



MOLECULAR STRUCTURE AND HYBRIDIZATION PATTERNS OF *Abramis brama* × *Rutilus rutilus* HYBRIDS FROM MODRAC RESERVOIR, BOSNIA AND HERZEGOVINA

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ABSTRACT

Interspecific hybridization in the Cyprinidae family has been recorded worldwide, with *Abramis brama* (bream) and *Rutilus rutilus* (roach) as one of the often-reported hybridizing pairs. The only account of such an event in Bosnia and Herzegovina has been in Modrac Reservoir. Using morphological and molecular markers, the presence of hybrids was surveyed, the hybridization direction was determined and the hybrid group structure in this ecosystem was evaluated. Our findings confirmed unhindered natural hybridization between roach and bream in Modrac Reservoir. Over 50% of the hybrid specimens were classified as F₂ hybrids by the NewHybrids software, while the rest were categorized as pure parental form, making it the first such finding in Europe. The analysis of mitochondrial cytochrome *b* showed that 90% of hybrid individuals were of bream maternal origin. The hybrid group expressed higher mean values of observed heterozygosity and gene diversity than both parental species. Signs of introgressive hybridization between parental species were detected. The hybrid zone of Modrac Reservoir appears to follow the intermediate or “flat” hybrid model based on the balanced distribution of parental and hybrid genotypes. Further investigation is needed to elucidate the factors that enable the survival and mating success of post-F₁ individuals.

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INTRODUCTION

An increase in hybridization rates, both interspecific and intraspecific, has become a major issue in conservation biology worldwide due to anthropogenically-influenced habitat modifications and species translocation (Frankham et al., 2002). Hybridization is more common in fish than in any other vertebrate group (Allendorf and Waples, 1996). Several factors drive the hybridization process between closely related fish taxa: a) loss or degradation of natural habitat, b) competition for common or limited habitat, feeding or spawning grounds, c) parental species occurring in unequal abundances, d) external mechanisms of fertilization, e) introduction of allochthonous species, f) weak isolating mechanisms, g) aquaculture and fisheries (Campton, 1987; reviewed in Scribner et al., 2001). Hybridization can result in progeny so poorly adapted to local conditions they do not reach maturity or that are fertile and able to backcross with parental species, leading to gene introgression (Leary et al., 1995).

Scribner et al. (2001) summarized the data from 158 articles reporting on the hybridization between freshwater fish species, mostly in North American rivers. Out of 168 species and 139 species pairs from 19 families, the most frequent hybridization was detected within the family Cyprinidae, accounting for 40% of all hybrids represented. One of the best-studied hybridization cases in this family is between bream *Abramis brama* (L. 1758) and roach *Rutilus rutilus* (L. 1758). This cross has been well-documented across Europe and the British Isles (Cowx, 1983; Wyatt et al., 2006; Kuparinen et al., 2014), being especially frequent in Ireland where these hybrids often exceed the abundance of both parental species (Hayden et al., 2010; Toscano et al., 2010). Temporal overlap of spawning activities and similar spawning habitats facilitate hybridization between these two parental species (Pitts et al., 1997). Under laboratory conditions, Wood and Jordan (1987) found that hybrids between roach and bream are fertile, showing F_1 -hybrid × roach (female named first), roach × F_1 -hybrid and F_1 -hybrid × F_1 -hybrid as hybrids or backcrosses that produced fry, and bream × roach cross as a hybrid that failed to produce fertile eggs. However, the crossing of bream and roach (female first) was successful under controlled conditions in a study by Matondo et al. (2008). Though the possibility of backcross of F_1 -hybrids with either parental species has been demonstrated under controlled conditions (Pitts et al., 1997), the frequency of occurrence of F_2 -hybrids in nature is low (Wyatt et al., 2006; Hayden et al., 2010; Kuparinen et al., 2014, Konopiński and Amirowicz, 2017). The variation in observable morphometric and meristic characteristics in the hybrid offspring is likely increased by backcrossing, aggravating the identification problems (Wood and Jordan, 1987).

Given the limitations of the morphological approach in detecting post- F_1 hybrids, hybridization and introgression are best investigated using heritable genetic markers. Since

hybrids carry nuclear DNA makeup from both parents, F_1 individuals can be detected based on a single locus displaying different alleles in parental species. However, to confidently identify post- F_1 and backcrossed individuals, at least three to five loci should be screened (Pacheco et al., 2002), to account for the effects of segregation during meiosis. The usual type of nuclear markers employed in the hybridization studies on cyprinids are microsatellites (Hamilton and Tyler, 2008; Dubut et al., 2010) and internal transcribed spacer 1 (*ITS1*) region of nuclear ribosomal DNA (nrDNA) (Wyatt et al., 2006; Hayden et al., 2010). Matrilinially inherited mitochondrial (mtDNA) cytochrome *b* (*cytb*) gene is commonly used to test the direction of hybridization (Wyatt et al., 2006; Perea et al., 2010).

