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Genetic Variation of *Salmo trutta* L. Populations from the Catchment Areas of the Rega, Parseta and Wieprza Rivers Evaluated by RAPD and SSR Markers

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By using simple sequence repeats (SSR) and random amplification of polymorphic DNA (RAPD) markers, the genetic variability of three *Salmo trutta* L. populations from three rivers, the Wieprza, the Rega and the Parseta, was determined. The investigated populations showed a high level of genetic variability. Microsatellites showed that observed heterozygosity (*Ho*) was higher than the expected heterozygosity (*He*), with most heterozygotes found in the population from the Parseta river and the fewest in the Wieprza population. The F_{IS} coefficient in all investigated populations of the sea trout indicate a high excess of heterozygotes. The highest genetic differentiation was observed between the sea trout from the Rega river and those from the Wieprza (0.366). The obtained results based on microsatellite and RAPD analysis showed that the investigated populations formed two groups. The first group consisted of the sea trout populations from the Wieprza and the Parseta rives, while the second group was formed solely by the Rega river population.

Key words: Trout, RAPD, SSR markers.

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Species from the Salmonidae family have been decreasing in recent years, caused by changes in their habitat, low survival rate in the sea, poaching and irresponsible fish introduction practices (PARRISH *et al.* 1998; BARTEL 2001; FRIEDLAND *et al*. 2003; JONSSON & JONSSON 2006). The occurrence of the sea trout (*Salmo trutta* m. *trutta* L., 1758) in some rivers of Pomerania (CHEŁKOWSKI 1966, 1970) is increasingly dependent on restoration (BARTEL 1988, 1993; BARTEL & DĘBOWSKI 1996). It is crucial to maintain genetic diversity in natural populations. Fish introduction into rivers may strongly affect the genetic integrity of native populations. Irresponsible introduction practices may lead to the loss of genes characteristic of a given fish population by introgression from farmed species (HINDAR *et al*. 1991; POTEAUX *et al*. 1999).

The genetic structure of every population is a result of many factors and mechanisms, such as genetic drift, natural selection and gene transfer (HEWITT 2000). Advances in molecular biology and the introduction of novel types of molecular markers permit the determination of genetic variability and diversity in the investigated populations. Genetic variability and diversity are the key features determining the adaptive capacities of a population or a species. Knowledge of these factors is extremely useful for establishing optimal culture conditions in the case of both fish farming and protection of endangered fish species. Populations that exhibit a high level of genetic diversity have a better chance of survival. Therefore, many studies aimed at determining genetic variability and diversity are conducted, especially in endangered populations. For the freshwater brown trout (*Salmo trutta* m. *fario*), isoenzyme (GUYOMARD 1989; GARCIA-MARIN *et al*. 1991; PRESA *et al*.

1994; GIUFFRA *et al*. 1996; LUCZYNSKI *et al*. 1997) and mtDNA (BERNATCHEZ *et al.* 1992; MACHORDOM *et al*. 2000; BERNATCHEZ 2001) analyses were used for this purpose, but the information on polymorphism obtained in this way was insufficient. Many studies indicate that microsatellite markers, used in this study as well, yield significant results in genetic diversity studies in fish (ESTOUP *et al*. 1993; PRESA *et al*. 1994; POTEAUX *et al*. 1999; HANSEN *et al*. 2001; WAS & WENNE 2002). They have been widely used in studies of salmonid phylogeography (BERNATCHEZ 2001; SÄISÄ *et al*. 2005) or in assessments of the genetic variability of wild and farmed fish populations (KOLJONEN *et al.* 2002; SÄISÄ *et al.* 2005; MACHADO-SCHIAFFINO *et al*. 2007).

