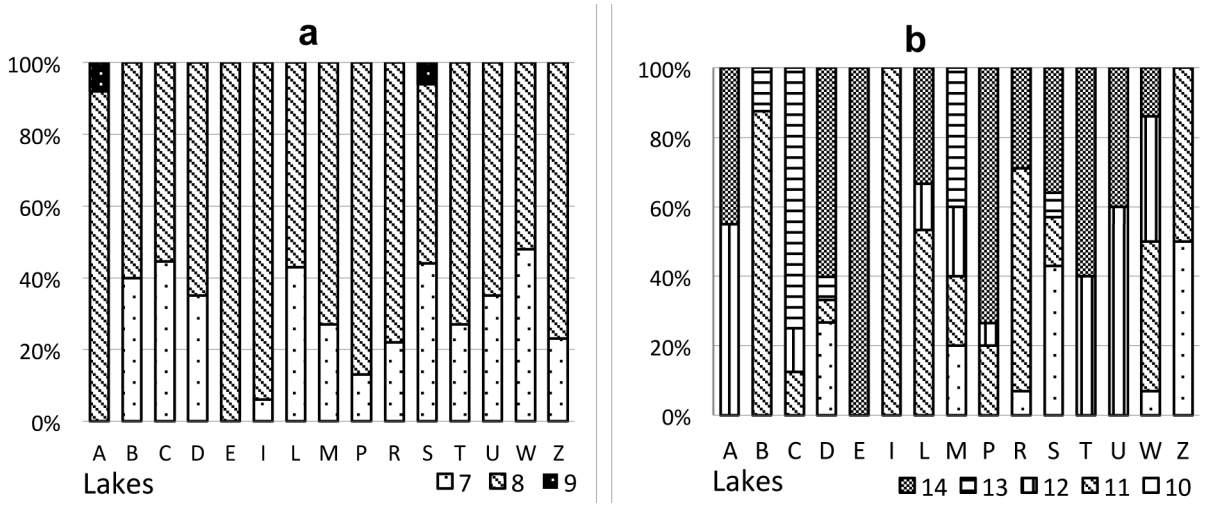

Figure 3. Distribution of haplotypes for the ND3/4 gene digested with *Rsa*I (a) and *Bsu*I (b) in the European cisco populations from the investigated lakes; legend: haplotype no. Site letter code as in Figure 1.

Table 3. Pattern of restriction fragments for European cisco after digestion of the ND1 gene with the endonucleases *Msp*I, *Rsa*I and *Bsu*I.

Restriction endonuclease	***Msp*I**	***Rsa*I**		***Bsu*I**	
No of haplotypes	1	2	3	4	5
Fragment sizes (bp)			693		
	485				
					400
		387			
				369	
	300				
	263				
		200			
	185			170	183
	142	138		126	133
	108				

