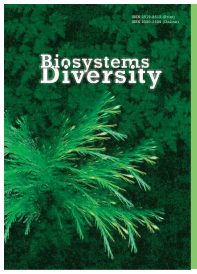

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Asymmetric hybridization of roach *Rutilus rutilus* and common bream *Abramis brama* in controlled backcrosses: Genetic and morphological patterns

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In most cases in natural environments, hybrids of roach *Rutilus rutilus* L. and bream *Abramis brama* L. possess mitochondrial DNA of bream. Presumably, the genetic basis for unidirectional hybridization of roach and bream is the high level of divergence in the genes of the mitochondrial electron transport chain (cytochrome *b* and cytochrome *c* oxidase subunits I, III). Disruption of the interaction of the products of these genes leads to nuclear-cytoplasmic incompatibility of alien genomes, what is shown in a decrease of viability and developmental abnormalities in hybrids. In the present work we studied the viability and morphology of hybrid underyearlings obtained by crossing of hybrid females of first generation (RA and AR) with males of roach *R. rutilus* and bream *A. brama*. The method of genotyping (ITS1 ribosomal DNA, cytochrome b mtDNA) and comparative analysis of the complex of 23 plastic and meristic characteristics of backcrossed hybrids are used. All progenies showed an increase in morphological variability compared to parental species and F1 hybrids. In progenies with introgression of mtDNA, a violation of associations between traits and the formation of transgressive phenotypes that go beyond the parental populations were found. In $_{R}$AA backcrosses (combining mtDNA of *R. rutilus* and nuclear genome of *A. brama*) a decrease in viability with impaired recovery of external traits of bream was found. Conversely, $_{A}$RR backcrosses (combining mtDNA of *A. brama* and the nuclear genome of *R. rutilus*) have a high viability and completely restore the morphotype of roach, which indicates the stable development of hybrids when they include alien genetic material. The differences in viability and morphology between backcrossed hybrids with the mtDNA of *R. rutilus* and *A. brama* evidence varying degrees of nuclear-cytoplasmic compatibility of the genomes of roach and bream. The complete interaction between mitochondrial and nuclear DNA from different species (wild-type-like) happens in direction of introgression mtDNA of *A. brama*, the less polymorphic of the two parental genomes. In the direction of introgression of highly polymorphic mtDNA of *R. rutilus* the formation of a reproductive barrier occurs. Our results show that the main reason for the asymmetry of hybridization of *R. rutilus* and *A. brama* is unequal rates of mitochondrial evolution and the priority of the introgression of mtDNA belongs a species with a lower rate of changes in mtDNA.

Keywords: backcrosses; Cyprinidae; introgression of mtDNA; morphological analysis; nuclear-cytoplasmic incompatibility.

Introduction

The Cyprinidae family is characterized by a great number of cases of interspecific hybridization (Schwartz, 1981). In particular, remote hybridization between roach *Rutilus rutilus* L. and common bream *Abramis brama* L. is widespread in Europe (Economidis & Wheeler, 1985). According to E. Mayr's classification (1963), it belongs to the category "from case by case", when hybrids of the first generation (F1) of both sexes are fertile and capable of crossing with parental species (Nikolyukin, 1952; Wood & Jordan, 1987). In sympatric habitats, hybridization between *A. brama* and *R. rutilus* is sporadic and does not significantly affect the population structure of species; there is a generally low incidence of interspecific hybrids (0.22–1.92% of parental species) (Kodukhova, 2011). Due to habitat changes resulting from the introductions or natural resettlement of species, outbreaks of hybridization between *A. brama* and *R. rutilus* occur, and the proportion of interspecific hybrids in such cases may exceed the frequency of occurrence of parental species (Fahy et al., 1988; Allendorf et al., 2001).

The studies of hybrid zones demonstrate that crosses between species occur predominantly in the direction female *A. brama* – male *R. rutilus* (Wyatt et al., 2006; Hayden et al., 2010; Toscano et al., 2010; Kuparinen et al., 2014; Konopinski & Amirowicz, 2017). According to the observations of Nikolyukin (1964), repeated backcrossing of F1 hybrids also occurs with *R. rutilus*. Experimental studies of reproductive behaviour confirmed these observations since F1 hybrid females preferably cross with males *R. rutilus*, not *A. brama* (Nzau Matondo et al., 2011), indicating assortative mating. In these circumstances, only mtDNA of *A. brama* can be included in the genome of *R. rutilus* that was recorded in the lakes of Ireland, where the species has been introduced artificially at different times (Hayden et al., 2010). It was previously considered that this scenario is characteristic only of the secondary contact zones and associated with the resettlement and adaptation of species to new habitat conditions (Rhymer & Simberloff, 1996; Hewitt, 2001; Toscano et al., 2010), so may differ from native habitats. However, genetic analysis of hybrids from southern Finland (Kuparinen et al., 2014) and the Rybinsk Reservoir (our unpublished data), which are within the natural range of these species, confirmed the asymmetry of hybridization of *R. rutilus* and *A. brama* in sympatry. Some authors attribute the presence of the reproductive barrier in the direction of hybridization of female *R. rutilus* – male *A. brama* to differences in spawning behaviour of males of these species, spawning time, and body sizes, which allows female *R. rutilus* to spawn in shallow waters inaccessible to *A. brama* (Poncin et al., 1996; Toscano et al., 2010). The terminal manifestation of such asymmetric hybridization during repeated absorption crosses can be complete replacement of the mtDNA of one species by that of another species, with preservation of external characteristics (Borkin & Litvinchuk, 2013).

A high level of interspecific differences of *R. rutilus* and *A. brama* in genes encoding the subunits of cytochrome c-oxidase I and III (Ludanniy, 2008) suggests the existence of an additional postzygotic mechanism of reproductive isolation in the direction of introgression of highly polymorphic mtDNA of *R. rutilus*, based on the nuclear-cytoplasmic incompatibility of alien genomes (Stolbunova, 2017). Incompatibility of cytochrome *c*-oxidase genes (COXI, COXIII), the subunits of which are encoded exclusively by nuclear genes, leads to dysfunction of the mitochondrial

electron transport chain, a decrease in the efficiency of respiration, and a significant loss in the viability of backcrossed hybrids (Ellison & Burton, 2006). The direction of nuclear-cytoplasmic interactions affects the indicators of recombination and energy exchange, which is an important factor in the variability of hybrid offspring (Marckmann, 1954; Zhuchenko & Korol, 1985). In these circumstances, it is of interest to study the direct phenogenetic effect in hybrids during introgression of alien mtDNA. The pronounced nuclear-cytoplasmic effects are observed in alloplasmic hybrids, combining a nuclear genome of one species and a cytoplasm of another species (Pershina et al., 2014). Such combinations are formed as a result of backcrossing of reciprocal hybrids of the first generation with the paternal species. Differences between hybrids with different mtDNA in viability, fertility and morphological features are a sign of nuclear-cytoplasmic conflict (Rand et al., 2004). The purpose of this work was to study the viability and the formation of morphological characters in hybrid combinations with varying degrees of nuclear-cytoplasmic compatibility of the genomes of *R. rutilus* and *A. brama*.