The RAPD technique is also commonly used in studies of genetic variability (BARTISH *et al*. 2000; ALI *et al*. 2004). This technique has also been used to assess the genetic variability and similarity of many fish species (BARDAKCI & SKIBINSKI 1994) including freshwater brown trout (*Salmo trutta* m. *fario*) and Atlantic salmon (ELO *et al*. 1997), carp (BARTFAI *et al*. 2003) and tilapia (HASSANIEN *et al*. 2004), and to identify hybrids (JAMSHIDI *et al*. 2009).

The aim of this study was to analyse the genetic variability of three *Salmo trutta* L. populations from the rivers Rega, Parseta and Wieprza, the three largest trout reservoirs among the rivers of north-western Poland. For approx. 40 years, these rivers have been subjected to reintroductions of *Salmo trutta* with material derived from spawning fish of each respective river. The aim of these activities is to restore the natural populations of this precious species and to preserve the individual characteristics of each river. Herein a comparison between the populations was conducted and, by means of microsatellite marker analysis and RAPD, the current level of genetic variability in these populations was determined.

Material and Methods

Sea trout individuals caught in November 2010 at three sites, the Rega, Parseta and Wieprza rivers, were subjected to analyses (Fig. 1). The fish were weighed and measured. Body length (*L. caudalis*) of the analysed fish ranged from 56 to 62 cm, while their body mass was between 1.82 and 2.51 kg. In total, 30 individuals were analysed (10 from each site) that belonged to the most prevalent age group of >1 year. Samples of muscle tissue were taken from every individual. The samples were subsequently frozen at -70°C.

Isolation of genomic DNA

The DNA was extracted from 0.2 g of muscle tissue of particular individuals of the investigated fish population. The tissue was put in 1.5 ml microtubes and 1 ml extraction buffer was added (100 mM Tris-HCl, 200 mM NaCl, 0.2% SDS, 5 mM EDTA and 100 μg/ml proteinase K). The mixture was incubated for 12 h at 55°C in a heating block and centrifuged at 6000 rpm for 15 min. Supernatant was transferred to a 1.5 ml microcentrifuge tube and 700 μl of isopropanol was added. The preparations were centrifuged at 6000 rpm for 15 min. After precipitation the DNA was washed with 400 μl 70% ethanol. The phases were separated by centrifugation at 6000 rpm for 5 min. Supernatant was discarded and the pellet was allowed to dry. The pellet was dissolved in 20 μl TE buffer and stored at -20°C. Quantity and purity of DNA was analysed by a spectrophotometer SmartSpecTMPlus (BioRad, USA).

RAPD analysis

All DNA preparations extracted from muscle tissues used in the reaction were diluted to 120 ng/μl.

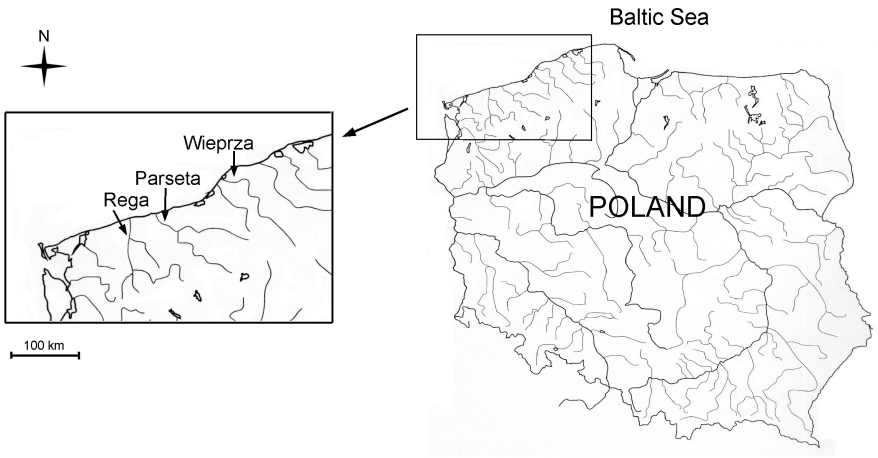

Fig. 1. Map of the sampling locations in Poland. Research area: Rega River, Parseta River, Wieprza River.

