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GENETIC DIVERSITY OF WILD AND FARMED KALIBAUS (*Labeo calbasu*, Hamilton, 1822) BY RAPD ANALYSIS OF THE GENOMIC DNA

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Summary

Genetic diversity of two wild Kalibaus, *Labeo calbasu* populations and one hatchery stock was studied using random amplified polymorphic DNA (RAPD) method. The three 10–mer random primers (OPA01, OPB02 and OPC03) yielded a total of 26 reproducible and consistently scorable RAPD bands of which 15 (57.69%) were considered as polymorphic (P95) indicating a high level of genetic variation in all the studied populations. Among the three populations, Padma population shows low level of genetic diversity (0.1238) compared to other two and it might be caused by habitat degradation in many ways which ultimately affects the genetic variation of Kalibaus. The UPGMA dendrogram based on Nei's (1972) original measures of genetic distance (D) indicated the segregation of two wild and hatchery populations of *L. calbasu* into two distinct clusters: the Hatchery and Padma populations produced one cluster whereas the Jamuna population belonged to another cluster. This indicates that hatchery brood stock is derived from Padma River. Nevertheless, the preliminary study revealed that RAPD technique could be an effective tool in the assessment of population genetic structure of Kalibaus.

Key words: Labeo calbasu, genetic diversity, RAPD markers, genomic DNA

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INTRODUCTION

Kalibaus (*Labeo calbasu*, Hamilton, 1822) is an important food fish and is a teleost (family: Cyprinidae, order: Cypriniformis) having its karyotype with 25 pairs of diploid (2n) chromosomes (R e d d y, 1990). *L. calbasu* enjoys wider distribution in many countries namely, Pakistan, India, Myanmar, Thailand, Yamuna (South China) (R e d d y, 1990) and Bangladesh (the Padma–Brahmaputra, i.e. Padma, Jamuna, Arial Khan, Kumar and Old Brahmaputra River as well as the Halda river systems in hilly Chittagong) (A l a m and I s l a m, 2005). Aquaculture now accounts for nearly 41% of the total fish production in Bangladesh (D o F, 2006–2007). Polyculture of Indian major carps including *L. calbasu* contributed approximately 945,812 mt which was about 34.36% of total fish production in Bangladesh (D o F, 2006–2007). But in the recent years, the natural breeding of *L. calbasu* has become uncertain due to continuous habitat degradation caused by environmental modification and human interventions (overfishing, dam construction, pollution etc.) affecting feeding migration and spawning which decreasing its population size in all Bangladeshi rivers (D a s and B a r a t, 1990). River contribution as a natural source of major carp species fry for aquaculture has been reduced to almost nil (1%) in 2003 as against 80% in the early 1980s (D o F, 2003). On the contrary, the demand for fish fry has increased several folds through aquaculture expansion; so, fry production in public hatcheries has intensified. But little attention has been given to genetic quality maintenance. Hybridization and inbreeding are very common practices in Bangladeshi hatcheries. As a result, the hatchery–produced seed are not performing in culture as in earlier years. Besides this, the mixing of hatchery–reared *L. calbasu* through escapes and the government's massive seed stocking program in open water bodies may have resulted in gene introgression into the pure wild stocks and may result in less well adapted fish in comparison with native stock. So it is essential to understand the genetic composition of *L. calbasu* for management of their natural populations in nature. Genetic marker identification is required for baseline studies for monitoring potential changes in genetic makeup and adaptive values as a result of interaction between wild and culture populations. Random amplified polymorphic DNA (RAPD) analysis is based on amplification of discrete regions of the genome by polymerase chain reaction (PCR) with short oligonucleotide primers of arbitrary sequence (W e l s h and M c C l e l l a n d, 1990; W i l l i a m s et al., 1990) to detect polymorphisms in the respective primer sites of the genome. Such polymorphisms inherit in a Mendelian fashion and can be used as genetic markers in discriminating different populations (H a d r y s et al., 1992).

In the present study, the RAPD method was applied to two river and one hatchery population of *L. calbasu* to assess intraspecific genetic variation and relatedness among the populations.