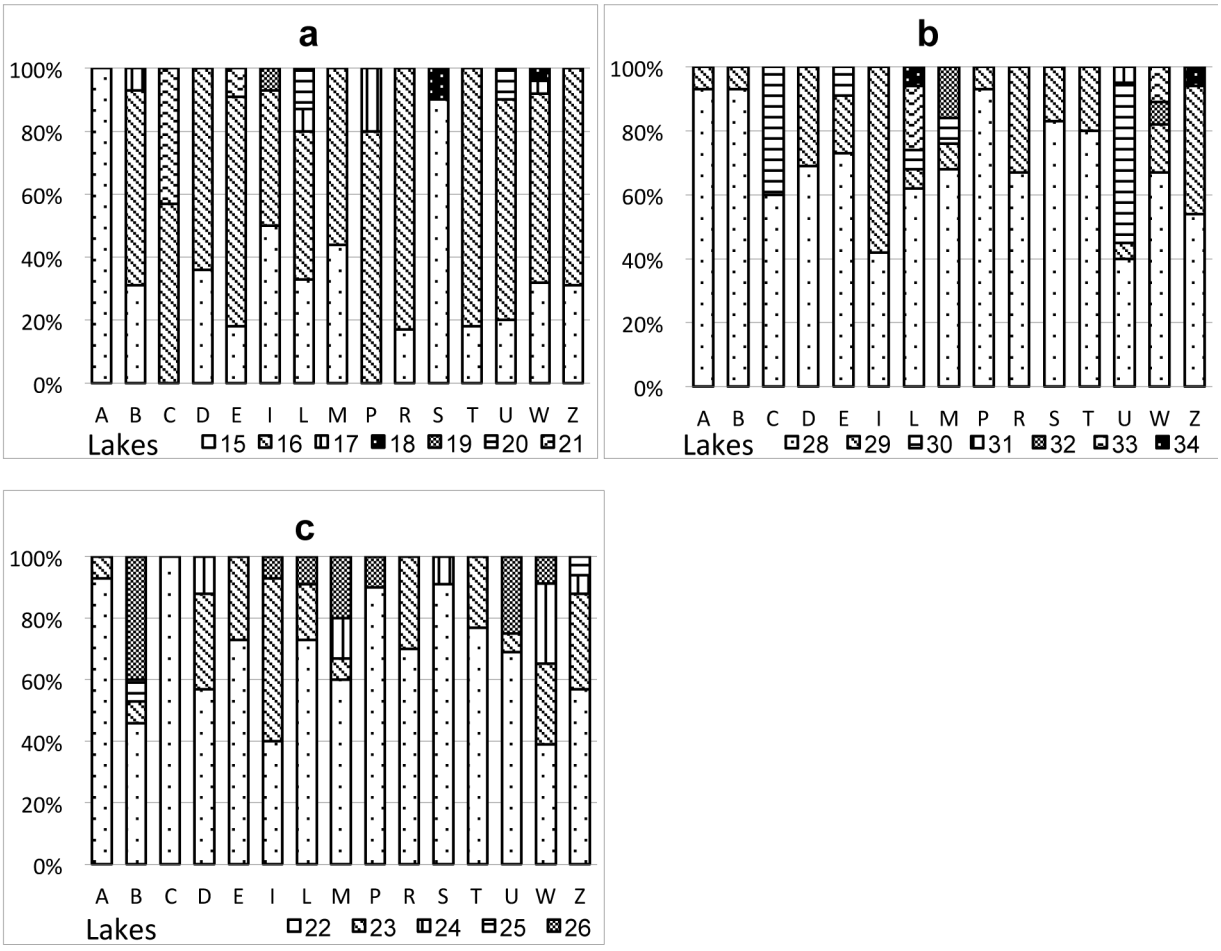

Figure 4. Distribution of haplotypes for the D-loop control region digested with *Msp*I (a), *Hha*I (b) and *Rsa*I (c) in the European cisco populations from the investigated lakes; legend: haplotype no. Site letter code as in Figure 1.

Table 4. Pattern of restriction fragments for European cisco after digestion of the ND3/4 gene with the endonucleases *Msp*I, *Rsa*I and *Bsu*I.

Restriction endonuclease	*Msp*I	*Rsa*I			*Bsu*I				
No of haplotypes	6	7	8	9	10	11	12	13	14
Fragment sizes (bp)			1600						
		915		915					
				669					
	559				560			560	560
						478			
							412	412	
		390	390		388	388			388
	375								
							300	300	300
	283				291	291			
		244	244						
	218			209			208		208
								126	