Among 10 RAPD primers (10 bases-long) tested (C02, C04, C06, C07, C08, C09, C11, C13, C16, F08) only 4 gave good quality results (C-02 GTGAGGCGTC, C-04 CCGCATCTAC, C-06 GAACGGACTC, C-11 AAAGCTGCGG, C-13 AAGCCTCGTC). The reaction volume of 25 μl was composed of 120 ngμl^{-1} of DNA, 1xGreen GoTaq™ Reaction Buffer (Promega, USA), 2 mM $MgCl_2$, 2 mM dNTP (Fermentas, Lithuania), 0.2 mM primer (Operon Technologies, USA), 0.2 U GoTaq™ DNA Polymerase (Promega, USA). The reaction profile was as follows: a) preliminary denaturation at 95°C for 2 min; b) 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, elongation at 72°C for 2 min; c) final elongation at 72°C for 10 min. Amplification was done in a Thermal Cycler MJ Mini (BioRad, USA). Reaction products were separated by electrophoresis in 1.5% agarose gels in 1 × TBE at 80 V. Gels were stained with ethidium bromide (0.35 μg ml^{-1}), and documented in GelDoc XR apparatus. The sizes of amplification products were determined by comparison with molecular weight standard GeneRuler (Fermentas, Lithuania).

SSR analysis

Out of twelve tested microsatellite loci including Str60INRA (ESTOUP *et al*. 1993), Str85INRA, Str591INRA (PRESA & GUYOMARD 1996), Strutta 12, Strutta 58 (POTEAUX *et al*. 1995), Ssa85, Ssa197 (O'REILLY *et al*. 1996), SsoSL417 (SLETTAN *et al*. 1995), SsoSL438 (SLETTAN 1995), Str43INRA, T3-13 and BS131 (ESTOUP *et al*. 1998), 4 were selected for analysis (Ssa 197, Str85INRA, Strutta 12 and Strutta 58). Each amplification reaction was carried out in a final volume of 20 μl containing 120 ng μl^{-1} of DNA, 1x PCR buffer (Promega, USA), 2.5 mM MgCl2, 0.2 mM of dNTPs (Fermentas, Lithuania), 0.5 mM primer (IBB PAN, Poland) and 0.2U GoTaq™ DNA Polymerase (Promega, USA). DNA amplifications for individual primers were done in a Thermal Cycler MJ Mini (BioRad, USA) with an initial denaturation of 4 min at 95°C, followed by 30 cycles of denaturation DNA for 30 s at 95°C, annealing at 55ºC or 60ºC for 45 s, elongation at 72C for 2.5 min and a final 5 min extension at 72C. PCR products were analysed for length variation on 6% polyacrylamide gels containing 7 M urea and documented in GelDoc XR apparatus. To check consistency of the results from gels, a series of rerun sessions was conducted.

Statistical analysis of RAPD results

The results were stored on a BioRad gel documentation system and analysed using Quantity-One® software (BioRad, USA). The presence or absence of a band was considered as a trait and noted as 1 or 0, respectively. A similarity matrix was computed using the Dice coefficient (DICE 1945): Dice coefficient = (x,y) = 2P(x, y)/(P(x) + P(y); where P(x) and P(y) is the probability of events x or y occurring together. A dendrogram of genetic similarity was constructed using the UPGMA method (Diversity Database, BioRad, USA).

Statistical analysis of SSR results

The number of alleles per locus (*Na*) and the number of effective alleles per locus (*Ne*) were calculated for each investigated population and for all populations at the same time. Genetic information was determined for four SSR loci in three populations using the following indices: number of private alleles per population (*Np*), Shannon diversity index (*I*), observed heterozygosity (H_O), expected heterozygosity (*He*), inbreeding coefficient (F_{IS}), total inbreeding coefficient per individual, estimated by determining the heterozygosity of an individual against the entire population (F_{IT}), and fixation index (F_{ST}) that became the basis for calculating the number of migrants (*Nm*). Tests of departure from Hardy-Weinberg equilibrium were calculated through the Chi-square test. The genetic similarity between the populations was expressed using Nei's genetic distance (NEI *et al*. 1983). Dendrograms were constructed based on the unweighted pair-group method with arithmetic mean (UPGMA) clustering method. All calculations were done with GenStat 15th Edition and GenAlEx v. 6.5b4.

