ICES Journal of Marine Science, 58: 230–237. 2001
doi:10.1006/jmsc.2000.1005, available online at http://www.idealibrary.com on IDEAL®

Mitochondrial DNA variation in sea trout from coastal rivers in the southern Baltic region

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Wlodarczyk, E. and Wenne, R. 2001. Mitochondrial DNA variation in sea trout from coastal rivers in the southern Baltic region. – ICES Journal of Marine Science, 58: 230–237.

Sea trout (*Salmo trutta* m. *trutta*) populations are intensely stocked in Poland. It is estimated that over 90% of smolts migrating from rivers to the sea are offspring of locally caught spawners, artificially bred for over one year. Mitochondrial DNA variation in the populations of sea trout from six Polish rivers was investigated by RFLP analysis of PCR-amplified NADH-dehydrogenase 1 and 5/6 segments of mtDNA. Sixteen composite haplotypes were identified. Limited variation in the frequency of haplotypes between populations ($\chi^2=116.46$, $p<0.001$) was observed. Only the Słupia River population differed from the other five. Close similarity between Vistula River and the Pomeranian Rega and Parseţa rivers in a neighbour-joining tree indicates the presence of Pomeranian trout in the Vistula River system. In order to explain the possible causes of low differentiation of mtDNA among these sea trout populations, a comparison has been made to that found among populations from geographically close rivers in Bornholm Island, Denmark based on the literature data.

Key words: mtDNA, PCR, RFLP, *Salmo trutta*, sea trout, southern Baltic.

Received 14 December 1998; accepted 1 September 2000.

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Introduction

The sea trout (*Salmo trutta* m. *trutta*) is an anadromous salmonid species of high commercial value, widely distributed in Europe. Its freshwater counterpart, the brown trout has been extensively researched, and a number of studies have shown a strong genetic differentiation of populations within the river systems (reviewed by Ferguson, 1989; Ryman, 1983). However, strong differentiation has also been observed within small geographic regions (Ferguson and Taggart, 1991; McVeigh *et al.*, 1995; Hansen and Loeschcke, 1996; Apostolidis *et al.*, 1997) as well as on a large geographic scale (Bernatchez *et al.*, 1992; Giuffra *et al.*, 1994; Garcia-Marin and Pla, 1996). Due to environmental degradation and stocking of hatchery-raised trout into the wild, the genetic make up of many populations has been changed (Largiader and Scholl, 1996; Skaala *et al.*, 1996). In the areas where intense stocking with non-indigenous or with local but mixed stocks has been performed the lack of pronounced genetic variation among geographically close freshwater trout populations has been observed (Hansen *et al.*, 1995; Garcia-Marin *et al.*, 1991). Landlocked pristine populations have retained genetic differentiation due to limited gene flow and genetic drift (Hindar *et al.*, 1991). In localities accessible from the sea, the level of genetic differentiation in trout populations has been found to be low (Hansen and Loeschcke, 1996). For naturally reproducing populations of the sea trout, high genetic diversity is observed among tributary populations, whereas genetic diversity is low among river systems. Family related structuring in upper tributaries has been reported (Hansen *et al.*, 1997). However, the present knowledge of population genetics of the sea trout is still limited.

In Poland, the estimated number of smolts migrating to the sea decreased from about 1.5 million at the turn of century to 80 000–100 000 in recent decades (Bartel, 1998). This decline has in part been attributed to the degradation of natural spawning sites and the construction of dams. Stocking activities to enhance populations have been performed to avoid a drastic decrease of sea

trout numbers and catches. During the 1970s the number of released smolts was 170 000–33 000 thousand per year. The number of releases has increased since to approximately 1.5 million (Bartel, 1997) with the number of released alevins approaching five million. After the Włocławek dam construction in 1968, trout from rivers in the west of Poland (a collection of small Pomeranian rivers) were used in restocking the Vistula River (Bartel, 1993). Since the early 1970s, coastal trout rivers in Poland have been stocked only with smolts and alevins of local origin (Bartel and Dębowski, 1996). Up to 100 000 smolts are released to each of the rivers in the west and one million to the Vistula River and its tributary Drwęca. Spawners for obtaining stocking material are caught in the lower parts of each river due to economical reasons (Bartel, 1997). Therefore, each river is treated as a separate management unit. It has been established that some of these artificially released trout return for natural reproduction to the upper tributaries of rivers (homing) (Dębowski and Bartel, 1995, 1996).