Material and methods

Controlled crosses and hybrid growing conditions. Two hybrid females of first generation (V maturity stage, aged 4+) and four *R. rutilus* and *A. brama* males were used for controlled crosses: ♀RA × ♂R1 and ♀RA × ♂A1; ♀AR × ♂R2 and ♀AR × ♂A2 (Fig. 1, explanation below). Hybrid females were obtained in reciprocal interspecific crosses (♀ *R. rutilus* × ♂ *A. brama*, RA; ♀ *A. brama* × ♂ *R. rutilus*, AR) and raised to maturity. The males were caught in separate spawning grounds during a natural spawning event in the Rybinsk Reservoir (Yaroslavl oblast, Russia). The species status of the males was established before the cross by general external appearance and was later confirmed by molecular analysis.

The method of dry fertilization was used (Ryabov, 1981). Eggs of each female were divided into two parts; one part was mixed with the sperm of male *R. rutilus* and the other with the sperm of male *A. brama*. After collection of sexual products, parental individuals were frozen for further analysis. Upon fertilization, eggs were placed in four jars (diameter 50 cm, height 15 cm) with water from the open pond, about 2500–3000 eggs in each jar. The mean ± s.d. water temperature during embryo incubation was 17.5 ± 1.5 °C, a temperature regime corresponding with the natural conditions. The incubation of eggs took place under conditions of constant water exchange (3x day), control of oxygen concentration, acidity and temperature. After reaching the first feeding stage, the larvae were fed with wild plankton until the yolk sac completely resorbed. The larvae were then placed in open ponds with preliminary acclimation to temperature conditions (600 specimens per 600 m^2). Progeny from each cross was kept in a separate pond until the age of 6 months (i.e. from May to October). At least 200 underyearlings (0+) from each pond were obtained and frozen. Six parental individuals (♀RA, ♀AR, ♂A1, ♂A2, ♂R1, ♂R2) and 190 individuals of backcrossed hybrids were subjected to molecular and morphological analysis.

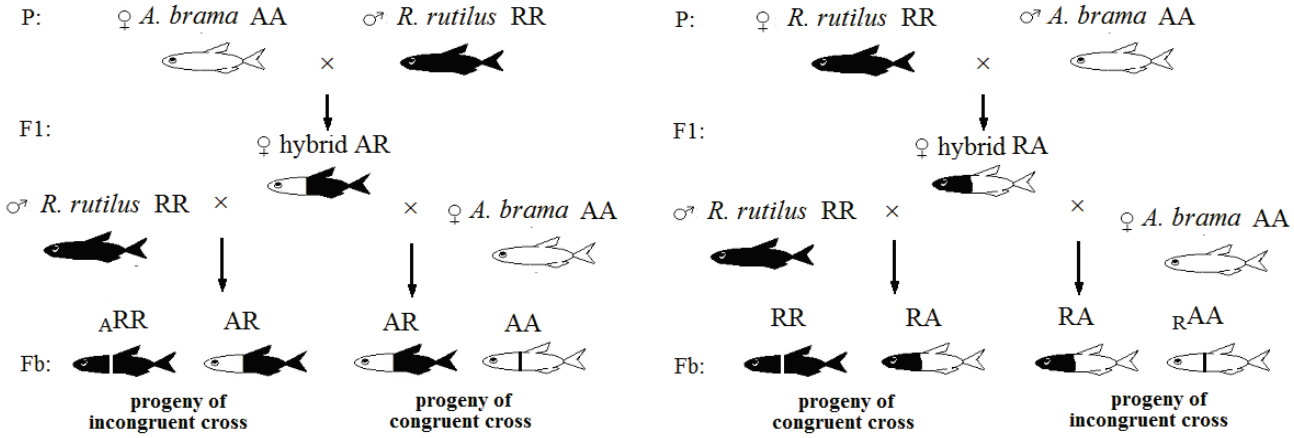

Fig. 1. Controlled mating scheme for obtaining of reciprocal hybrid females F1 (RA – *R. rutilus* × *A. brama*, AR – *A. brama* × *R. rutilus*) and backcrossed hybrids Fb in incongruent (♀RA × ♂A1, ♀AR × ♂R2) and congruent (♀RA × ♂R1, ♀AR × ♂A2) crosses. R and A – haploid genomes of *R. rutilus* and *A. brama* marked with black and white colours, correspondingly; the marker of haploid genome is species-specific fragment of ITS1 rDNA; P – parental species, F1– first generation hybrids, Fb – backcrossed hybrids

Molecular analysis of fish. DNA was isolated from the skeletal muscles of the parent individuals and hybrid underyearlings by the phenol-chloroform extraction method (Mathew, 1984). A preliminary analysis of the offspring was carried out on three microsatellite loci (*CypG53*, *CypG48*, *CypG24*) (Baerwald & May, 2004) to confirm kinship and exclude the introduction of accidental genetic material, since the hybrids were kept in open ponds. Only individuals with both maternal and paternal alleles were selected for further work (for details see Stolbunova, 2017). Nuclear (the ITS1 region of ribosomal DNA) and mitochondrial (cytochrome *b*) markers were used to ascertain genotype. Locus-specific PCRs were carried out for each marker type according to the method and primers outlined in Wyatt et al. (2006). Amplification of species-specific fragments of ITS1 of *A. brama* (147–152 bp) and *R. rutilus* (385–386 bp) was performed using two forward primers (for *A. brama*, Abi:5'-CCATGCCTCGGTTGTGTCT-3'; for *R. rutilus*, Rbi: 5'-AGGTCCCAGGAACAAAACAAC-3') and one reverse universal primer for both species (ITR: 5'-AGTGTCGATGATCAATGTGTCCT G-3'). Species-specific fragments of the mitochondrial cyt b gene of *A. brama* (672 bp) and *R. rutilus* (450 bp) were amplified using two forward primers (for *A. brama*, Abb: 5'-TGTTTATTACCCAAATCCTCACG-3'; for *R. rutilus*, Rbb: 5'-AACATTGTGTGGTTCTATTCCTC-3') and one reverse primer (Ubb: 5'-CACGAGTG GGTTTGCTGG-3').