Results

RAPD

Of the 10 RAPD primers used in the study, only 4 yielded polymorphic loci in the three investigated populations. Repetitive and intensive amplification products, as well as those differing in size compared with the neighbouring fragments, were analysed. The number of analysed PCR products was 758 and included polymorphic loci whose size ranged from 103 to 1478 base pairs. The number of polymorphic products amplified by one primer ranged from 98 in C02 to 309 in C13, with a mean of 166.5 polymorphic bands per primer (Table 1). The percentage of polymorphic bands in the populations of the Wieprza and Rega rivers was similar: 27% and 28%, respectively. In the fish from the Parseta river, the value was 33%. The reactions performed using the obtained DNA yielded 88% polymorphic products (Table 1). The results demonstrate that the genetic diversity of the analysed fish populations is very high.

Table 1

Characteristics of RAPD marker polymorphism

Primer sequences 5'→3'	Number of fragments		Number of polymorphic fragments		
	Total	Polymorphic	Rega	Parseta	Wieprza
C02	98	98	43	32	23
C04	164	164	61	45	58
C06	187	187	70	69	48
C13	309	217	34	101	82
Total	758	666	208	247	211
% Polymorphic bands		88	27	33	28

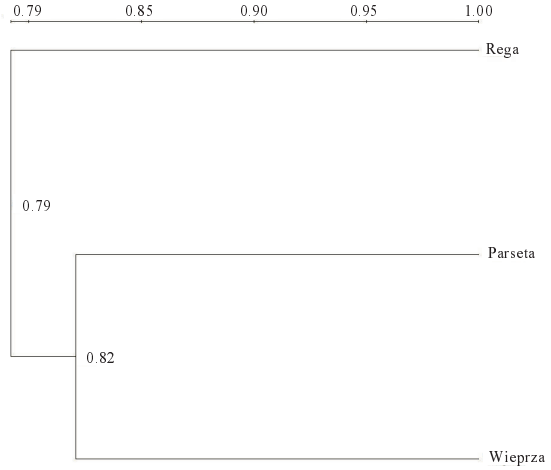

Fig 2. Genetic similarity dendrogram (UPGMA) of the investigated population based on the PCR-RAPD analysis.

Based on the results of RAPD analyses, similarity matrices were constructed and genetic similarity dendrograms were generated. RAPD results were used to calculate genetic similarity between the analysed fish populations ranging from 78.1% between the Rega and the Wieprza populations to 82.0% between the Parseta and the Wieprza populations.

The UPGMA-based dendrogram presents a division of the investigated populations into two similarity groups. The fish from the Parseta and Wieprza rivers form one group. The other group consists of the Rega population (Fig. 2).

SSR

The investigated populations were characterized by a high level of genetic variability (Tables 2 & 3).

Table 2

Genetic coefficients of variation of the investigated populations based on the 4 SSR primers

Population	*Na*	*Ne*	*I*	*Np*	*Ho*	*He*	F_{IS}
Wieprza	1.750	1.740	0.517	0.750	0.700	0.373	-0.879
Parseta	2.250	2.045	0.733	0.500	0.950	0.510	-0.867
Rega	2.750	2.332	0.853	1.750	0.775	0.539	-0.510

Na – number of alleles per locus; *Ne* – number of effective alleles per locus; *I* – Shannon diversity index; *Np* – number of private alleles per population; *Ho* – observed heterozygosity; *He* – expected heterozygosity; F_{IS} – inbreeding coefficient for an individual relative to the subpopulation.