In Bosnia and Herzegovina, bream and roach overlap in their distributions, both species being common in the lowland rivers of the Black Sea basin (Sofradžija, 2009). The only report of *R. rutilus* × *A. brama* hybrids in Bosnia and Herzegovina was given by Adrović et al. (2009a). In that study, 65 specimens of bream, 41 of roach and 54 of hybrid individuals were caught in Modrac Reservoir (the Sava river drainage) near Tuzla. Out of 22 biometric characters, two morphological traits most prominently discriminated hybrids from parental species: the length of the anal fin base and the number of rays in the anal fin. Compared to bream, hybrid individuals displayed a shorter anal fin base and fewer rays in the anal fin but had longer anal fin with more rays than in roach. The authors concluded that molecular genetic studies should be conducted to confirm the results of morphological analysis.

This study aimed to test the discrimination power of morphological traits in detecting roach × bream hybrids from Modrac Reservoir (northern Bosnia and Herzegovina) in comparison to the nuclear *ITS1* region and seven microsatellite markers. Also, mitochondrial cytochrome *b* was analyzed to determine the maternal origin of hybrid individuals. Finally, the sample was assessed for the presence of post- F_1 individuals using NewHybrids software.

MATERIALS AND METHODS

Sampling

In total, 161 fish were caught from Modrac Reservoir in 2009 using the sets of three gillnets (mesh sizes 10, 30 and 50 mm). Fish were initially identified as bream, roach or hybrid, according to the Vuković and Ivanović (1971) determination key. Hybrid individuals were further confirmed based on morphological observations of the length of the anal fin base and the number of rays in the anal fin, following Adrović et al. (2009a). Clippings of abdominal fins were taken and stored in individual tubes with 96% ethanol until further analysis.

DNA isolation

DNA was extracted from abdominal fins following the salting-out protocol (Miller et al., 1988) and optimized for DNA isolation from fin tissue by Durmić-Pašić et al. (2005). The quality and yield of genomic DNA were tested by horizontal gel electrophoresis in SB buffer (Brody and Kern, 2005).

Analysis of ITS region, the mitochondrial cytochrome *b* and microsatellite markers

Primer sequences and protocols for the amplification of *ITS1* (product size of 385 bp and 152 bp for bream and roach, respectively) and cytochrome *b* (product size of 672 bp and 450 bp for bream and roach, respectively) regions were as in Wyatt et al. (2006). Analyses of PCR products were done on 1.5% agarose gel stained with ethidium bromide. Based on previous reports of successful cross-amplification in different cyprinid species (Hamilton and Tyler, 2008), seven microsatellite loci - *Ca1*, *Ca3* (Dimsoski et al., 2000), *CypG24*, *CypG27*, *CypG30* (Baerwald and May, 2004), *Rru2* (Barinova et al., 2004) and *Z21908* (<http://zfin.org/>) - were selected. All loci were amplified in 15 µl multiplex PCRs containing 1× PCR buffer, 3 mM MgCl₂, 0.5 mM dNTPs, 1 U Taq polymerase, 50 ng of DNA, and primers in final concentrations of 0.05 µM for *CypG24*, 0.1 µM for *Ca1*, *Rru2* and *Z21908*, 0.2 µM for *CypG30*, and 0.5 µM for *Ca3* and *CypG27*. Touchdown reaction was employed, consisting of 2 min of initial denaturation at 95°C, followed by 30 sec at 95°C, 30 sec at the annealing temperature and 45 sec elongation at 72°C, with 10 min of final extension at 72°C. The annealing temperature was decreased every five cycles (62°C, 58°C, 55°C, 53°C, 51°C, 49°C, 47°C), as suggested in Hamilton and Tyler (2008). Allele sizing was performed using GeneMapper ID v3.2 software on ABI PRISM™ 310 Genetic Analyzer.

Biostatistical data analysis and population-genetic analysis

To estimate the difference in the percentage of detected roach, bream and hybrid individuals based on morphological traits and molecular *ITS1* marker, the concordance correlation coefficient (Lin, 1989; Lin, 2000) was used within MedCalc for Windows, version 19.4 (MedCalc Software, Ostend, Belgium). GenAEx 6.5 (Peakall and Smouse, 2012) was used to calculate genetic indices in order to estimate the diversity of three analyzed groups (bream, roach and hybrids). These indices included a number of detected and effective alleles, allele frequencies based on the direct counting, observed (Hartl and Clark, 1997; Meirmans and Hedrick, 2011) and expected (Nei, 1987) heterozygosity and intrapopulation inbreeding (fixation index) (Hartl and Clark, 1997). A simple ratio between the number of effective and detected alleles (A_E/A_N) was used as suggested by Pojskić and Kalamujić