PCR: 25 μL of reaction mixture contained 10x buffer ("Fermentas", Lithuania); 2.0 mmol $MgCl_2$; 200 μmol dNTPs; 3.2 pmol primers; 0.9 U Taq polymerase ("Bionem", Moscow); and 50 ng template DNA. Initial DNA denaturation was performed at 94 °C for 5 min, followed by 30 cycles of synthesis for ITS1, and 35 cycles for cyt *b*: denaturation – 94 °C for 45 s, annealing – 64 °C (cyt *b*) and 67 °C (ITS1) for 80 s, elongation – 72 °C for 60 s, final elongation at 72 °C for 5 min. Cyt *b* and ITS1 amplification products were fractionated by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized by UV.

The first internal transcribed spacer ITS1 of multicopy rDNA in *R. rutilus* and *A. brama* is located in the nucleolar organizer regions (NORs) at the ends of a single chromosome pair (Bianco et al., 2004; Ocalewicz et al., 2004). Due to the topological separation of parental genomes in intergeneric hybrids (Bennett, 1982), ITS1 is a marker of the haploid genome of the parent species with a co-dominant mode of inheritance. In hybrids of first generation of *R. rutilus* and *A. brama*, amplification of both parental fragments of ITS1 is expected (Fig. 1). In the progeny of each backcross, two hybrid classes are expected: 1) with one fragment of ITS1 (as in the parental species), which indicates the restoration of the nuclear genome of *R. rutilus* (RR) or *A. brama* (AA), and 2) with two fragments of ITS1, which indicates the hybrid nuclear genome (RA, AR, as in F1 hybrid).

In the study, two progenies from each female were obtained: one in a cross where the mtDNA of female and male coincided in species status – RA × R1, AR × A2 (congruent cross), and the other in a cross with intergenomic conflict, in which the parents had mtDNA of different species – RA × A1, AR × R2 (incongruent cross). Figure 1 shows the genotypes formed in the progeny of each cross. The first letter in the designation of the hybrid genotype (AR, RA) indicates the maternal species. In the designation of the genotype of alloplasmic hybrids ($_R$AA and $_A$RR), combining alien nuclear and mitochondrial genomes, an additional subscript letter was introduced to denote alien mtDNA. Taking into account that the expected ratio of genotypes in backcross is 1:1 (Mendel, 1923), the number of individuals of different classes was counted in each progeny. Significance of differences between theoretical and empirical distribution of genotypes was estimated using a χ^2 criterion (Zhivotovsky, 1991).

Morphological analysis. Comparative analysis of the complex of 23 plastic and meristic characteristics was performed in underyearlings of backcrossed hybrids (46–48 in each of four crosses) and in underyearlings of *A. brama* (A×A, n = 40), *R. rutilus* (R×R, n = 40), F1 hybrids – siblings of hybrid females (female×male: A×R, n = 50 and R×A, n = 29), obtained in inter- and intraspecific crosses. Only the most distinctive meristic characteristics were studied in parental individuals.

Meristic features were the number of scales in the lateral line (l.l.), the number of rows of scales above (SD) and under (SA) the lateral line, the number of rays in the anal (Ab) and dorsal (Db) fins, the number of vertebrae in the truncal (Va), transitional (Vi), and caudal (Vc) regions of the vertebral column, the total number of vertebrae (Vert), number of pores of the cephalic lateral line canal system in the cranial bones (frontal (CSOfr + par), parietal (CSTpar), praeoperculum (CPMpop), dental (CPMdn)) (Disler, 1960), and pharyngeal tooth formula (d.ph.). The ratio of number of vertebrae in the abdominal and caudal regions was estimated: Va ≥ Vc was defined as a "roach" type of axial skeleton, and Va < Vc as a "bream" type. The following plastic features were measured and expressed as a percentage of standard length (LS): head length (c), length of anal fin base (lA), length of dorsal fin base (lD), anteanal distance (aA), postanal distance (pA), antedorsal distance (aD), the maximum body height (H), and the smallest body height (h). Counting of vertebrae and pores of the cephalic lateral line canal system was performed on dry skeletons according to a standard procedure (Pravdin, 1966). The coefficient of variation (CV) and standard deviation (SD) were used to determine the variability of the features. For the most distinctive quantitative characteristics (Ab, l.l., Vert, SD, SA), the hybrid index HI was calculated from the average values of hybrids and parental species, using the formula (Hubbs & Kuronuma, 1942) HI = 100 * (Hi–Mi1) / (Mi2–Mi1), where Hi was average of hybrids for characteristic I, Mi1 was average of female parent species/form (for F1: female *R. rutilus* or *A. brama*; for Fb: hybrid females AR or RA), Mi2 was average of species representing the males (*A. brama* or *R. rutilus*). For characteristic I, the value of HI was interpreted as follows: for F1: from 45 to 55, intermediate characteristic to two species; < 45, characteristic close to female parent's species; > 55, characteristic close to male parent's species; for Fb: < 50, characteristic close to female hybrid F1; > 50, characteristic close to male parent's species (*A. brama* or *R. rutilus*). To compare Fb hybrids with the parent species and F1 hybrids and to identify the discrete groups in the backcross progeny, the principal component analysis (PCA) based on 18 morphological features was performed. Significance of differences of traits between progenies of one direction of hybridization was calculated by one way ANOVA, Tukey HSD test. Statistical processing was carried out using the Statistica 8.0 (StatSoft Inc., USA).

Results

Genotyping of parental individuals and backcrossed hybrids. One species-specific fragment for both ITS1 and cyt b was amplified in each male *R. rutilus* (♂R1, ♂R2) and *A. brama* (♂A1, ♂A2), confirming the species status. In each hybrid female ♀AR and ♀RA, both parental fragments of ITS1 and one species-specific fragment of cyt b of the corresponding length were amplified.

Deviations from the maternal inheritance of mtDNA were not detected in any of the backcross progeny. Genotyping (ITS1 and cyt *b*) showed two expected classes of individuals in each of the four backcrosses (Table 1). In incongruent crosses, the alloplasmic hybrids of the $_R$AA class (combining the nuclear genome of *A. brama* and mtDNA of *R. rutilus*, Fig. 2) and of the $_A$RR class (combining the nuclear genome of *R. rutilus* and mtDNA of *A. brama*) were obtained (Table 1).