Table 3

Genetic variation at four microsatellite loci in the analysed samples

Locus	No. alleles	*He*	*Ho*	F_{ST}	F_{IT}	F_{IS}	*Nm*
Ssa197	4	0.325	0.567	0.519	0.160	-0.744	0.232
Strutta12	6	0.587	0.800	0.170	-0.132	-0.364	1.222
Strutta 58	4	0.498	0.967	0.304	-0.350	-0.940	0.572
Str85INRA	5	0.485	0.900	0.372	-0.165	-0.856	0.421
Mean	4.75			0.341	-0.122	-0.726	0.612

He – expected heterozygosity; *Ho* – observed heterozygosity; F_{ST} – the effect of the subpopulation relative to the total population; F_{IT} – inbreeding coefficient for an individual relative to the total population; F_{IS} – inbreeding coefficient for an individual relative to the subpopulation; *Nm* – number of migrants per generation.

In all of them, the mean number of alleles per locus (*Na*) was higher than the mean number of effective alleles per locus (Ne) (Table 2). Shannon diversity index (*I*) was lower in the sea trout population from the Parseta river compared to the other investigated populations. The observed heterozygosity (*Ho*) was lower than the expected heterozygosity (*He*) in all populations and most heterozygotes were observed in the sea trout population from the Parseta, while the fewest number of heterozygotes were observed in the Wieprza population (Tables 2 & 3). Chi-square test did not show (P>0.01) significant deviations from Hardy-Weinberg equilibrium for any of the analyzed loci. Private alleles were observed in all the investigated populations, in all loci. The highest number of private alleles occurred in the sea trout population from the Rega river, while the fewest occurred in the Parseta river (Table 2). Statistical analyses revealed that the investigated populations are characterized by inbreeding coefficient (F_{IS}) below zero, which indicates a high excess of heterozygotes (Tables 2 & 3).

The observed heterozygosity values generated by each SSR primer were between 0.567 and 0.967, while the values of expected heterozygosity ranged from 0.325 to 0.587. The fixation index was the highest for the Ssa 197 primer, and the lowest for the Strutta 12 primer (Table 3). The level of genetic differentiation between the populations was determined using the F_{ST} parameter. The genetic diversity between the investigated populations is very high (0.230-0.366). The highest genetic diversity was observed between the sea trout from the Rega river and those from the Wieprza (0.366) (Table 4).

Table 4

Genetic differentiation among populations (F_{ST})

	Wieprza	Parseta	Rega
Wieprza	0.000		
Parseta	0.244	0.000	
Rega	0.366	0.230	0.000

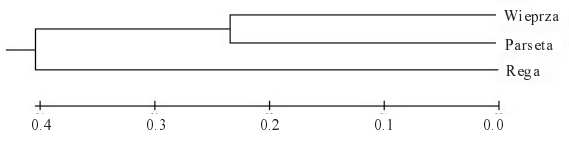

Fig. 3. Dendrogram (UPGMA) showing the genetic relationships between analysed populations *Salmo trutta* L. based on SSR polymorphism.

All polymorphic loci were detected in the Rega and Parseta population, while 75% of them were detected in the Wieprza population. A UPGMA dendrogram was constructed based SSR data in which two groups could be distinguished. The first group consisted of the sea trout populations from the Wieprza and the Parseta rives, while the second group was formed solely by the Rega river population (Fig. 3).