Cytogenetic studies of sea trout populations in Poland revealed polymorphism of the sixth pair of metacentric chromosomes and differences in the frequency of cytotypes of the eleventh pair between the Pomeranian and Vistula River populations (Woźnicki *et al.*, 1998). Results of allozymatic studies indicate differentiation in the frequencies of some alleles between populations from two Pomeranian rivers (Rega and Parsęta) and those from the Vistula and Słupia rivers (Luczynski *et al.*, 1997). The authors conclude that it could be due to the management practices. Only preliminary studies of mtDNA polymorphism in populations from two rivers in Poland have so far been performed (Włodarczyk and Wenne, 1998). Mitochondrial DNA has successfully been used as a molecular marker in studies of variation among sea trout populations in the western Baltic (Hansen and Loeschke, 1996).

Here, we present results of restriction analyses (RFLP) of PCR-amplified mtDNA coding regions (ND1 and ND5/6) from spawning populations of sea trout from six rivers on the Polish Baltic coast. The main aim of this work was to determine genetic characteristics of these fish and to study their genetic population structure towards stock identification at the mtDNA level. Due to the historical release of Pomeranian trout to the Vistula River system in the course of stocking activities, a similarity between those populations could suggest survival and return for spawning of Pomeranian trout in the Vistula River. A comparison of mtDNA polymorphism in sea trout from Polish rivers to the geographically closest populations studied to date (i.e. from Bornholm, Denmark) has been performed in order to provide a better understanding of possible management impact on its populations in the southern Baltic.

Materials and Methods

Sample collection

Due to low natural spawning in the upper tributaries of our study rivers and with the management practice to treat each river as a separate unit, samples for this research were collected from the lower parts of rivers. Between September and December 1996, tail fin clippings were collected from sea trout spawners entering six Polish rivers: Rega, Parsęta, Wieprza, Słupia, Vistula and Drwęca – a tributary to the Vistula River (Figure 1). Fish from the rivers Rega, Wieprza, Słupia and Drwęca were caught in traps positioned at a fixed distance from the river mouth (3–28 km) whilst the samples from the Vistula River consisted of fish caught by commercial fishermen, close to the river mouth. The trout from the Parsęta River were caught by electrofishing at a location 40 km up the river mouth. Forty individuals were sampled at each site. Fin clippings were preserved in 95% (v/v) ethanol and kept at 4°C after collection.

MtDNA analysis

Total genomic DNA was extracted with the use of the "Genomic DNA Prep Plus" kit (A&A Biotechnology, Gdynia). The ND-1 (NADH dehydrogenase 1) and ND-5/6 (NADH dehydrogenase 5 and 6) coding regions of mtDNA were amplified with the primers described by Nielsen *et al.* (1998), following the PCR protocol of Hansen and Loeschcke (1996). Approximately 250 ng of total genomic DNA was added per each reaction tube containing 50 µl of PCR mix. The amplified ND-1 segment was digested with the restriction endonucleases *Alu*I, *Ava*II, *Hae*III, *Hin*fI, *Hpa*II, and the ND-5/6 segment with *Ava*II, *Hae*III, *Hin*fI, *Taq*I and *Xba*I. For each sample 3–8 µl of the PCR products were digested with 1–2 units of the appropriate restriction enzyme and separated by electrophoresis in 8% (29:1) polyacrylamide gels followed by silver-staining (Sambrook *et al.*, 1989). The molecular mass standard, a 100 bp ladder (Gibco BRL), was used to size the restriction fragments. Each restriction morph was assigned a capital letter.