Table 1
Distribution of ITS1 rDNA genotypes in incongruent and congruent crosses

Crosses		n	Genotypes, No/Ne		$\chi^2\varphi$, χ^2st, d.f.
Incongruent	♀RA × ♂A1	48	$_R$AA 16/24	RA 32/24	5.32 > 3.84 (1) (P < 0.05)
	♀AR × ♂R2	48	$_A$RR 30/24	AR 18/24	3.00 > 2.71 (1) (P = 0.10)
Congruent	♀RA × ♂R1	46	RR 25/23	RA 21/23	0.34 < 3.84 (1) (P > 0.05)
	♀AR × ♂A2	48	AA 24/24	AR 24/24	0.00 < 3.84 (1) (P > 0.05)

Note: R – *R. rutilus*, A – *A. brama*, RA and AR – reciprocal hybrid F1, n – number of hybrids, No-observed number of individuals, Ne-expected number of individuals, $\chi^2\varphi$-actual chi-square, χ^2st – statistical chi-square, d.f. – degrees of freedom.

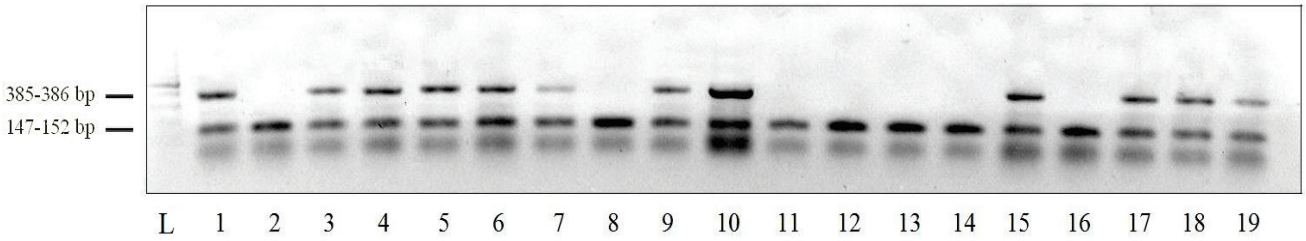

Fig. 2. Electrophoretic patterns of ITS1 rDNA in the progeny of backcrossed hybrids RA × A1: 1 – hybrid female ♀RA, 2 – male *A. brama* ♂A1; 3–7, 9, 10, 15, 17–19 – backcrossed hybrids of RA class with a combination of hybrid nuclear genome and mtDNA of *R. rutilus*; 8, 11–14, 16 – backcrossed hybrids of $_R$AA class with a combination nuclear genome of *A. brama* and mtDNA of *R. rutilus*; L – 100 bp DNA ladder; lengths of ITS1 fragments of *A. brama* (147–152 bp) and *R. rutilus* (385–386 bp) are given on the left

Segregation analysis on ITS1 rDNA in Fb progeny. Following Mendel's second law, the expected ratio of homo-and heterozygotes in the backcrossed progeny should be 1:1. This ratio was observed only in congruent crosses with the absence of intergenomic conflict (Table 1). Deviation from theoretical distribution of different descendant classes was found in incongruent crosses where hybrid female and male have mtDNA of different species. As Mendel noted, deviation from expected equilibrium in hybrids and their progeny occurs as a result of unequal viability of zygotes with different gene combinations. Accordingly, the deficit of homozygotes of $_R$AA class in cross ♀RA × ♂A1 is considered as a decrease in viability of these alloplasmic hybrids, which indicates low compatibility of the nuclear genome of *A. brama* and mtDNA of *R. rutilus*. In cross ♀AR × ♂R2, the viability of homozygotes of the $_A$RR class was higher than those of siblings with AR genotype, which indicates higher compatibility of the nuclear genome of *R. rutilus* and mtDNA of *A. brama*. In the second progeny of each hybrid female, where there were no alloplasmic combinations, hybrids of both classes had equal viability (congruent crosses).

Morphology of parental individuals and backcrossed hybrids. The most distinctive meristic characteristics in male *R. rutilus* and

A. brama ♂R1, ♂R2, ♂A1, and ♂A2 correspond to their species status and fell into the range of values of the artificial samples of *R. rutilus* (R×R) and *A. brama* (A×A) (Table 2). The values of meristic characters in hybrid females (RA and AR) were intermediate between those from intraspecies crosses (A×A, R×R) and fell into the range of values for artificial F1 hybrids (A×R, R×A). All F1 hybrids had the axial skeleton of "bream" type (Va < Vc).

Based on the similarity of mean values of morphological characters, two progeny of hybrid females were combined according to the direction of crosses: F1 × A (RA × A1 and AR × A2) and F1 × R (RA × R1 and AR × R2, Table 3). Mean values of the main diagnostic characters in backcrossed hybrids F1×A were close to those of F1 hybrids (Tables 2 and 3) and in hybrids F1×R – to those of the paternal species, *R. rutilus*. This is confirmed by hybrid indexes calculated for the key morphometric characteristics (Table 4) and by PCA results (Fig. 3). According to the regularities of inheritance of quantitative traits, the ellipses of distribution of individuals from crosses of F1 hybrid to each of the parents should be shifted closer to the distribution ellipses of the parental form involved in the cross.

In the direction F1 × R, despite the different cytoplasm, the 95% confidence ellipses of RA × R1 and AR × R2 samples almost completely overlap with each other, and partially overlap with the *R. rutilus* sample (Fig. 3a). Significant differences between these samples were revealed in 6 traits (Table 3). The axial skeleton of the "roach" type (Va ≥ Vc) was observed in 92% of RA × R1 hybrids and 94% of AR × R2 hybrids. Similar to *R. rutilus* (Table 2), the polymorphism of the variants of pharyngeal tooth formula with the dominant phenotype 6–5 was shown in these backcrossed hybrids (Table 3).