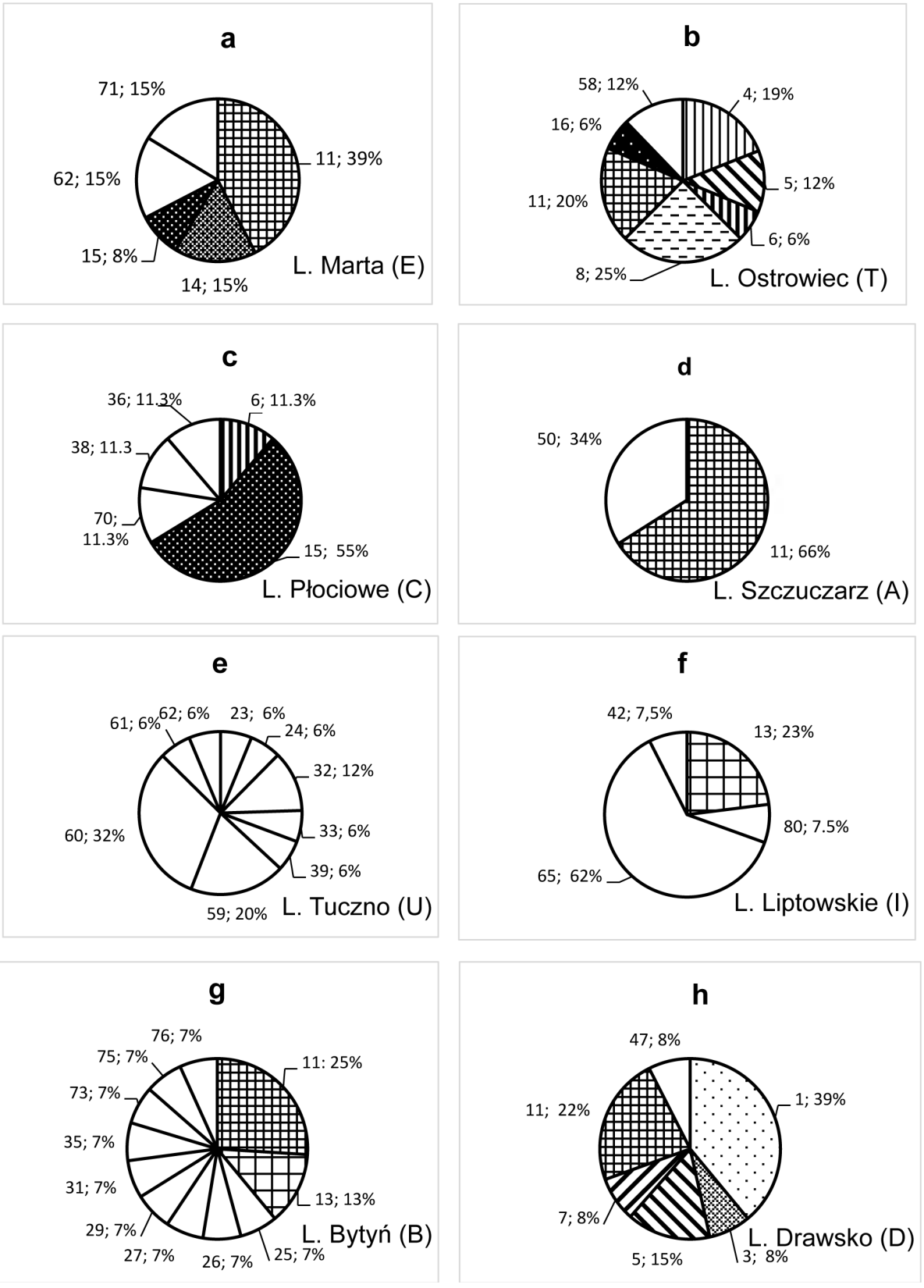

Figure 5. List of haplotype compositions in the investigated population of the European cisco (*Coregonus albula*). Haplotype compositions 1–6 (with filling) were found at multiple sites; compositions 17–80 (without filling) were only found in individuals from single sites. Labelling: combination no., % individuals with the haplotype composition.