Restriction patterns were checked for the presence of fragments smaller than 100 bp by 15% (29:1) polyacrylamide gel electrophoresis and visualized by silver-staining. In order to compare haplotypes identified in the studied populations with the Bornholm (Denmark) ones, agarose gel electrophoresis was used as in Hansen and Loeschcke (1996), along with restriction fragments size estimation. Direct comparison of haplotypes identified through restriction analysis of the ND-1 region has been performed by Dr Linda Laikre (University of Stockolm, Sweden) within the EU (FAIR CT 97 3882) "Concerted Action on Identification, Management and Exploitation of Genetic Resources in Brown Trout

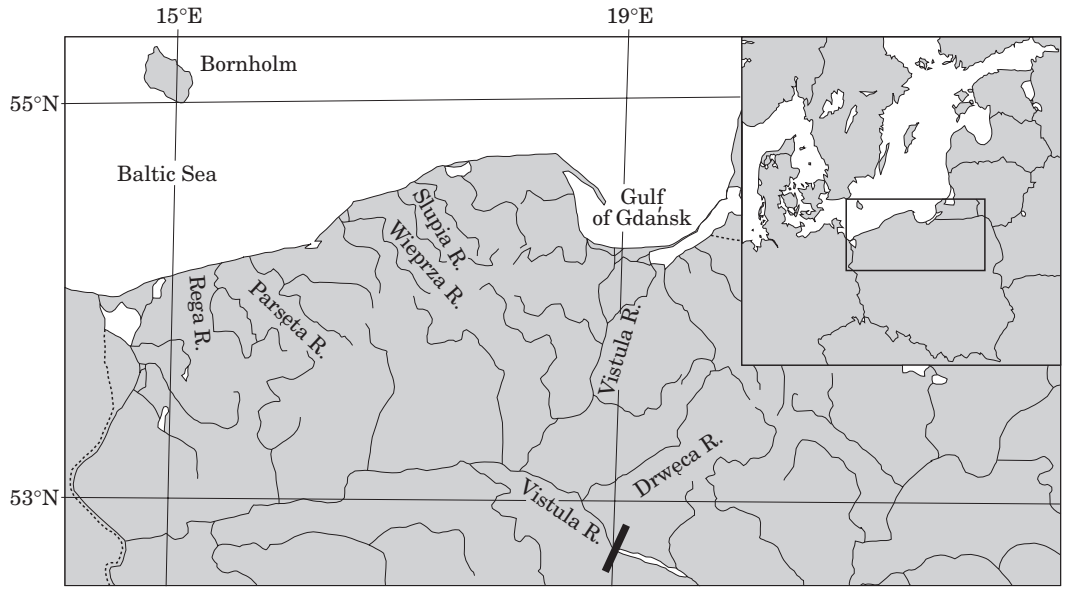

Figure 1. Map of northern Poland. Marked are the rivers from which samples of sea trout were collected in the fall of 1996. Black bar indicates the Włocławek dam.

(*Salmo trutta*)". The site-by-site comparison has confirmed that most haplotypes observed in Sweden, Poland and Denmark are identical, as it had been assumed from the literature data.

Statistical treatment

The genetic structuring among haplotypes was analyzed by calculating the mean number of substitutions per site for all pairs of haplotypes from site data (Nei and Tajima, 1981), followed by constructing a tree according to the maximum parsimony method. The programs D from the REAP package (McElroy *et al.*, 1991) and Dollo from the PHYLIP package (Felsenstein, 1993) were used.