Table 2

Comparison of the most distinctive meristic characteristics in the parental individuals (♀F1 hybrid – RA and AR, ♂R – *R. rutilus* and ♂A – *A. brama*) and in samples of underyearlings F1 hybrids (A×R, R×A – female×male), *A. brama* (A×A), and *R. rutilus* (R×R) (x ± SE, range)

		l.l.	S_D	S_A	*Ab*	*Db*	*d.ph.*†	*Va*	*Vc*	*Vert*
Parents	♀AR	48	10	5	17	10	5-5	15	16	41
	♀RA	48	10	5	15	10	6-5	15	16	42
	♂R1	42	9	4	10	10	6-5	16	14	40
	♂R2	43	8	4	11	10	6-5	16	14	40
	♂A2	54	13	6	27	9	5-5	15	18	44
	♂A1	58	13	7	25	9	5-5	14	18	44
Hybrids F1	A×R	46.51 ± 0.23 44–52	9.93 ± 0.04 9–10	4.93 ± 0.04 4–5	14.82 ± 0.12 13–17	9.01 ± 0.02 9–10	6–5 (94%) 5–5 (6%)	14.71 ± 0.07 14–16	16.34 ± 0.09 15–18	41.33 ± 0.11 40–43
	R×A	49.09 ± 0.36 47–53	10.14 ± 0.05 10–11	5.03 ± 0.03 5–6	16.18 ± 0.18 15–18	9.39 ± 0.09 9–10	6–5 (90%) 5–5 (10%)	15.60 ± 0.11 14–16	16.82 ± 0.08 16–17	42.74 ± 0.10 41–43
A. brama	A×A	55.60 ± 0.21 50–56	11.74 ± 0.08 10–13	6.12 ± 0.06 5–7	25.83 ± 0.14 21–28	9.00 ± 0 8–10	5–5 (100%)	14.44 ± 0.07 14–15	17.81 ± 0.09 18–20	43.51 ± 0.08 43–45
R. rutilus	R×R	42.92 ± 0.11 39–44	8.12 ± 0.04 7–9	4.14 ± 0.04 3–5	10.32 ± 0.08 9–11	10.11 ± 0.05 9–11	6–5 (90.9%) 5–5 (9.1%)	16.52 ± 0.11 15–18	14.64 ± 0.12 14–16	41.31 ± 0.13 39–42

Note: † – pharyngeal teeth formula, frequency of occurrence is given in parenthesis.

Table 3

Comparison of the plastic (as % from LS) and meristic characteristics (x ± SE / range) in the backcrossed hybrids according to the direction of crosses F1 × R and F1 × A

Features	Direction	F1×R		F1×A	
	Cross	♀AR×♂R2	♀RA×♂R1	♀AR×♂A2	♀RA×♂A1
Plastic	L_S, mm	76.21 ± 0.59 64–87.3	52.08 ± 0.56 45.7–60.2	77.53 ± 0.51 69.4–86.6	52.61 ± 0.66 43.8–64.5
	C	24.83 ± 0.14 / 23.1–27.1	25.31 ± 0.15 / 22.1–28.0	24.53 ± 0.10 / 22.5–25.6	24.56 ± 0.14 / 22.7–27.2
	lA	14.68 ± 0.19 / 11.3–17.5	14.61 ± 0.22 / 11.3–18.7	20.91 ± 0.22 / 16.8–24.5	20.57 ± 0.24 / 17.1–23.9
	lD	14.49 ± 0.15 / 11.9–16.4	14.02 ± 0.18 / 11.0–16.7	13.70 ± 0.27 / 11.2–15.0	12.78 ± 0.16 / 10.4–15.4
	aA	71.33 ± 0.40 / 65.3–78.9	68.28 ± 0.29 / 64.5–74.3***	67.67 ± 0.23 / 65.0–73.5	64.66 ± 0.40 / 60.6–78.2***
	pA	14.91 ± 0.29 / 10.3–19.9	16.44 ± 0.31 / 12.3–21.0**	12.59 ± 0.19 / 10.0–16.1	14.51 ± 0.23 / 11.6–17.8***
	aD	52.38 ± 0.26 / 47.9–55.8	52.45 ± 0.24 / 49.3–56.2	54.23 ± 0.20 / 51.1–56.80	53.36 ± 0.24 / 50.6–57.6
	H	27.83 ± 0.26 / 24.5–34.0	26.94 ± 0.19 / 23.2–29.6*	30.24 ± 0.23 / 27.1–33.7	27.28 ± 0.25 / 23.8–33.6***
	h	9.42 ± 0.10 / 7.4–1.6	9.31 ± 0.13 / 7.7–11.5	10.14 ± 0.08 / 9.0–11.8	9.42 ± 0.09 / 7.8–10.6***
Meristic	*l.l.*	44.31 ± 0.25 / 42–48	44.93 ± 0.27 / 42–49	51.44 ± 0.33 / 47–58	47.52 ± 0.29 / 39–51***
	S_D	9.36 ± 0.08 / 8–10	8.64 ± 0.11 / 7–10***	11.10 ± 0.12 / 9–12	10.22 ± 0.08 / 9–11***
	S_A	4.95 ± 0.05 / 4–6	4.56 ± 0.07 / 4–6*	5.17 ± 0.07 / 4–5	4.93 ± 0.04 / 4–5**
	Ab	12.64 ± 0.11 / 11–14	11.76 ± 0.14 / 9–14**	19.11 ± 0.22 / 16–22	18.01 ± 0.16 / 15–21***
	Db	9.87 ± 0.04 / 9–10	9.90 ± 0.05 / 9–11	9.23 ± 0.07 / 8–10	9.12 ± 0.05 / 9–10
	Va	15.56 ± 0.13 / 13–18	15.30 ± 0.10 / 14–17	14.76 ± 0.11 / 13–16	15.09 ± 0.09 / 14–17
	Vi	3.50 ± 0.07 / 3–4	3.67 ± 0.07 / 3–4	4.22 ± 0.07 / 3–6	3.81 ± 0.06 / 3–5***
	Vc	14.43 ± 0.10 / 13–6	14.73 ± 0.09 / 13–16	16.59 ± 0.11 / 15–18	17.08 ± 0.08 / 16–18***
	Vert	40.57 ± 0.15 / 37–42	40.72 ± 0.12 / 39–42	42.62 ± 0.16 / 41–45	43.08 ± 0.10 / 42–45
	CSO_{fc+par}	10.01 ± 0.12 / 8–12	9.40 ± 0.11 / 8–11	10.54 ± 0.16 / 5–12	10.10 ± 0.14 / 7–12
	CST_{par}	3.31 ± 0.08 / 2–5	3.04 ± 0.06 / 2–4	3.61 ± 0.11 / 2–5	3.49 ± 0.12 / 2–6
	CPM_{pop}	11.78 ± 0.17 / 10–14	10.41 ± 0.12 / 9–12	11.28 ± 0.14 / 10–13	11.04 ± 0.12 / 9–13
	CPM_{dn}	6.33 ± 0.13 / 5–8	5.37 ± 0.09 / 4–7	6.42 ± 0.12 / 4–8	5.91 ± 0.09 / 5–7
	d.ph.†	6-5 / 51.17% 5-5 / 35.42% 5-6 / 8.33% 6-6 / 2.08%	6-5 / 72.92% 5-5 / 18.75% 6-6 / 8.33%	5-5 / 91.67% 6-5 / 8.33%	5-5 / 56.25% 6-5 / 43.75%

Note: F1 – reciprocal hybrid F1, R – *R. rutilus*, A – *A. brama*; † – pharyngeal teeth formula, frequency of occurrence is given in parenthesis; differences between progenies of one direction of hybridization were estimated using ANOVA, Tukey HSD test; asterisks designate significance level: * – P < 0.05, ** – P < 0.01, *** – P < 0.001.