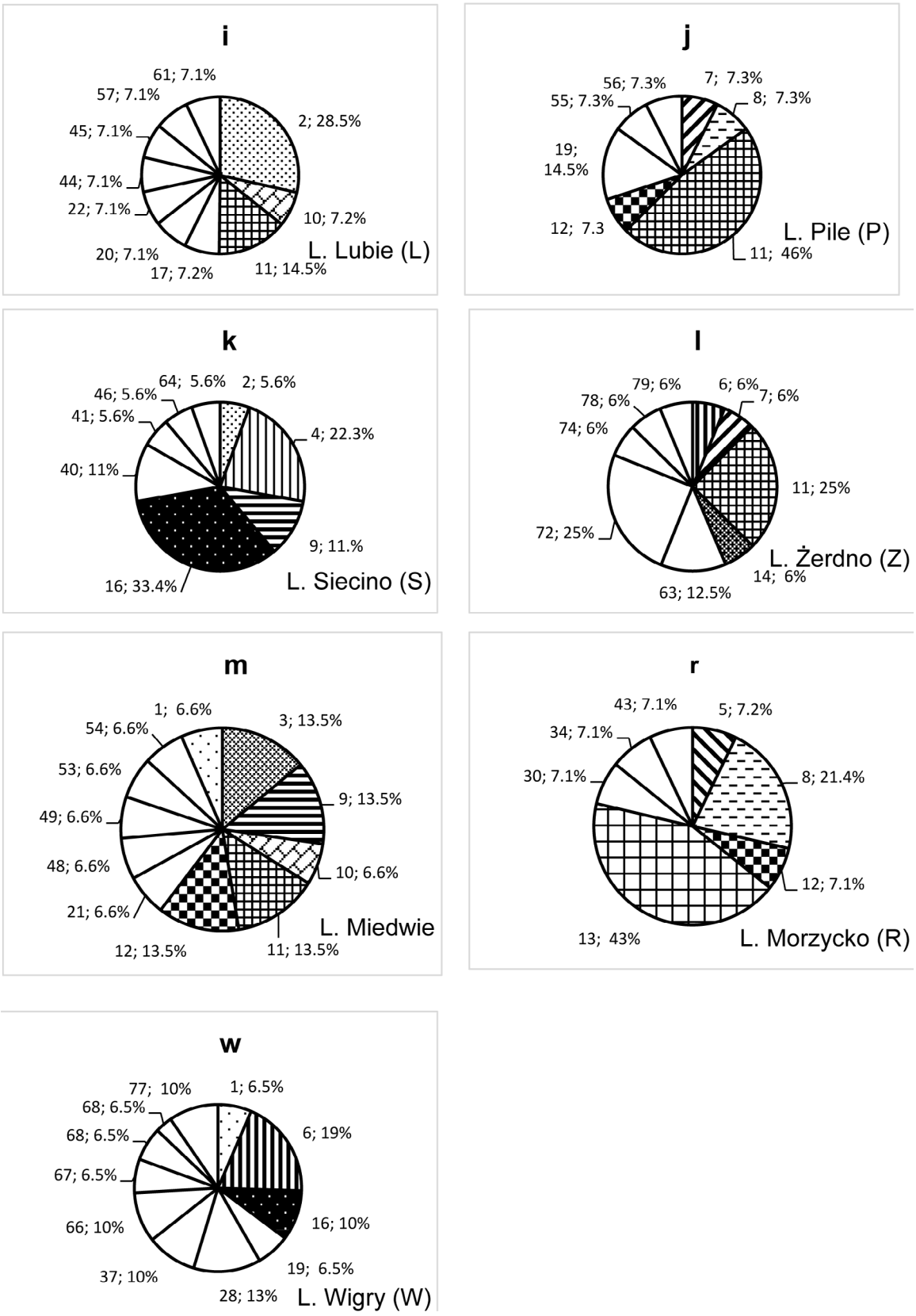

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Table 5. Pattern of restriction fragments for European cisco after digestion of the D-loop gene with the endonucleases *Msp*I, *Rsa*I, *Bsu*I and *Hha*I.

Restriction endonuclease	*Msp*I							*Rsa*I					*Bsu*I	*Hha*I						
No of haplotypes	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Fragment sizes (bp)																	1300			
																		865	865	865
					830									824	824	824				
										742	742	742								
								691	691											
	606	606	606	606		606														
																500				
							480											465		
				450		450														
										417				428						
								400					405							
					370															
			356												345				330	
									319		319									300
							301													
												280								
							269													
		243																		
	228		228			228				150	220		220							
				200																
														190						142
												150		142						
														101						

Table 6. List of repeated haplotype compositions (16) in the investigated population of the European cisco.