(2015). This measure indicates a possible degree of deviation between the effective number of alleles and the number detected by direct counting. Ratio and its *P* values were implemented within the ALRATIO R script (Pojskić, 2019). A Z-score of $P < 0.01$ was considered statistically significant. Deviation from Hardy-Weinberg equilibrium was tested by calculating χ^2 (Hedrick, 1999) with the significance level set to $P < 0.05$. Wright's *F* statistics (Hartl and Clark, 1997; Meirmans and Hedrick, 2011), pairwise *F*_{st} and AMOVA (Excoffier et al., 1992) were estimated in order to measure the genetic differentiation among analyzed groups. The population assignment test (Paetkau et al., 1995; Paetkau et al., 2004) was conducted using GENECLASS2 software (Piry et al., 2004) to investigate the relationship between the hybrids and parental species. Principal Coordinates Analysis (PCoA) was performed on the basis of the results of genetic distance between analyzed groups using GenAEx 6.5 (Peakall and Smouse, 2012). Estimation of the *K* value (ΔK) was performed using STRUCTURE HARVESTER software (Dent and von Holdt, 2012). A STRUCTURE test (Pritchard et al., 2000) was performed applying Markov chain Monte-Carlo (MCMC) analysis (admixture model, *K* = 2, burn-in period 100,000 and 200,000 iterations) to estimate possible hybridization. Assignment of individuals to clusters was provided by a probability of membership *qi* set at 90% for parental species and $0.1 < qi < 0.9$ for hybrids (Vähä and Pimmer, 2006). NewHybrids (Anderson and Thompson, 2002) was used to classify hybrid individuals into different categories (*F*₁, *F*₂ and backcross). The threshold *qi* values of belonging to a certain hybrid class were set up to $qi > 0.5$, following Aboim et al. (2010). To avoid overfitting, the following parameters were used: 100,000 MCMC sweeps after an initial burn-in period of 50,000 sweeps.

RESULTS

Based on morphological screening, 161 individuals were classified as 62 roach, 55 bream and 44 hybrids. Amplification of the *ITS1* region was successful for 152 samples, giving the expected band sizes for roach and bream, 152 bp and 385 bp, respectively, while hybrid individuals displayed both bands. Analysis of the *ITS1* region revealed that out of 40 samples that produced two bands, 87.5% of them were previously morphologically identified as hybrid individuals, while 2.5% and 10% were classified as roach and bream, respectively. Further, nine individuals, identified as hybrids based on morphological criteria, produced the *ITS1* band characteristic of roach. There was a substantial concordance in the percentage of detected roach, bream and hybrid individuals based on morphological traits and molecular *ITS1* marker (Pearson $P = 0.961$) (Fig. 1).

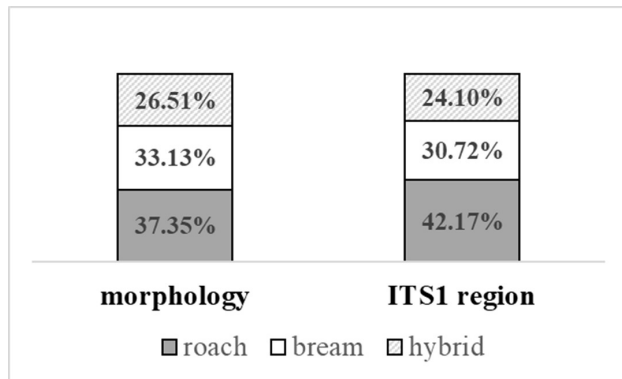


Fig 1. Agreement of detected species and hybrids based on morphological and molecular markers (*ITS1* region)

To determine the maternal origin of hybrid individuals, the mitochondrial cytochrome *b* was amplified using allele-specific amplification. Four of the *roach* × *bream* hybrids (10%) were found to have roach maternal origin, while 36 specimens (90%) were of bream maternal origin. Of seven investigated microsatellite loci, five were polymorphic in both bream and roach, as well as in hybrids (Table 1). Locus *Rru2* displayed only one allele (92 bp) in bream, while locus *CypG30* failed to give reproducible amplification in roach from Modrac Reservoir. Therefore, loci *Rru2* and *CypG30* were excluded from further analysis. Private alleles were observed for all five loci used in genetic indices calculations (Table 1).

Locus *Rru2* produced the smallest number of detected alleles (A_N) in all three investigated groups (9, 1 and 5 alleles in roach, bream and hybrids, respectively) while locus *Ca3* was most polymorphic across the sample. However, the number of effective alleles (A_E) was significantly lower than detected alleles (A_N), with P value lower than 0.01 for *CypG24* in the bream group. Observed heterozygosity and gene diversity were high (mean 0.749 and 0.858, respectively) in roach, while both indices varied across loci in bream and had reduced mean values (0.592 and 0.731, respectively).