Table 4

Estimation of the degree of intermediacy of backrossed hybrids between the parental forms (hybrid index HI) and the variability of the most distinctive characteristics (coefficient of variation CV and standard deviation SD)

Progeny	l.l.			S_D			S_A			Ab			Vert		
	HI	CV	SD	HI	CV	SD	HI	CV	SD	HI	CV	SD	HI	CV	SD
♀RA×♂A1	24	4.26	2.02	62	5.33	0.54	9	5.68	0.28	30	6.17	1.11	50	1.65	0.71
♀AR×♂A2	54	4.5	2.31	67	7.16	0.80	25	9.03	0.47	39	7.97	1.53	65	2.55	1.09
♀RA×♂R1	68	4.82	2.17	75	8.83	0.76	45	10.58	0.49	68	8.13	0.96	100	2.01	0.82
♀AR×♂R2	61	3.86	1.70	28	6.13	0.58	12	7.58	0.37	48	6.31	0.79	100	2.49	1.01
A×R	72	3.56	1.66	50	3.06	0.30	60	6.18	0.30	71	5.57	0.83	100	1.82	0.75
R×A	49	3.93	1.93	56	2.56	0.26	45	3.69	0.19	38	5.97	0.97	64	1.27	0.54
A×A	–	2.80	1.49	–	6.22	0.71	–	8.33	0.50	–	5.46	1.05	–	1.05	0.46
R×R	–	2.60	1.08	–	4.97	0.40	–	10.64	0.42	–	4.39	0.45	–	1.51	0.62

Note: R – *R. rutilus*, A – *A. brama*, RA and AR – hybrid F1, underyearlings of parental forms: F1 hybrids (A×R, R×A, female×male), *A. brama* (A×A), and *R. rutilus* (R×R).

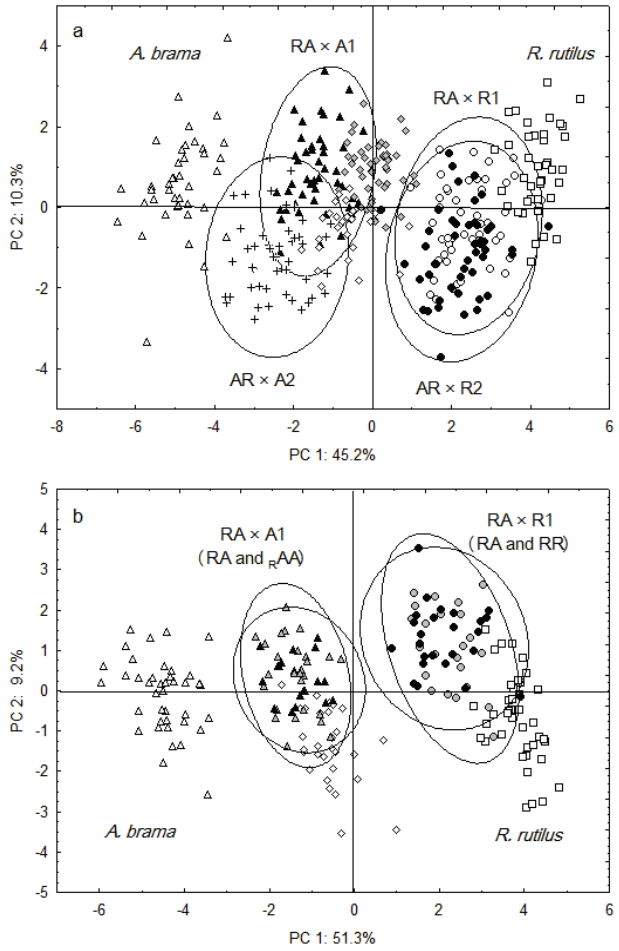

Fig. 3. Scatterplot of the first two axes of principal component analysis based on the set of morphological features for four progenies of backcrossed hybrids (shown with 95% confidence ellipses) and samples of parental species and F1 hybrids: *a* – morphological similarity between progeny groups of one direction of hybridization: F1×R (female×male: RA × R1 and AR × R2) or F1×A (RA × A1 and AR × A2); *b* – the absence of differentiation between backcrossed hybrids of two genotypic classes on ITS1 rDNA in the crosses RA × R1 and RA × A1; R – *R. rutilus*, A – *A. brama*, RA and AR – reciprocal hybrid F1; parental species/forms: △ – *A. brama*; □ – *R. rutilus*; ◇ – AR hybrid; ◇ – RA hybrid; backcrossed hybrids: F1×R: ● – AR × R2; ○ – RA × R1 (in panel *b*, different genotypes in this progeny are shown as follows: ● – RR genotype, ○ – RA genotype); F1×A: + – AR × A2; ▲ – RA × A1 (in panel *b*, different genotypes in this progeny are shown as follows: ▲ – $_R$AA genotype, △ – RA genotype)

In the direction F1 × A, there is a discrepancy to the above regularities of inheritance: 1) sample of backcrossed hybrids RA × A1 is shifted to the hybrid maternal form, and not to the parental species; 2) there are differences in distribution between backcrossed hybrids of congruent RA × R1 and incongruent RA × A1 crosses (Fig. 3a). Significant differences between these progenies were revealed in 10 traits (Table 3). The axial skeleton of the "bream" type (Va < Vc) was inherited by 96% of RA × A1 hybrids and 90% of AR × A2 hybrids and only two variants of the pharyngeal tooth formula with the dominance of *A. brama* phenotype 5–5 were revealed.

All backcrossed hybrids exhibited continuous distribution, with higher variance compared to samples of parental species and F1 hybrids (Fig. 3a). The differences between homo-and heterozygotes for ITS1 were not established (Fig. 3b). The highest phenotypic variability (estimated as CV and SD) was recorded by key diagnostic features: the number of scales in the lateral line, the total number of vertebrae and the number of rays in the anal fin (Table 4). In both progeny of incongruent crosses, variability was lower than in the congruent crosses, probably due to endogenous selection (Table 1). However, in hybrids of incongruent crosses transgressive phenotypes that went beyond the variability of the parental species were established.