To analyze genetic structuring among sampled populations, a pairwise matrix of diversity (Nei, 1987) was computed using the DA program from the REAP package (McElroy *et al.*, 1991). Pairwise tests for homogeneity of haplotype frequencies between populations were performed using a Monte Carlo simulation approach (Roff and Bentzen, 1989) with the MONTE program from the REAP package (McElroy *et al.*, 1991). Multiple test significance levels were calculated using the Bonferroni method (Rice, 1987). Finally, a neighbour-joining tree was constructed to summarize the genetic relationships among populations, based on the nucleotide divergence matrix and using the NEIGHBOUR program from the PHYLIP package (Felsenstein, 1993).

To quantify genetic differentiation, the AMOVA (Analysis of Molecular Variance) was used (Excoffier *et al.*, 1992). The variance components were calculated for two groups, where the five rivers were compared to the Słupia River sample. The Φ-statistics and the corresponding p-values were calculated by performing 4000 permutations of the original data matrices and comparing the results to the original values.

Results

The sizes of restriction fragments based on the results of polyacrylamide gel electrophoresis are shown in Table 1. The number of restriction sites in each restriction morph was inferred from the fragment size data. The presence of small fragments was successfully confirmed through electrophoresis on 15% (w/v) polyacrylamide gels and silver staining.

A total of 16 composite haplotypes were identified among 240 individuals sampled from six locations. Genetic relationships among those haplotypes are shown in Figure 2. No relationship between phylogeny of haplotypes and their geographic origin was observed. The number of haplotypes per population ranged from four to eight (Table 2). Three common haplotypes were found in all six populations. Twelve rare haplotypes were observed (frequency ≤ 0.075) and nine of these were only observed in one sample. The highest number of rare haplotypes (five) was found in the sample from the Słupia River while no rare haplotypes were detected in the Drwęca River. All populations were fixed at one morph for the ND-1 segment digested with *Hin*fI. The levels of haplotype diversity within populations were high and ranged from 0.71–0.80. The screening of the

Table 1. Approximate fragment sizes of restriction morphs for ND-1 and ND-5/6 regions of mtDNA as assessed from polyacrylamide gels. Fragments smaller than 100 bp not shown.

(a) ND-1

Restriction endonuclease	*Alu*I		*Ava*II		*Hae*III			*Hpa*II		*Hin*fI
Restriction morph	A	B	A	B	A	B	C	A	B	A
Fragment sizes (bp)	700	700	950		700	700	700		800	1150
		490		750	540	540		550	550	580
	390	390	425	425			415	480		530
	290		320	320	290	290	290	460	460	
	235	235	225	225		220	220	265		
	185			160	200	200	200	185	185	
	180	180	150	150	185			130	130	
	100	100					105			

(b) ND-5/6

Restriction endonuclease	*Ava*II			*Hae*III				*Hin*fIII				*Taq*I				*Xba*I	
Restriction morph	A	B	C	A	B	C	D	A	B	C	D	A	B	C	D	A	B
Fragment sizes (bp)	1 900	1 900		950	950	950	950		1 200				800			2 600	
			1 000	830			830	1 050		1 050	1 050	670	670	670	670		2 350
	950				800	800		475	475		475	650			650		250
			900	650	650					460				630			
		600	600			630	630	320	320	320		325	325	325	325		
		250	250		200						315	315	315	315			
		125	125	175	175	175	175	275	275	275	275				300		
				135		135	135	240	240	240	240	290	290	290	290		
				120	120	120	120	185	185	185	185	175	175	175	175		
								150		150	150	140		140	140		
												120	120	120	120		

ND-1 and ND-5/6 regions with the restriction enzymes *Dra*I, *Bcl*I and *Mbo*I in the limited number of specimens did not detect polymorphism.

Statistically significant differences in haplotype frequencies were found among the six rivers ($\chi^2=116.46$, $p<0.001$). Pairwise tests for homogeneity of haplotype frequencies between populations using a Monte Carlo simulation approach with 1000 randomizations yielded statistically significant results for the Słupia River. The p values ranged from 0.006–0.028. The application of sequential Bonferroni procedure did not reveal significant differences among groups of rivers. In the neighbour-joining trees, the Słupia River consistently clustered with the geographically close Wieprza River (Figure 3). The second cluster consisted of the two neighbouring rivers Rega and Parsęta with the Vistula River.