Number of combinations of haplotypes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Lakes	M, W^{3} D	LS	MD	T^{1} S	D, T^{1}	RC^{1},E^{1}, T^{1},W^{3}Ż	T^{1},P	E^{1},A^{2} B, L,M,P, R,T^{1},Ż	MP	B, I, Ż	C^{1}E^{1}	P MR	B,IR	E^{1}Z	C^{1}E^{1}	T^{1}W^{3}

Signatures as in Figure 1

and Lake Wigry (W) (Figure 5w), the latter of which was the only one of the investigated lakes that was located in north-eastern Poland (WNP). Ten different combinations were identified in Lake Lubie (L) (Figure 5h), while among the 9 haplotype compositions identified in Lake Tuczno (U), all of them were unique for that site (Figure 5e). In other lakes of north-western Poland, 4 to 9 haplotype compositions were observed (Figure 5f, h, i–l, r).

For all 34 haplotypes, genetic similarity was calculated according to Nei and Li's (1979) (Table 6). The genetic diversity between the European cisco populations was between 0.0% and 7.0 %, with the upper limit observed in only one case, the populations of Lake Morzycko (R) and Lake Lubie (L), located at the opposing ends of Western Pomerania in relation to all other investigated lakes. Among the remaining European cisco populations, the largest genetic distance was observed between the individuals from Lake Szczuczarz (A) (area around DNP) and those from Lake Drawsko (D) and Lake Liptowskie (I) (4.5%), (Figure 6, Table 7). As expected, the populations inhabiting the DNP lakes showed small genetic diversity: from 0.1% between the fish from Lake Marta (E) and Lake Płociowe (C) to 1.5% between the fish from Lake Płociowe (C) and Lake Ostrowieckie (T). Small genetic distance was

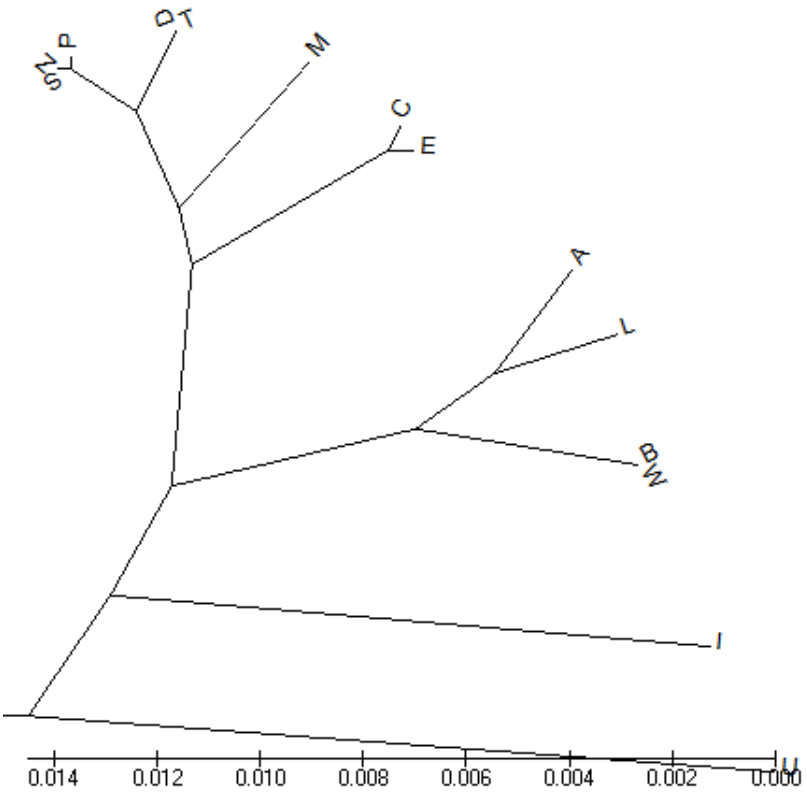

Figure 6. A dendrogram showing the genetic distances [37] between the vendance populations studied (UPGMA).