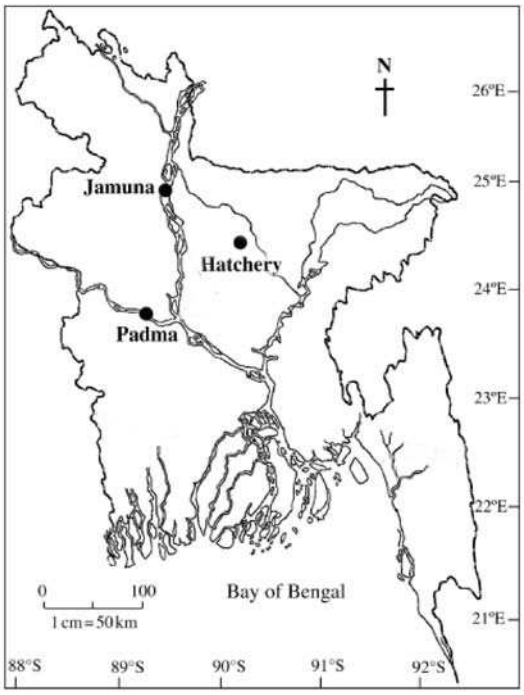

Fig. 1. Map of Bangladesh showing the different sampling zones (•) of Labeo calbasu
Slika 1. Karta Bangladeša sa područjima uzorkovanja (•) Labeo calbasu

MATERIALS AND METHODS

Fish sample sources

A total of 75 live fish (*L. calbasu*), twenty–five from each population, were collected using net from three different sources: two from wild or natural sources such as the Padma River (Hardinge Bridge, Ishwardi) and the Jamuna River (Jamuna Bridge, Sirajgonj), during the month of July, and one from hatchery source that is Vhai Vhai Hatchery, Trishal, Mymensingh (Fig. 1).

Genomic DNA isolation

Genomic DNA was isolated from approximately 30 mg of caudal fin tissue using proteinase–K digestion, phenol–chloroform–isoamyl alcohol purification and ethanol precipitation method (A l a m et al., 1996). In brief, approxi–mately 30 mg of fin tissue was cut into small pieces, homogenized and digested with proteinase K in extraction buffer [100 mM Tris–HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 250 mM NaCl and 1% sodium dodecyl sulphate (SDS)] overnight at 37ºC. DNA was purified by successive extraction with phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v/v) and chloroform : isoamyl alcohol (24 : 1, v/v), respectively. DNA was precipitated first using 0.6

volume of isopropanol, pelleted by centrifugation, then re–suspended in TE buffer (10 mM M Tris–HCl, 1 mM M EDTA, pH8.0). DNA was re–precipitated by adding two volumes of absolute ethanol in the presence of 0.3 M sodium acetate, and pelleted by centrifugation. The pellets were then washed with 70% ethanol, air–dried, re–suspended in an appropriate volume of TE buffer and lastly stored in freezer at –18ºC. DNA quality was checked by 1% agarose gel electrophoresis and quantified using a spectrophotometer (SPECTRONIC® GENESYS™5, Spectronic Instruments Inc., USA).

Primer selection

Eighteen decamer primers of random sequences were screened in this study which were purchased from Pharmica LKB — Gene Assembler Special by Oligo Synthesis Department, Bangalore Genei Pvt. Ltd., India. Firstly, primers were divided into three groups and each group was screened on the sub sample of one fish from the each population. Finally, three primers out of eighteen that exhibit the highest quality banding patterns and sufficient variability for population analysis were then retained for further analysis (Fig. 2).

PCR amplification

The amplification conditions were based on Williams et al. (1990), with some modifications. PCR reactions were performed on each DNA sample in a l0 μl reaction mix containing 1 μl of l0x Taq DNA Polymerase buffer, 0.25 μl of 0.4 μM primer, 1 μl of l0mM dNTPs (Genei, India), 0.2 μl of 3u/μl of Taq DNA polymerase (Genei, India), 4μl of fish genomic DNA (25ng/μl) and a suitable amount of sterile deionized water. DNA amplification was performed in an oil–free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94ºC for 3 min followed by 40 cycles of 1 min denaturation at 94ºC, 1 min annealing at 36ºC and elongation or extension at 72ºC for 2 min. After the last cycle, a final step of 7 min at 72ºC was maintained to allow complete extension of all amplified fragments followed by holding at 4ºC.

Electrophoresis of amplified products

An aliquot of l0 μl of amplified product from each sample was separated electrophoretically on 1.4% agarose gel containing ethidium bromide in 1x TBE buffer. Two DNA molecular weight markers (l00 bp DNA Ladder, Genei, India and Low Range DNA Ruler, Genei, India) were electrophoresed alongside the RAPD reactions. DNA bands were observed on UV–transilluminator and photographed with a Gel Cam Polaroid camera.