The AMOVA results showed that the regions did not statistically differ from each other. When the Słupia River was used as a separate region and contrasted against the remaining five sites, the ''between two groups'' variance component was 1.46% but the Φ_{st} value was not statistically significant. The ''within populations'' variance approached 100%.

Discussion

On the basis of sequence analysis of the PCR-amplified mtDNA control region, Bernatchez *et al.* (1992) concluded that sea trout from the Polish rivers Słupia and Vistula belong to the Atlantic grouping. Karyotype analysis led Woźnicki *et al.* (1998) to a suggestion that a recognizable genetic divergence between the trout from the two investigated rivers might exist. The allozyme study of sea trout from the rivers Vistula, Rega, Słupia and Parsęta revealed very short genetic distances (Nei's D) among the sampled populations, ranging from 0–0.001 (Luczynski *et al.*, 1997). Heterozygote deficits, indicating the possibility of mixed populations in Polish rivers, as detected by allozymes have not been observed. The level of genetic polymorphism in Polish populations

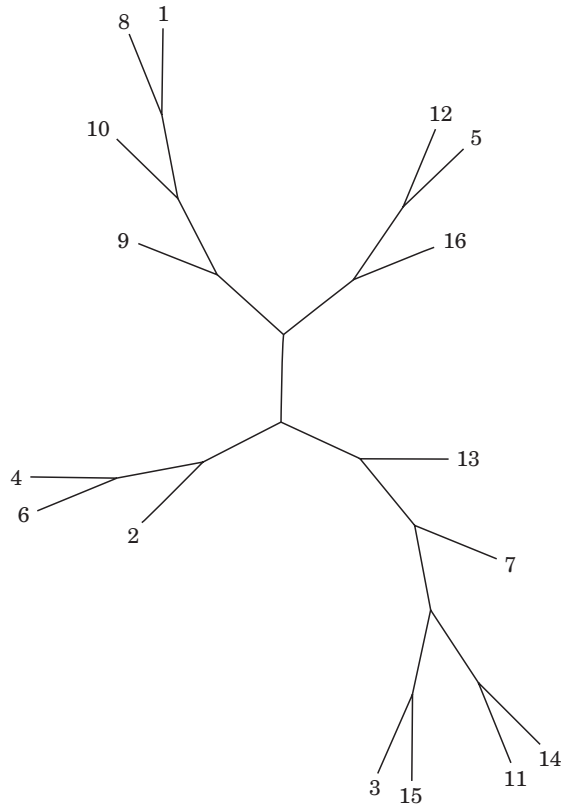

Figure 2. Phylogenetic tree showing the relationships among the observed haplotypes. The tree was constructed according to the maximum parsimony method using the program Dollo from Felsenstein's PHYLIP package.

of sea trout is similar to those from other regions (Luczynski *et al.*, 1997). In the present study, samples of sea trout from six rivers displayed a similar frequency of the most common mtDNA haplotypes. Pairwise comparisons of haplotype frequencies indicated that the Słupia River population might differ from the other five.