Table 7. Pairwise Population Linearized PhiPT Values. Analysis of molecular variance analysis (AMOVA) was used to compute the distribution of genetic variability among European cisco populations.

Locality	A	B	C	D	E	I	L	M	P	S	T	U	W	Z	R
A	0.000														
B	0.004	0.000													
C	0.029	0.006	0.000												
D	0.045	0.018	0.005	0.000											
E	0.019	0.011	0.001	0.020	0.000										
I	0.045	0.015	0.025	0.025	0.020	0.000									
L	0.005	0.008	0.028	0.018	0.021	0.024	0.000								
M	0.023	0.019	0.024	0.009	0.016	0.028	0.007	0.000							
P	0.023	0.013	0.009	0.003	0.004	0.018	0.007	0.002	0.000						
S	0.043	0.019	0.013	0.000	0.006	0.028	0.010	0.000	0.001	0.000					
T	0.031	0.010	0.003	0.000	0.015	0.017	0.023	0.017	0.014	0.002	0.000				
U	0.028	0.019	0.032	0.030	0.044	0.025	0.023	0.029	0.030	0.042	0.027	0.000			
W	0.009	0.000	0.009	0.018	0.014	0.014	0.014	0.013	0.008	0.023	0.015	0.011	0.000		
Z	0.033	0.010	0.000	0.000	0.003	0.021	0.008	0.010	0.000	0.000	0.002	0.037	0.017	0.000	
R	0.031	0.009	0.020	0.008	0.017	0.040	0.070	0.011	0.022	0.033	0.030	0.029	0.015	0.017	0.000

Signatures as in Figure 1

also observed between the populations from Lake Wigry and those from other lakes (0.0% to 2.3%).

In the phenogram illustrating the genetic distance between populations from different lakes, two groups are distinguished: group I, containing the populations from most Western Pomeranian lakes, including the protected lakes of DNP, and group II, formed by the populations from Lake Wigry (north-eastern Poland) and the lakes located in the south of the investigated area, including Lake Szczuczarz (A) in the area around DNP.

4 Discussion

In the presented study, the genetic characteristics of the European cisco populations from lakes located in northern Poland are described, based on the analysis of restriction sites in several regions of mitochondrial DNA, i.e. genes encoding cytochrome b, ND1 (NADH dehydrogenase, subunit 1) and ND3/4 (NADH dehydrogenase, subunit 3–4), as well as D-loop, a control region. The analysis of 450 individuals caught in natural and commercially exploited lakes, as well as the use of several restriction enzymes for the cleavage of marker fragments of mtDNA, resulted in a rich research material.

The RFLP-PCR analysis of the cytochrome *b* gene did not reveal any variability for any of the restriction enzymes used. Possible DNA variability could be demonstrated by sequencing, however, due to the conservative nature of the gene, variability within the species is small and of neutral nature. According to Churikov *et al.* [35], among the species of the *Oncorhynchus* genus, intermediate levels of nucleotide diversity were observed in the cytochrome *b* gene due to the relatively slow evolution of this gene. Therefore, the mitochondrial cytochrome *b* gene, along with the *nuclear rhodopsin* gene sequences, is used for genetic barcoding that allows the identification of more than 200 fish species [36].

The cytochrome *b* gene is an excellent marker for species differentiation, which has already been used numerous times for the taxonomic identification within the *Coregonidae* genus [26]. This is of particular importance in the case of species whose phenotypic differences are slight and their taxonomic identification based on morphological characteristics can be difficult. As noted by Cantatore *et al.* [37], the analysis of species of the Perciformes order, cytochrome *b* is useful as a phylogenetic marker at a higher taxonomic level, between families or orders. The cytochrome *b* gene analysis has been widely used in the biogeographic studies of fish of the *Sardinops* [38], *Oncorhynchus* [39] and *Osmerus* [40] genera.