In the progeny RA × A1, there is an increase in the range of variability of the number of scales in the lateral line (39–51), which exceeds twice the number of variants shown for each of the samples of *A. brama* (50–56), *R. rutilus* (39–44), F1 hybrids (AR 44–52, and RA 47–53) and AR × R1 (42–48). In the AR × R2 progeny, the high variability of Vert, associated with the output of Vert values beyond the range of parental species (39–45) was found, which is a negative transgression. In both progeny AR × R2 and RA × A1, transgressions for combinations of characters were found: the number of vertebrae (Vert) and body length (LS) (Fig. 4), which are functionally linked in the parental species (Lindsey, 1975).

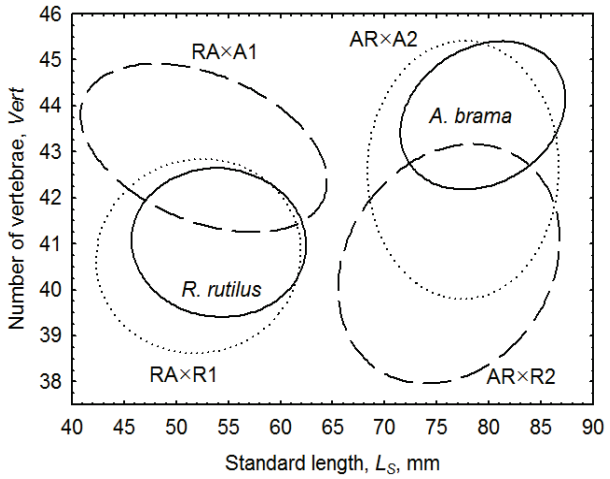

Fig. 4. Relationship between traits of standard length, LS and number of vertebrae, Vert in underyearling *R. rutilus*, *A. brama*, and backcrossed hybrids (shown as 95% confidence ellipses): positive correlation of LS and Vert is shown in samples of *R. rutilus*, *A. brama* (solid lines) and in hybrids of congruent crosses (RA × R1, AR × A2, dotted lines); in hybrids of incongruent crosses (RA × A1, AR × R2, dashed lines) negative association of LS and Vert with formation of two new combinations of traits is shown; AR, RA – reciprocal hybrid females F1, R – male *R. rutilus*, A – male *A. brama*

Based on the similarity in body length between underyearlings of *A. brama* (LS 79.42 ± 0.59 mm) and backcrossed hybrids with *A. brama* mtDNA (AR × R1 and AR × A1), as well as between *R. rutilus* (LS 54.08 ± 0.51 mm) and hybrids with mtDNA of *R. rutilus* (RA × R2 and RA × A2), maternal inheritance of LS in Fb progeny was established (Table 3). Consequently, hybrids from incongruent crosses had body length of the maternal species, and a number of vertebrae inherited from the paternal species. Significant interspecific differences between roach and bream in LS and Vert lead to the formation of two new combinations of traits in backcrosses (Fig. 4), with new functional relationships between them, which distinguish them from both parental species.

Discussion

The backcrossed hybrids of two genotypic classes (ITS1 marker) obtained in four controlled breedings of hybrid females F1 with males *R. rutilus* or *A. brama* do not differ in morphology (Fig. 3b). Most of them have intermediate characteristics between F1 hybrid and the parent involved in the cross (Fig. 3a). The backcrossed hybrids possess the greater variance of meristic and plastic traits compared to parental species and F1 hybrids and show a near-continuous distribution without discrete groupings. The main reasons for the high variability of hybrids are considered the intraspecific plasticity, the polygenic nature of quantitative traits with an additive character of interaction, revealing the latent diversity of species (recessive signs) during the decomposition of heterozygotes, and recombination (Vavilov, 1922; Mather & Jinks, 1982; Cowx, 1983; Bell & Travis, 2005; Dittrich-Reed & Fitzpatrick, 2013). Intermediate characteristics of backcrossed hybrids were shown in previous works (Pitts et al., 1997; Yakovlev et al., 2000). However, Pitts et al. (1997) studied only congruent crossings AR × A and RA × R that do not allow discovery of mechanisms of asymmetric hybridization. And Yakovlev et al. (2000) obtained the discrete groupings within the backcrosses of ♀ parent species × ♂ hybrid F1. Different morphological effects are obviously due to use of different parental forms as females.

In this work, for the first time, the morphological differences between the hybrids of congruent (RA × R1, AR × A2) and incongruent crosses (RA × A1, AR × R2) are shown; they are more pronounced between the offspring of the direction F1 × A (Fig. 3a). These differences are due to the fact that the genetic variation generated in incongruent crosses leads to the sudden appearance of transgressive phenotypes that go beyond the parental populations. Transgressions were established in individual traits (l.l., Vert, Table 3), combination of traits (LS and Vert, Fig. 4) and viability of backcrosses of different genotypic classes (Table 1). It is obvious that the formation of new phenotypes in crosses with a mismatch in the plasma of the parental forms is associated not only with the genetic divergence of the genomes of the parental species but also with nuclear-cytoplasmic interactions that affect recombination parameters, endogenous selection, energy exchange, the sex determination system and inheritance of individual traits (Zhuchenko & Korol, 1985; Werren & Beukeboom, 1998; Sulo et al., 2003). Alien cytoplasm reduces the relationship between the traits in hybrids of incongruent crosses since the inheritance of some traits is matroclinal. Thus, hybrids of congruent crosses maintain the positive correlation between body length (LS) and the number of vertebrae (Vert, Fig. 4), characteristic of parental species, and hybrids of incongruent crosses demonstrate the violation of this pattern.

The success of mtDNA introgression is assessed by the ability of hybrids to restore the species traits after alien genetic material is included (Formozov, 2007). The hybrid morphotype of backcrossed hybrids indicates that the parental species will not be able to maintain stability during introgressive hybridization, since blurring of species boundaries will occur (Wirtz, 1999). Among incongruent crosses AR × R2 hybrids show greater similarity with paternal species – *R. rutilus* and of RA × A1 hybrids – with F1 hybrids as revealed by hybrid indexes calculated for the key morphometric characteristics (Table 4) and results of PCA (Fig. 3). Obviously, in the direction of introgression of mtDNA of *R. rutilus*, hybrids RA × A1 ($_{R}$AA) are not able to restore the morphotype of the parent species – *A. brama*, what contradicts to the general regularities of inheritance of quantitative traits (Rokitsky, 1978). The violation of the restoration of the morphology of bream in progeny RA × A1 could result from low nuclear-cytoplasmic compatibility of a nuclear genome of *A. brama* and mtDNA of *R. rutilus* revealed from a decrease in viability of alloplasmic backcrossed hybrids $_{R}$AA (Table 1). In similar controlled cross roach-bream × bream only 9 of 40 hybrids survived and they possessed intermediate morphology (Nikolyukin, 1952).