Table 1. Size ranges (in bp) for investigated microsatellite loci in bream, roach and hybrids. Numbers in brackets represent the observed number of private alleles

Locus name	<i>Abramis brama</i>	<i>Rutilus rutilus</i>	<i>Abramis</i> × <i>Rutilus</i>
<i>Ca1</i>	93-133	99-129 (4)	91-133 (1)
<i>Ca3</i>	231-305 (2)	235-333 (10)	231-341 (4)
<i>CypG24</i>	186/190/196/201 (1)	176-234 (3)	186-222
<i>CypG27</i>	279-333 (3)	237-287 (7)	241-331 (5)
<i>Z21908</i>	141-159	139-177 (4)	139-169
<i>CypG30</i>	173-231	-	173-231
<i>Rru2</i>	92	88-104	84-104

The mean values of heterozygosity (0.759) and gene diversity (0.884) were quite high in hybrid individuals. The level of inbreeding (F) varied among loci, with mean values of 0.123, 0.247 and 0.147 in roach, bream and hybrids, respectively (Table 2).

Table 2. Mean values over loci for each group. N – number of individuals, A_N – number of detected alleles, A_E – number of effective alleles, H_o – observed heterozygosity, H_e – gene diversity, F – inbreeding index

Group	N	A_N	A_E	H_o	H_e	F
Roach	63.000	18.800	9.252	0.749	0.858	0.123
Bream	50.000	11.200	5.582	0.592	0.731	0.247
Hybrid	39.000	18.600	9.369	0.759	0.884	0.147

In all analyzed groups, at least half of the loci significantly deviated from HWE. Also, Wright's F -statistics pointed to the increased inbreeding, with elevated values of F_{IS} and F_{IT} that varied across loci. Overall mean F_{ST} was moderate and equalled 0.081 (Table 3). These findings were corroborated by AMOVA that revealed that most variation was within individuals (75%), while variation between individuals and among groups was 14% and 11%, respectively. Analysis of pairwise F_{ST} (pF_{ST}) revealed clear genetic differentiation between bream and roach (0.110), low differentiation between roach and hybrids (0.027), as well as between bream and hybrids (0.050).

Table 3. Overall F -statistics for each analyzed locus

	<i>Ca1</i>	<i>CypG24</i>	<i>Ca3</i>	<i>Z21908</i>	<i>CypG27</i>	Mean
F_{IS}	0.034	0.322	0.179	0.281	-0.024	0.158
F_{IT}	0.104	0.447	0.207	0.323	0.032	0.223
F_{ST}	0.073	0.184	0.033	0.058	0.055	0.081

Table 4. Putative hybrid specimens as diagnosed using morphology, *ITS1*, *cytb* and microsatellite genotypic class assignments using STRUCTURE and NewHybrids. A – *Abramis brama*, R – *Rutilus rutilus*, H – hybrid, P1 – 1st parental species, P2 – 2nd parental species, F1 – F₁ generation, F2 – F₂ generation, B1 – backcrossed with 1st parental species, B2 – backcrossed with 2nd parental species

Sample	Morphology	ITS1	cytb	STRUCTURE qI values				NewHybrids qI values			
				R	A	P1	P2	F1	F2	B1	B2
H59	H	H	R	0.177	0.823	0.859	0.011	0	0.120	0.009	0.002
H88	H	H	A	0.247	0.753	0.020	0.009	0.007	0.893	0.014	0.058
H89	H	H	A	0.837	0.163	0.953	0	0	0.038	0.009	0
H91	H	H	A	0.576	0.424	0.393	0.024	0.002	0.537	0.024	0.020
H119	H	H	A	0.364	0.636	0.704	0.002	0.003	0.255	0.030	0.006
H139	H	H	A	0.412	0.588	0.901	0	0	0.090	0.009	0.001
H140	H	H	A	0.552	0.448	0.025	0.046	0.006	0.851	0.009	0.062
H141	H	H	A	0.365	0.635	0.160	0.003	0.001	0.786	0.019	0.030
H143	H	H	A	0.264	0.736	0.881	0.000	0.001	0.099	0.018	0.001
H144	H	H	A	0.471	0.529	0.403	0.036	0.003	0.521	0.015	0.023
H145	H	H	A	0.081	0.919	0.933	0	0	0.056	0.010	0
H146	H	H	R	0.706	0.294	0.033	0.026	0.002	0.905	0.013	0.021
H147	H	H	A	0.362	0.638	0.024	0.010	0.001	0.943	0.011	0.011
H149	H	H	A	0.199	0.801	0	0.006	0.006	0.912	0.007	0.069
H151	H	H	A	0.361	0.639	0.871	0	0.001	0.114	0.013	0.001
H152	H	H	A	0.396	0.604	0.721	0.001	0	0.248	0.027	0.002
H156	H	H	A	0.560	0.440	0.030	0.052	0.001	0.842	0.001	0.074
H157	H	H	A	0.253	0.747	0.212	0.011	0.003	0.701	0.066	0.007
H158	H	H	A	0.288	0.712	0.138	0	0.029	0.760	0.055	0.018
H159	H	H	A	0.565	0.435	0.014	0.022	0.001	0.935	0.003	0.026
H160	H	H	A	0.436	0.564	0.429	0	0	0.555	0.010	0.006
H162	H	H	A	0.516	0.484	0.897	0	0	0.091	0.008	0.004
H163	H	H	A	0.686	0.314	0.556	0	0	0.432	0.009	0.003
H167	H	H	A	0.563	0.437	0.327	0.017	0.001	0.580	0.072	0.003
H168	H	H	A	0.479	0.521	0.036	0.002	0.004	0.919	0.010	0.029
H170	H	H	A	0.498	0.502	0.053	0.164	0	0.692	0.003	0.089
H171	H	H	A	0.294	0.706	0.439	0.010	0	0.540	0.007	0.003
H172	H	H	A	0.293	0.707	0.533	0.003	0	0.418	0.005	0.041
H173	H	H	A	0.340	0.660	0.035	0.004	0.004	0.870	0.002	0.085
H174	H	H	A	0.062	0.938	0	0.041	0.001	0.882	0.001	0.074
H175	H	H	A	0.265	0.735	0.001	0.040	0.001	0.885	0.002	0.071
H177	H	H	A	0.460	0.540	0.403	0.030	0.002	0.523	0.024	0.017
H178	H	H	A	0.333	0.667	0.875	0.015	0	0.104	0.006	0.001
H179	H	H	R	0.528	0.472	0.607	0.001	0	0.380	0.005	0.006
B125	R	H	R	0.425	0.575	0.031	0.039	0	0.881	0.002	0.047
D75	A	H	A	0.891	0.109	0.613	0.012	0	0.345	0.028	0.002
D79	A	H	A	0.843	0.157	0.921	0	0	0.065	0.011	0.002
D82	A	H	A	0.760	0.240	0.888	0	0	0.105	0.006	0
D87	A	H	A	0.961	0.039	0.872	0.007	0	0.105	0.014	0.001