To date, in the studies of the Polish populations of the European cisco, the analysis of the ND1 gene variability has not been performed. Our study demonstrated that the digestion of an mtDNA gene encoding NADH dehydrogenase, subunit 1, with three enzymes (*Rsa*I, *Msp*I and *Bsu*I) revealed 5 haplotypes of the European cisco from lakes in northern Poland. Some of the haplotypes were rare and occurred only at single sites (e.g., haplotype 3 obtained with *Rsa*I), in a commercially exploited lake. In the literature worldwide, there have been reports of the use of the ND1, e.g., for the assessment of the degree of hybridization between coregonid species with highly variable morphological characteristics [41]. Based on the molecular-genetic analysis of the mitochondrial ND1 fragment of *C. albula* and *C. peled*, the authors demonstrated that these species are very closely related. In an earlier study [23] using ND1, two genetic variants referred to as E-3 and S-2 for *C. sardinella* and *C. albula*, respectively, originating from Vodlozero (Kareli, Russia) were observed. One of them was characteristic of the Syberian populations (S-2), while the other was characteristic of the European cisco populations from European lakes (E-3). High variability of this gene among the various species of *Coregonidae* was demonstrated by Politov *et al.* [24] who analysed intra- and interspecific variability. Analyses employing *Rsa*I are successfully used for the identification of the species of *Coregonidae* [24]. The obtained PCR-RLFP restriction patterns of the ND1 gene were most useful for the discrimination between the Coregonidae species.

Politov *et al.* [24] also noticed that unusual mtDNA haplotypes found in a species might be a result of introgression that occurred in the distant past or recently. A confirmation for this claim may be the fact that the reintroduction of individuals from Lake Morzycko (R) into the same lake, and similar practices in the case of some DNP laes (E, A, C) and Lake Liptowskie (I) results in a high percentage of one haplotype composition.

The mtDNA fragment including the genes encoding NADH dehydrogenase, subunits 3 and 4 (ND3/4), of more than 2000 bp, is rarely used to describe the genetic structure of *Coregonidae*. Nielsen *et al.* [42], using the same primers as those described in this paper, successfully amplified the DNA of Atlantic salmon. Their PCR-RFLP analysis involving digestion with *Rsa*I produced two haplotypes in *Salmo salar*. Kovpak *et al.* [43] investigated the genetic divergence among smelts of the *Osmerus* genus originating from the European and Asian regions of Russia, using the PCR-RFLP analysis on the ND3–4 gene. The authors demonstrated high interspecific divergence values with low intraspecific differentiation values between smelts

originating from different regions of the Pacific Ocean. In the *C. albula* populations of north-eastern Poland, PCR-RFLP analysis of ND3–4 was carried out by Brzuzan *et al.* [32]. Two of the restriction enzymes used by these authors were also used in our study, namely *Msp*I and *Rsa*I, and the same set of primers was used for the amplification. Brzuzan *et al.* [32] obtained 2 haplotypes with *Msp*I and 4 haplotypes with *Rsa* I. In our study, 1 and 3 restrictive patterns were obtained with these enzymes, respectively, but only two haplotypes obtained with *Rsa*I were the same as in the study by Brzuzan *et al.* [32], while the remaining ones were unique.

In the population studies of fish, D-loop located in mtDNA is the most commonly used control region. Because of the noncoding nature of this fragment, it is highly variable as has been shown in many studies [20,21,33,44]. Schulz *et al.* [27] based on the nucleotide sequence of a short D-loop segment (328 bp) in *Coregonidae*, identified as many as 10 haplotypes. Reed *et al.* [46] by analysing the complete D-loop, reported 4 length variants resulting from duplication of single D-loop segments in the investigated *Coregonus* species, but also demonstrated a high variability of this region resulting from nucleotide substitutions and indel mutations.