Discussion

A high level of genetic variability of *Salmo trutta* L. populations was observed in this study using microsatellite sequences. In the three investigated populations, only the Wieprza river population demonstrated a lower percentage of polymorphic loci (75%), while the remaining populations carried 100% polymorphic loci. Similar results were obtained in 9 populations of the brown trout in Spain (CORUJO *et al*. 2004), with as many as 7 populations carrying all polymorphic loci. The mean values of expected heterozygosity in the investigated *Salmo trutta* L. populations ranged between 0.373 to 0.539 and are similar to the values measured in populations from northwestern Spain (CORUJO *et al*. 2004) and France (POTEAUX *et al*. 1999), analysed using microsatellites. A comparable *He* level was also found in populations of Atlantic salmon, in Europe and in North America (SANCHEZ *et al*. 1996; MCCONNELL *et al*. 1997; GARANT *et al*. 2000; KING *et al*. 2001; LETCHER & KING 2001). Somewhat higher values of the expected heterozygosity (between 0.562 and 0.928) were reported by WAS and WENNE (2002) in the sea trout from the Rega and Vistula rivers or STELKENS *et al*. 2012 (between 0.73 and 0.81). However, the same study revealed lower values of the observed heterozygosity, suggesting a lower number of heterozygotes in the populations (WAS & WENNE 2002), as compared with this study. High similarity among individuals, and thus an increasing degree of inbreeding, is a serious problem in small populations, but also in bigger populations undergoing intense selection. The values of the inbreeding coefficient obtained for the sea trout populations from the Vistula, Rega and Parseta rivers were between -0.51 and -0.879. Higher values of inbreeding coefficient were found in populations of sea trout from Spain (ANTUNES *et al*. 2006), France (POTEAUX *et al*. 2001) and Greece (KARAKOUSIS & TRIANTAPHYLLIDIS 1990), resulting in a lower number of heterozygotes per population.

The results presented in this paper demonstrate that the investigated sea trout populations carry private alleles. Most of the characteristic alleles

were found in the sea trout population of the Rega river, while the lowest number of private alleles was found in the Parseta population. The Rega population belonged to a separate group distinguished in dendrograms generated using the results of the RAPD and SSR analyses, as opposed to fish from the Wieprza and Parseta rivers that formed the other group. Earlier studies employing allozymes (LUCZYNSKI *et al*. 1997), mtDNA (WŁODARCZYK & WENNE 2001) and microsatellites (WAS and WENNE 2002) demonstrated only an insignificant level of diversity between the sea trout population from the Rega and Parseta rivers.

The genetic differentiation between the investigated populations from the Rega, Parseta and Wieprza rivers is very high (0.230-0.366). The highest genetic differentiation was observed between sea trout from the Rega river and those from the Wieprza (0.366). A high level of genetic differentiation among sea trout populations inhabiting rivers was confirmed by numerous studies (RYMAN 1983; FERGUSON 1989). Nonetheless, such high values among populations are not always observed: HANSEN *et al*. (2001), in their studies of wild and the farmed populations of *Salmo trutta*, revealed a moderate level of genetic differentiation (0.065), while values determined by HANSEN and MENSBERG, (2009) were low (0.005-0.053). WAS and WENNE (2002), compared the genetic differentiation of the sea trout populations of the Rega and Vistula rivers, and obtained low values as well (approx. 0.03). The authors claim that such high genetic similarity between the sea trout populations from the Vistula, Rega and Parseta rivers is in accordance with the occurrence of the Pomeranian brown trout in the Vistula (WAS & WENNE 2002). This study, by employing RAPD markers, demonstrated a high genetic similarity between the investigated populations, ranging from 78.1% between the fish from the Rega and the Wieprza rivers to 82.0% between the fish from the Parseta and the Wieprza. Such a high interpopulation similarity of the sea trout was also shown between populations from the Vistula, Rega and Parseta rivers using isoenzyme analysis (LUCZYNSKI *et al*. 1997). It is thought that the wild populations of *Salmo trutta* and salmon are temporarily genetically stable (HANSEN *et al*. 2002; VÄHÄ *et al*. 2007), and the level of genetic polymorphism in the Polish populations of the sea trout is similar to that of fish from other regions (LUCZYNSKI *et al*. 1997).

The higher level of genetic differentiation between populations from the Rega, Parseta and Wieprza rivers, observed in this study, may be related with the fragmentation of these small populations, demographic processes, such as genetic drift, that occur within populations, but also with a restricted exchange of genes between these populations.

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