The assignment test (Paetkau et al., 1995; Paetkau et al., 2004) showed clear differentiation between bream and roach, but also the genetic similarity between parental species and the hybrid group. When all three groups were compared, the hybrid group was positioned between bream and roach, with apparent dispersion and admixture of hybrid individuals with the other two clusters. These were confirmed by the results of PCoA analysis (Fig. 2). STRUCTURE analysis based on the MCMC method also supported such relations (Fig. 3). Assuming $K=2$, STRUCTURE revealed that 97.5% of bream individuals

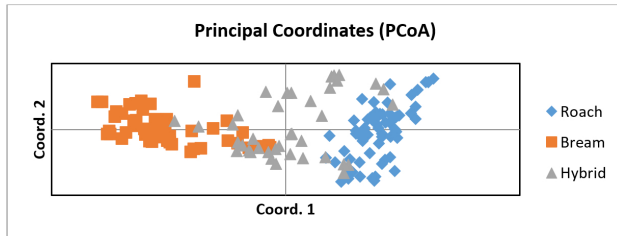


Fig 2. Results of the Principal Coordinates Analysis (PCoA)

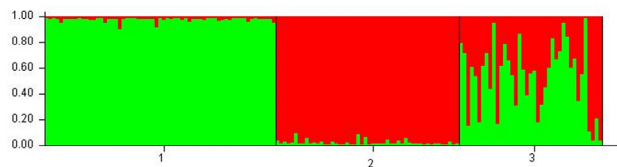


Fig 3. Results of STRUCTURE analysis based on MCMC method (cluster I - bream, green; cluster II - roach, red; Groups: 1 - bream, 2 - roach, 3 - hybrids). Each column represents one individual.

belonged to cluster I (roach), while 97.8% of roach fell into cluster II. Most of the hybrids were distributed relatively equally between both clusters (Table 4).

Four individuals assigned as bream and one specimen assigned as roach according to morphology and microsatellite data displayed an *ITS1* profile characteristic of hybrids (Table 4). Analysis of cytochrome *b* showed that those four putative bream individuals were mothered by bream, while the roach had roach maternal origin. Nine samples, described as hybrids based on morphology and microsatellites, gave only one band for *ITS1*, specific for roach. Cytochrome *b* showed that six of them had roach maternal origin, while three displayed a bream profile. Finally, ten samples were thought to be hybrids according to morphology and *ITS1* profiles displayed bias towards parental clusters in STRUCTURE analysis (71.2% – 93.8%). Nine of them were assigned to the roach cluster, while one was placed in the bream group. The applied analysis showed concordance with the results of *ITS1* profiling in most of the cases. Out of 40 samples that appeared to be hybrids when analyzed with *ITS1* marker, 92.5% were confirmed by MCMC analysis as well.