PCR-RFLP analysis usually covers several mtDNA regions and the haplotypes are grouped into combinations, therefore authors do not always refer to the variability within D-loop. Our study confirmed that the number of restriction sites, and thus the number of modifications in the control region nucleotide sequence, is high. Digestion with four restriction enzymes (*Msp*I, *Rsa*I, *Bsu*I, *Hha*I) of the complete D-loop shows that the populations from natural lakes (C, E, T) are characterized by a lower variability (1–3 haplotypes). Only in the case of digestion with *Rsa*I, 2 haplotypes corresponding to the restriction patterns by Brzuzan *et al.* [32] were obtained.

Our study covered the analysis of 3 genes and an mtDNA control region, and involved 15 *C. albula* populations, including 2 originating from national parks. The large number of investigated individuals allowed the observation of rare haplotypes in unique combinations. Gordeeva *et al.* [20] noted that the sufficient number of investigated individuals is necessary for the correct interpretation of the results in a population analysis.

Based on the RFLP analysis of mtDNA, two groups were identified in the population of the European cisco: one originating from north-eastern Poland and the other originating from the western and central part of Pomerania [32]. Brzuzan *et al.* [32] suggested that the ancestral populations of the European cisco might have developed in two separate glacial refugia and then colonized lakes in the postglacial period. However, our analysis demonstrated that the western populations are not homogeneous and the fish from lakes located near the south-western borders of Pomerania (Lubie, Bytyń, Liptowskie and Tuczno), from a genetic point of view, are more closely related to those from north-eastern Poland (Lake Wigry) than those from Western Pomerania. There may be several causes of this phenomenon. It may be a result of human activity, when reintroduction was arbitrary and was performed without a clear plan. However, the division of the European cisco populations into two groups may have a more complex background and result from a single or repeated bottleneck effect, which may have occurred in the ancestral populations of this species. Our analysis revealed a small genetic distance between the European cisco population from Lake Wigry and the populations from the other investigated lakes. Reintroduction is being carried out since 1930s in Lake Wigry using the juvenile individuals from the fish inhabiting that lake has contributed to the restoration of the European cisco population [45]. Due to the above, the population from Lake Wigry is probably the most widespread European cisco population in Poland [10]. Apparently, this is confirmed by the results of our study suggesting that the population from Lake Wigry might have spread in the Pomeranian lakes in the past. However, as noted by Schulz *et al.* [27] based on the studies of *Coregonidae*, it cannot be excluded that the genetic similarity between the populations is due to processes, which occurred, in the more distant past.

Populations from the lakes in which no catches of the European cisco are performed, due to their localization in the strict protection zone of DNP, are characterized by a relatively low genetic diversity. This can be explained by the reintroduction using material from the spawning fish from those lakes, performed in order to maintain the natural populations in 2004–2008. In the subsequent years, it is planned to carry out further reintroduction in the lakes in the strict protection zone of DNP.

The European cisco is a valuable species, for many years covered by programmes of renewal and reintroduction, e.g., in Poland, Scotland, England and Germany [46-48]. This is necessary, as the natural populations are reduced due to the deteriorating quality of water and currently constitute a small fraction of the total population [10]. Extinction of the European cisco has also been observed in some lakes in Poland [3,4]. Thanks to the reintroduction combined with improving the quality of water, it is possible to restore and strengthen the existing populations of the European cisco. Therefore, in order to protect this valuable species and maintain the biodiversity of the lakes

(ecosystems) inhabited by the species, it is important to perform the genetic characterization of the population. Continuous genetic monitoring is necessary in the process of renewal of endangered species in order to prevent the disruption of the genetic structure of the population, which was investigated by Fopp-Bayat [49] in the common whitefish. Similarly, Pamminger-Lahnsteiner *et al*. [15], who investigated *Coregonidae* from Austria, emphasize the importance of elucidating the genetic structure of the population for the protection of the species and indicate the need to learn and characterize this aspect in as many species as possible. Our study and the obtained results are a valuable characterization of selected populations and may be a starting point for further genetic monitoring.

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