Based on the tagging experiments conducted by Dębowski and Bartel (1995), it has been concluded that smolts released within the Vistula River system home with high efficiency (10% of return) in comparison with Pomeranian rivers (0.7–3.9% of return). 98.6% of trout released and recaptured in the Vistula River and 45.3% (mean for four rivers) of those released to Pomeranian rivers were caught in the natal river (Dębowski and Bartel, 1995). 89% of trout released to the Parsęta River and 53% of trout released to the Słupia River were recaptured in the Wieprza River, whereas 75% of trout released to the Wieprza River were recaptured there. Of the trout released to the Wieprza River and the Słupia River, 20% and 17.6%, respectively, were recaptured in the Vistula River. In general, fish from Pomeranian rivers were going astray much more often than those from the Vistula River. The Vistula River is the largest Polish river. Factors such as high river runoff can facilitate sea trout homing. Runoffs from the Pomeranian rivers (Rega, Parsęta, Słupia and Wieprza) are low (Jasińska, 1991). Thus it is justifiable to make an assumption that each year a certain number of straying individuals from the Pomeranian rivers ascend the Vistula River more often than in the opposite direction. Survival and reproductive success of straying individuals may not be reduced in Polish rivers as in populations reproducing only naturally, due to artificial reproduction performed as part of stocking activities. Spawners for collection of eggs and milt used in artificial reproduction are caught in the low parts of rivers (see Materials and Methods). Therefore gene flow among Pomeranian and Vistula Rivers can be assumed.

All six rivers in Poland have been intensely stocked since the 1950s. Construction of a dam in Włocławek resulted in a dramatic decrease in sea trout and other fish numbers. In the 1960s, insufficient amounts of stocking material were being obtained from the sea trout ascending the Vistula River (Bartel, 1993). To alleviate this situation, for nearly a decade the hatchery-reared smolts originating from the Pomeranian rivers (including Rega and Parsęta) were used to supplement the Vistula River population (Borzęcka, 1997; Bartel R., pers. comm.). The survival rates of the released smolts were not estimated, although an increase in catches of sea trout in the Vistula estuary in the years following the release can suggest their successful survivorship (Bartel, 1993).

Close similarity between Vistula and Pomeranian Rega and Parsęta Rivers in a neighbour-joining tree based on mtDNA haplotype frequencies indicates the presence of Pomeranian trout in the Vistula River (Figure 3). Whereas, in the Vistula tributary (Drwęca) the population most probably represents native Vistula River trout (Bartel and Bontemps, 1995; Borzęcka, 1997). The Drwęca population is isolated from the Vistula estuary population by the Włocławek dam. Based on allozyme data, it has been found that populations from Słupia and Vistula Rivers were not genetically differentiated, whereas both Parsęta and Rega populations were slightly differentiated from each other, as well as in comparison to the two former rivers (Luczynski *et al.*, 1997). The allozyme analysis also indicated the presence of Pomeranian trout (from the Słupia River) in the Vistula River. Therefore, the allozyme and mtDNA data are not in agreement and different genetic relationships among trout populations in Poland can be inferred, as is the situation in Denmark (Hansen and Loeschcke, 1996). In conclusion, the effect of straying spawners combined with intense stocking with artificially performed reproduction could cause mixing of populations and in consequence, it could

Table 2. Frequencies of composite haplotypes in sea trout samples from six Polish rivers, based on the RFLP of the ND-1 and ND-5/6 regions of mtDNA in polyacrylamide gels. Haplotypes are denoted by capital letters in the following order: (ND-1) *Ava*II, *Hin*fI, *Alu*I, *Hae*III, *Hpa*II; (ND-5/6) *Ava*II, *Hin*fI, *Hae*III, *Taq*I, *Xba*I. In parentheses, the number of individuals displaying a given haplotype; n=40. River populations are arranged according to their geographical proximity in the eastward direction.