Whether any of the analyzed specimens represented a post- F_1 individual was further tested by NewHybrids software. Based on the allele frequencies of multilocus genotypes, this software estimates the posterior probability (q_i) that a given individual falls in each of the set categories: parent species (i.e. P1 and P2), F_1 , F_2 and backcrosses (i.e. B1 or B2). Of all samples that displayed the hybrid *ITS1* profile, 56.4% were classified as F_2 hybrids when the posterior probability threshold of

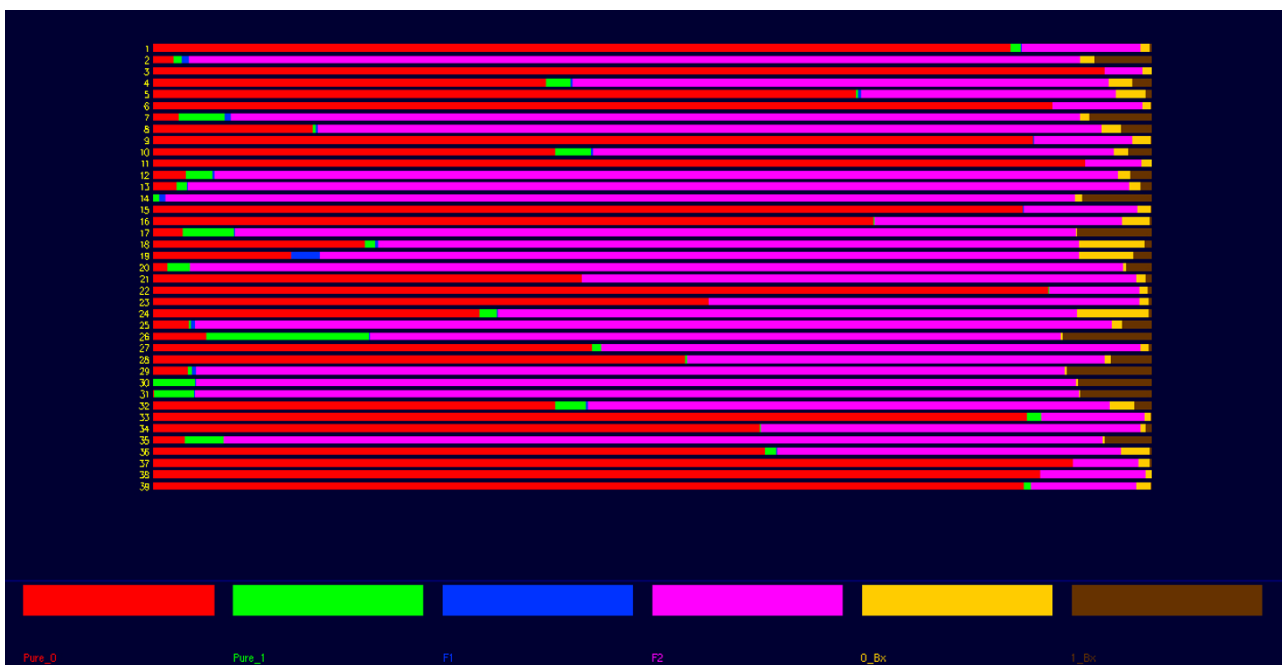


Fig 4. The classification of hybrid individuals based on the NewHybrids analysis to six set groups: Pure_0 – 1st parent species (red), Pure_1 – 2nd parent species (green), F1 – F_1 generation (blue), F2 – F_2 generation (purple), 0_Bx – backcrossed with 1st parental species (yellow), 1_Bx – backcrossed with 2nd parental species. Each line represents one individual (brown)

0.5 was applied, while 43.6% were categorized as pure parental form. No F_1 or backcross categories were found (Fig. 4, Table 4). Loci *Ca3*, *CypG27* and *Z21908* were most informative for determining the hybrid category.

DISCUSSION

Intergeneric hybridization in the Cyprinidae family has been known since as early as the late 19th century (Kodukhova, 2010). The most common hybridizing pair within the cyprinids appears to be the roach × bream, especially in lakes and reservoirs (Kuparinen et al., 2014). Modrac Reservoir was established in 1964 by constructing a dam across the River Spreča to supply water for the surrounding industry. The reservoir flooded parts of riverbeds of the rivers Spreča and Turija, merging their fish communities in the new habitat and facilitating hybridization events. Notwithstanding more than 50 years of its existence, the data on the ichthyological structure of Modrac Reservoir have been very scarce (Habeković et al., 1981; Adrović et al., 2009a; Adrović et al., 2012). A study from the 1980s showed the prevalence of common bleak *Alburnus alburnus* (L. 1758) (64.34% of the total catch; Habeković et al., 1981). However, some 30 years later, the dominance shifted to roach *Rutilus rutilus* and bream *Abramis brama* (31.25% and 19.56%, respectively; Adrović et al., 2009a). The first report of natural hybridization between these two species in Modrac Reservoir and in Bosnia and Herzegovina, in general, was given by Adrović et al. (2009a, b). Based on the analyses of several morphometric characteristics, most prominently the length of the anal fin base and the number of rays in the anal fin, the authors structured their catch as 24% bream, 16.4% roach and 21.6% hybrids. The present study offers the first insight into the genetic composition of the hybrid individuals and both parental species from this location, as well as the hybridization direction.