High viability of alloplasmic hybrids $_{A}$RR indicates compatibility of a nuclear genome of *R. rutilus* and mtDNA of *A. brama*, which does not preclude the restoration of the morphology of roach in backcrossed hybrids AR × R2 (Fig. 3a). Such hybrids, with the morphology and ITS1 of *R. rutilus* and cyt *b* of *A. brama* were recorded in Lake Lough Ramor (Ireland) following the establishment of an invasive *R. rutilus* population in waters containing resident *A. brama* stocks (Hayden et al., 2010). Because the mitochondrial genome often contains region-specific adaptive polymorphisms (Wallace, 2007), the inclusion of mtDNA of *A. brama* in the genome of *R. rutilus* allows hybrids to quickly adapt to a new habitat and gives an additional directionality of nuclear genome evolution. Based on the data received, during the inclusion of bream mtDNA in AR × R2 hybrids, the most complete (wild-type-like) interaction mitochondria with a nucleus of a different species occurs. This is supported by the similarity of the morphotypes of these hybrids with the second progeny of the direction F1 × R (RA × R1), where the nuclear genome and roach mtDNA are restored. However, during the inclusion of roach mtDNA in RA × A1 hybrids a low degree of nuclear-cytoplasmic compatibility of genomes leads to the instability of ontogenetic trajectory, which may be due to the low viability of hybrids (Grodnitsky, 2002). Endogenous selection against $_{R}$AA genotype is the main reason for the more pronounced differences between hybrids of congruent and incongruent crosses in the F1 × A direction when compared with the F1 × R direction (Fig. 3a).

The nuclear-cytoplasmic incompatibility of alien genomes in $_{R}$AA combination may be due to the high rate of changes in mtDNA of *R. rutilus* compared to *A. brama* in cyt *b* and cytochrome *c* – oxidase genes (interspecific differences amount to 11.4% for COXI, 6.2% for COXII, and 24.9% for COXIII) (Ludannyi, 2008; Hayden et al., 2011). As predicted, species with accelerated mitochondrial evolution tend to be the worse maternal parent not only for F1 hybrids (Bolnick et al., 2008), but also for backrossed hybrids. It has been shown that the breakdown of concerted interaction between alien genomes occurs due to unequal rates of mitochondrial evolution in diverging species and a high level of interspecific differences by genes of the mitochondrial respiratory chain that leads to suppression of cell respiration, changes in energy metabolism and a reduction in the viability of backcrossed hybrids (Burke & Arnold, 2001; Ellison & Burton, 2006; Bolnick et al., 2008).

A hypothesis explaining the breakdown of mitochondrial-nuclear interactions has been presented by Hill (2015). The mitochondrial genome is subjected to much higher mutation rates than the nuclear genome. Complementary changes in nuclear genes, functionally related to mtDNA genes (N-mt genes), can compensate for deleterious changes in mitochondrial genes (mt genes), thus increasing fitness of individuals. Following this hypothesis, we assume that the low mutation rate in mtDNA and the absence of necessary compensatory changes in N-mt genes of *A. brama*, during the interaction with highly polymorphic mtDNA of *R. rutilus*, leads to functional incompatibility of the products of N-mt and mt genes and thus to a decrease in the viability of alloplasmic $_{R}$AA hybrids. The hybrid morphotype of this progeny $_{R}$AA (RA × A1) (Fig. 3) indicates that nuclear-cytoplasmic incompatibility affects nuclear-nuclear (epistatic) interactions, since morphological characters are polygenic in nature. Perhaps this is due to a change in energy metabolism or induction of translocations in incongruent crosses that can lead to reorganization of the ontogenetic regulation system through a position effect, heterochrony or new gene constructs (Zhuchenko & Korol, 1985; Golubovsky, 2000). However, we cannot rule out the possibility that violation of the restoration of the morphology of bream in progeny RA × A1 is associated with other endogenous factors. The high compatibility of alien genomes in $_{A}$RR combination may be due to the conservative nature of the mtDNA of *A. brama* (Hayden et al., 2011), or to the capacity of the N-mt genes of *R. rutilus* to keep pace with exchanges in mt genes of *A. brama* through rapid recombination of nuclear genes to find compensatory combinations for mitonuclear complexes (as shown in Havird et al., 2015). Apparently, the asymmetry of both the viability and developmental stability of the $_{R}$AA and

$_A$RR alloplasmic hybrids is associated with unequal rates of mitochondrial evolution of *R. rutilus* and *A. brama* resulting to nuclear-cytoplasmic incompatibility and reproductive isolation in the direction of introgression of mtDNA of *R. rutilus*. The best maternal parent is *A. brama* with a lower rate of changes in mtDNA that determines the direction of hybridization.

Conclusion

In general, the results clearly demonstrate the differences in viability and morphology between progeny of incongruent and congruent crosses and between alloplasmic backcrossed hybrids $_R$AA and $_A$RR. The inclusion of alien cytoplasm in the hybrids of the incongruent cross violates the relationship between traits, results in the formation of transgressive phenotypes and affects the viability of individuals. Differences between alloplasmic hybrids with the cytoplasm of *R. rutilus* and *A. brama* indicate varying degrees of nuclear-cytoplasmic compatibility of the genomes of roach and bream in reciprocal directions that are associated with unequal rates of mitochondrial evolution and a high level of divergence in mitochondrial genes (cyt *b* and cytochrome *c* – oxidase subunits I, III). The most complete (wild-type-like) interaction between mitochondrial and nuclear genomes from different species is established in the direction of introgression of less polymorphic mtDNA of *A. brama* (AR × R2). They are viable and restore morphology of roach. Upon introgression of more polymorphic mtDNA of *R. rutilus*, less-fit hybrids are formed ($_R$AA class alloplasmic hybrids), which are likely eliminated by natural selection due to their low viability. Combining genetic and morphological data allows us to conclude that the unidirectional (asymmetric) character of the hybridization between *R. rutilus* and *A. brama* is supported not only by prezygotic isolation but also a postzygotic reproductive barrier in the direction of introgression of mtDNA of *R. rutilus* (endogenous selection against $_R$AA genotype).

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