Population	AAABA ABABB 1	AABAA CAAAA 2	BAAAB BDBCA 3	AAABA CBABA 4	AAABA ACDDB A 5	AAACA CAAAA 6	AAABA BDBCA 7	BAAAB ABAABB B 8	AABAA ABABB 9	BAAAA ABABB 10	BAAAB BBBCA 11	AAABA ACCDB 12	BAAAB BBBBA 13	BAAAB BBBBA 14	BAAAB BDBCA 15	AAABA AAAAB 16	Hapl. diversity
Rega	0.325	0.275	0.175	0.125	0.025	0	0	0	0.025	0	0	0	0.050	0	0	0	0.779
	(13)	(11)	(7)	(5)	(1)				(1)				(2)				
Parsęta	0.450	0.225	0.150	0.050	0.025	0	0	0	0	0	0	0	0	0.025	0.050	0.025	0.727
	(18)	(9)	(6)	(2)	(1)									(1)	(2)	(1)	
Wieprza	0.325	0.325	0.100	0.100	0.075	0.075	0	0	0	0	0	0	0	0	0	0	0.767
	(13)	(13)	(4)	(4)	(3)	(3)											
Słupia	0.350	0.325	0	0.100	0	0.025	0	0	0.025	0.025	0.075	0.075	0	0	0	0	0.758
	(14)	(13)		(4)		(1)			(1)	(1)	(3)	(3)					
Vistula	0.325	0.250	0.150	0.125	0	0.050	0.075	0.025	0	0	0	0	0	0	0	0	0.795
	(13)	(10)	(6)	(5)		(2)	(3)	(1)									
Drwęca-tributary of Vistula	0.400	0.325	0.150	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0.705
	(16)	(13)	(6)	(5)													

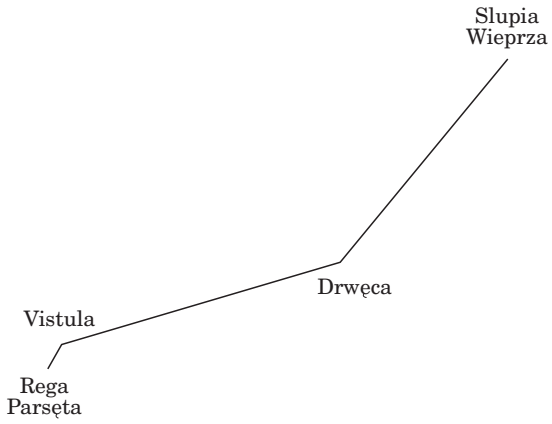

Figure 3. Neighbour-joining tree showing the genetic relationships among the sea trout populations from six Polish rivers.

contribute to the low differentiation of frequencies in sea trout populations within the studied area.

Hansen and Loeschcke (1996) reported low genetic differentiation among sea trout populations in a small geographic region close to the Polish coast. The higher level of genetic structuring of the Lake Hald (Jutland) trout has been explained by low N_e numbers and genetic drift (Hansen and Loeschcke, 1996). Ninety-three-point-one percent of the variance in the Bornholm Island (Denmark) anadromous trout was attributable to the "within population" variation. Homing of Bornholm trout is less efficient in comparison with that observed in the Jutland rivers due to dramatic changes in water level including a periodical drying up of rivers.

A comparison of six samples from Polish and three Danish coastal rivers from the Bornholm Island revealed that most probably, four common haplotypes are shared by sea trout in all locations. The haplotype frequencies in the trout populations from the three Bornholm rivers were significantly different from those of the six Polish locations. In AMOVA, for the comparison of Polish and Danish groups, the "between two groups" variance component was 16.3% and a highly significant Φ_{st} statistic equalled to 0.166 ($p<0.001$). Danish samples from the rivers Tejn, Vellens and Dyndals showed more variability than Polish samples, in spite of considerably smaller sizes. The closest genetic distance between the Polish and Danish locations was found for the western Polish Rega River and the Danish Dyndals River and it can be connected with interference of homing.

Glaciation history and the eastward recolonization of the Baltic, most probably have affected the pattern of genetic differentiation observed in the sea trout populations. Breaking up homing is another natural factor to be considered, as well as human activities such as mixing of populations by transplantation and stocking. To understand further these patterns and causes, it is necessary to carry out more research with the application of other molecular markers.

Acknowledgements

This study was partially supported by a grant to R.W. from the Polish National Committee for Scientific Research (5P06D00714). The results were presented as CM 1998/K:4 paper during the ICES Annual Science Conference in Cascais, Lisbone, Portugal. The authors are indebted to Dr Michael M. Hansen (The coordinator of the EU "TROUTCONCERT") for his valuable comments on the manuscript.

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