Our results confirmed unhindered natural hybridization between roach and bream in Modrac Reservoir, regardless of the marker system used. Since there was no significant difference between the results of morphological and molecular analysis, two biometric traits (i.e. base length and the number of rays in the anal fin) proposed by Adrović et al. (2009a) as the best discriminators for bream × roach hybrids from Modrac Reservoir proved to be reliable (Fig. 1).

According to morphological observations, 44 individuals were characterized as hybrids, of which 35 specimens (79.5%) displayed the hybrid profile when analyzed with *ITS1* marker. Finally, 32 (72.7%) were assigned as hybrids based on $0.1 < q_i < 90\%$ parameter (Vähä and Primmer, 2006) in STRUCTURE analysis. There was a concordance of 91.4% in the results of hybrid discrimination when only molecular markers (i.e. *ITS1* and microsatellites) were concerned. Our results are in line with those reported by Konopiński and Amirowicz (2017) who investigated the genetic composition of bream × roach hybrids in Dobczyce

Reservoir in southern Poland, based on 16 microsatellites derived for the species *Leuciscus leuciscus* (Cyprinidae).

Of seven microsatellite loci used in this study, *Rru2* was monomorphic in bream while *CypG30* did not amplify in roach, despite the previous reports of successful amplification in *R. rutilus* from the River Bourne, Surrey, UK (Hamilton and Tyler, 2008). Although it could be argued that the number of employed microsatellite markers was low, private alleles were observed for all five loci used in calculations (Table 1), proving their informativeness for the delineation of parental species and hybrid individuals. The mean gene diversity for hybrids was high (0.884) in our study, as it was in Konopiński and Amirowicz (2017), in which the mean genetic diversity in hybrids (0.716) was slightly lower than in *R. rutilus* (0.731), but higher than in *A. brama* (0.370). For the chosen microsatellite set, hybrids from our survey displayed higher gene diversity than both parent species (Table 2).

Analysis of the mitochondrial *cytb* region revealed that hybrids in Modrac Reservoir originate predominantly from the mating between bream females and roach males. This is in agreement with previous studies (Wyatt et al., 2006; Hayden et al., 2010; Kuparinen et al., 2014; Konopiński and Amirowicz, 2017) and appears to be a universal direction of hybridization of these two species, across their overlapping range in Europe.

When all markers and analyses were concerned, 16 specimens appeared as undoubted hybrid individuals. However, similar to other studies (Aboim et al., 2010), the hybridization rate depended highly on the investigated marker system. This finding further justifies the recommendation of using different marker systems when conducting hybridization surveys (Scribner et al., 2001).

The most surprising results of our study were those obtained by NewHybrids software. Over 50% of the specimens with *ITS1* hybrid profile were classified as F_2 hybrids when the threshold of 0.5 was applied (Table 4). This contradicts the most available reports on bream × roach hybridization in Europe that showed either the complete presence of F_1 individuals in the hybrid population (Kuparinen et al., 2014; Konopiński and Amirowicz, 2017) or their prevalence of over 90% (Toskano et al., 2010; Hayden et al., 2010). However, despite their abundance in most habitats where they occur, no incontestable evidence of hybrid swarming has been found so far. As shown by Tominaga et al. (2018), such distribution of hybrid classes in a population (no F_1 , all F_2 , no backcrosses) could be seen in those habitats where the pure parental species currently rarely come into direct contact around the hybrid zone and all gene flow is conducted via hybrid swarms. They further showed that NewHybrids assigned the majority (77.3%) of simulated individuals from posterior hybrid generations (i.e. F_3 , F_4 and F_5) into F_2 class. Such findings were expected due to the limitation of the software (six a priori assumed classes) to predict post- F_2 classes individually. Therefore, when our results are considered, the possibility of having

posterior hybrid generations in our sample as well cannot be disregarded, which would indicate the existence of the hybrid swarm in the investigated part of Modrac Reservoir. Some previous reports considered bream × roach hybrids to be fertile and able to produce offspring with either of parental species, both under experimental conditions (Pitts et al. 1997; Wood and Jordan, 1987) and in the wild (Toscano et al., 2010). Implications of post-F₁ hybridization and backcrossing, based only on discrepancies between morphological and molecular data, were given in some studies (Wyatt et al., 2006; Hayden et al., 2010; Toscano et al., 2010) but were disputed by some authors (Konopiński and Amirowicz, 2017) on the basis of the need for further confirmation using more complex marker systems and analyses. As far as it is known, our results are the first indication of F₂ bream × roach hybrid individuals in the natural habitat when following the approach suggested by Konopiński and Amirowicz (2017). However, to further confirm such findings, analysis of a larger sample and additional microsatellites would be beneficial. Although speculative, a possible explanation for the occurrence of the F₂ generation (and putative posterior hybrid generations) could be an ecological adaptation of hybrid individuals in Modrac Reservoir and a lack of selection pressure against their phenotype. It was reported that the intermediate morphology of *A. brama* × *R. rutilus* hybrids does not pose any significant disadvantage compared to parental species (Toscano et al., 2010). Furthermore, these hybrids have been shown to be more flexible in food choice than the parental species (Hayden et al., 2011). This could make them more resistant to fluctuations in available food, allowing for faster growth and conquest of new niches in the habitat. Such resilience is particularly advantageous in habitats under extensive anthropogenic pressure such as Modrac Reservoir (water level fluctuations, pollution, overfishing, etc.). If that is the case, then the existence of the hybrid swarm cannot be excluded, justifying the result of NewHybrids analysis seen in this study.

The observed distribution of parental and hybrid genotypes (43.6% and 56.4%, respectively) indicates that the hybrid zone of Modrac Reservoir follows the intermediate or “flat” hybrid model (Jiggins and Mallet, 2000), at least in the case of investigated species. Although a study by Konopiński and Amirowicz (2017) on bream × roach hybridization in Dobczyce Reservoir showed a clear unimodal hybrid zone, the occurrence of transitional hybrid zones with “flat” genotype distribution is not unusual for cyprinid fish (Meagher and Dowling, 1991; Costedoat et al., 2005, McKenzie et al., 2016). In an exogenous, post-zygotic selection, parental genotypes are favored in their typical habitats, but hybrids display higher fitness in intermediate habitats. Whether this type of selection is the driving force in Modrac Reservoir is still to be confirmed with further experimental data.

Our data also indicated a possible introgressive hybridization between two parental species. Four

specimens, that were assigned as bream according to morphological and mitochondrial screening, clearly grouped into roach clusters based on the STRUCTURE results ($q_i > 0.9$) or displayed high q_i values (i.e. 0.891, 0.843 and 0.760). Although mostly evidenced by mitochondrial introgression, introgressive hybridization between distant species has been previously documented in cyprinids (Sousa-Santos et al., 2014; Almodóvar et al., 2012; Aboim et al., 2010). In our survey, findings indicated the unidirectional type of introgression, probably driven by backcrossing of hybrid individuals, with bream mtDNA, with roach males. However, bidirectionality cannot be excluded until a larger sample set of all three groups is investigated. Varying hybridization rates, seen in the hybrid group in this study (Table 4), speak in favor of lasting and dynamic interspecific relations in Modrac Reservoir fish community.

Our results confirmed the existence of bream × roach hybrids based on various markers, but reliable data on the hybrid abundance in the fish community of Modrac Reservoir are still lacking. To explain whether the survival and mating success of post-F₁ individuals is driven by the extended spawning season, the possible broader ecological valence of hybrids, abundance in habitat or the combination of various causes, further studies on their ecology are needed. It would be valuable to investigate if similar hybridization and introgression scenarios are present in other cyprinid species in Modrac Reservoir fish community. The hybridization between *Abramis brama* and *Rutilus rutilus* clearly represents a convenient model for studying adaptation and hybridization patterns in European cyprinids.

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MOLEKULARNA STRUKTURA I OBRASCI HIBRIDIZACIJE HIBRIDA *Abramis brama* × *Rutilus rutilus* IZ AKUMULACIJE MODRAC, BOSNA I HERCEGOVINA

SAŽETAK

Interspecijska hibridizacija u obitelji Cyprinidae zabilježena je širom svijeta, a *Abramis brama* (deverika) i *Rutilus rutilus* (bodorka) su jedan od često prijavljenih hibridizirajućih parova. Jedini opis takvog događaja u Bosni i Hercegovini potječe iz akumulacije Modrac. Koristeći morfološke i molekularne markere, istražili smo prisutnost hibrida, odredili smjer hibridizacije i procijenili

strukturu hibridnih skupina u ovom ekosustavu. Naša su otkrića potvrdila nesmetanu prirodnu hibridizaciju bodorke i deverike u akumulaciji Modrac. Primjenom NewHybrids softvera, preko 50% hibridnih primjeraka klasificirano je kao F₂ hibridi, dok su ostali kategorizirani kao čisti roditeljski oblik, što je prvi takav nalaz u Europi. Analiza mitohondrijskog citokroma *b* pokazala je da 90% hibridnih jedinki ima majčinsko podrijetlo od deverike. Hibridna skupina pokazala je veće srednje vrijednosti promatrane heterozigotnosti i raznolikosti gena u odnosu na obje roditeljske vrste. Otkriveni su znakovi introgresivne hibridizacije između roditeljskih vrsta. Čini se da hibridna zona akumulacije Modrac slijedi intermedijarni ili "ravni" hibridni model zasnovan na uravnoteženoj raspodjeli roditeljskih i hibridnih genotipova. Potrebna su daljnja istraživanja kako bi se rasvijetlili čimbenici koji omogućuju preživljavanje i uspjeh u parenju jedinki poslije F₁ generacije.

Gljučne riječi: deverika, bodorka, NewHybrids, post-F₁, introgresija

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