RAPD data analysis

All distinct bands or fragments (RAPD markers) were given identification numbers according to size and scored visually on the basis of their presence

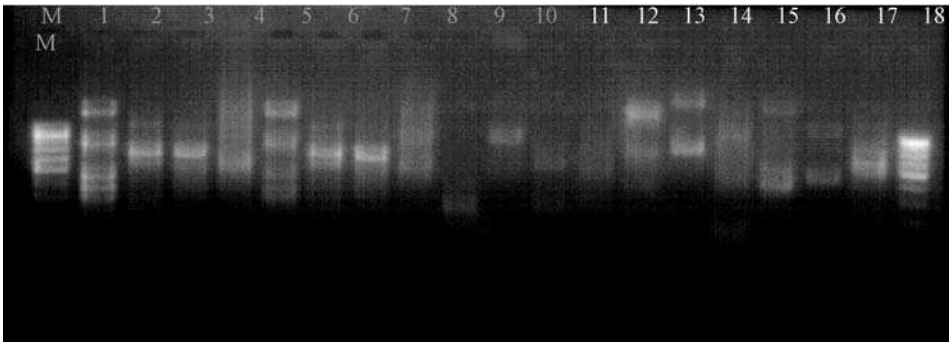

Fig. 2. Primer screening of L. calbasu *of Hatchery population. Lane 1–3: Kit A; lane 4–10: Kit B; lane 11–18: Kit C: M: Molecular weight marker (100 bp DNA ladder)*
Slika 2. Skrining primera za mrjestilišnu populaciju L. calbasu*: Trake 1–3: Kit A; trake 4–10: Kit B; trake 11–18: Kit C; M: marker molekularne težine (100 bp DNA ljestvica)*

(1) or absence (0), separately for each fish for each primer. For more accuracy, band scoring was performed by two independent persons. Bands or RAPD markers not identified by all two persons or readers were considered as non–scorable. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used to estimate polimorphic loci, Nei's (1973) gene diversity, gene flow (N_m), genetic distance (D) and to constructing an unweighted pair group Method of arithmetic mean (UPGMA) dendrogram among populations with 1000 simulated samples using the POPGENE (Version 1.31) (Yeh et al., 1999) computer program. The sizes of the RAPD markers were estimated by using the software DNAfrag (Version 3.03) (Nash, 1991).

RESULTS

Among the 18 primers initially tested, three primers (OPA01, OPB02 and OPC03) yielded comparatively maximum number of amplification products with high intensity and minimal smearing (Table 1). Each primer produced an unique fragment pattern of amplified DNA (Fig.3). All three primers (OPA01, OPB02 and OPC03) yielded a total of 26 reproducible and consistently scorable RAPD bands of which 15 (57.69%) were considered as polymorphic (P_{95}).

Polymorphic loci

The proportion of polymorphic loci was relatively high in the population of Jamuna river (42.31%) whereas those for other two populations were found to be low (Table 2). The within–population gene diversity was found to be the

highest in the population from Jamuna river followed by Padma river population whereas the lowest gene diversity was observed in Hatchery population (Table 2). Similarly, the highest and the lowest values of Shannon's Information index were observed in the Jamuna population and Hatchery populations, respectively (Table 2).

Genetic identity and genetic distances

The values for intra–population genetic identity were 0.8733 for Padma and Hatchery populations, 0.8668 for Padma and Jamuna populations and 0.8165 for Hatchery and Jamuna populations (Table 3). The genetic distances were 0.1355, 0.1429 and 0.2028 between Padma and Hatchery populations, Padma and Jamuna populations and Hatchery and Jamuna populations respectively.

UPGMA dendrogram

UPGMA dendrogram based on Nei's (1972) original measures of genetic distance (D) was constructed (Fig. 4). This measurement indicated the segregation of two wild and hatchery populations of *L. calbasu* into two distinct clusters: the Hatchery and Padma populations produced one cluster whereas the Jamuna populations belonged to another cluster.

DISCUSSION

Among the 18 decamer random primers, three produced considerable reproducible and polymorphic amplification with DNA of *L. calbasu* populations. In this study, no specific markers were found to discriminate kalibaus populations but the results analyzed from the data, scored from existing polymorphic bands (15), revealed some degree of divergence among the populations. The substantial difference in the number of polymorphic bands suggests that the level of genetic variation among the three kalibaus populations may be sufficient for developing an intra–specific populations.

Though three random primers were finally used, 57.69% of polymorphic loci detected during this study indicating the effectiveness of RAPD technique to study polymorphism. The percentage polymorphic loci in the studied three different populations of *L. calbasu*, which highly deviated from the result drawn by Das et al. (2005), where they obtained 75% of polymorphic loci after screening four RAPD primers in three wild populations of *L. calbasu*. In contrast, Barman et al. (2003) detected on an average 45% polymorphic loci in four Indian major carps like *Labeo rohita*, *C. catla*, *L. calbasu* and *Cirrhinus mrigala*. In another studies, Islam and Alam (2005) investigated four different populations of Indian major carp, *L. rohita* and found 46.5% of polymorphic loci by five RAPD primers, and Bielawski and Pumo (1996) detected 32% RAPD markers in Atlantic Coast striped bass. Mamuris et al.

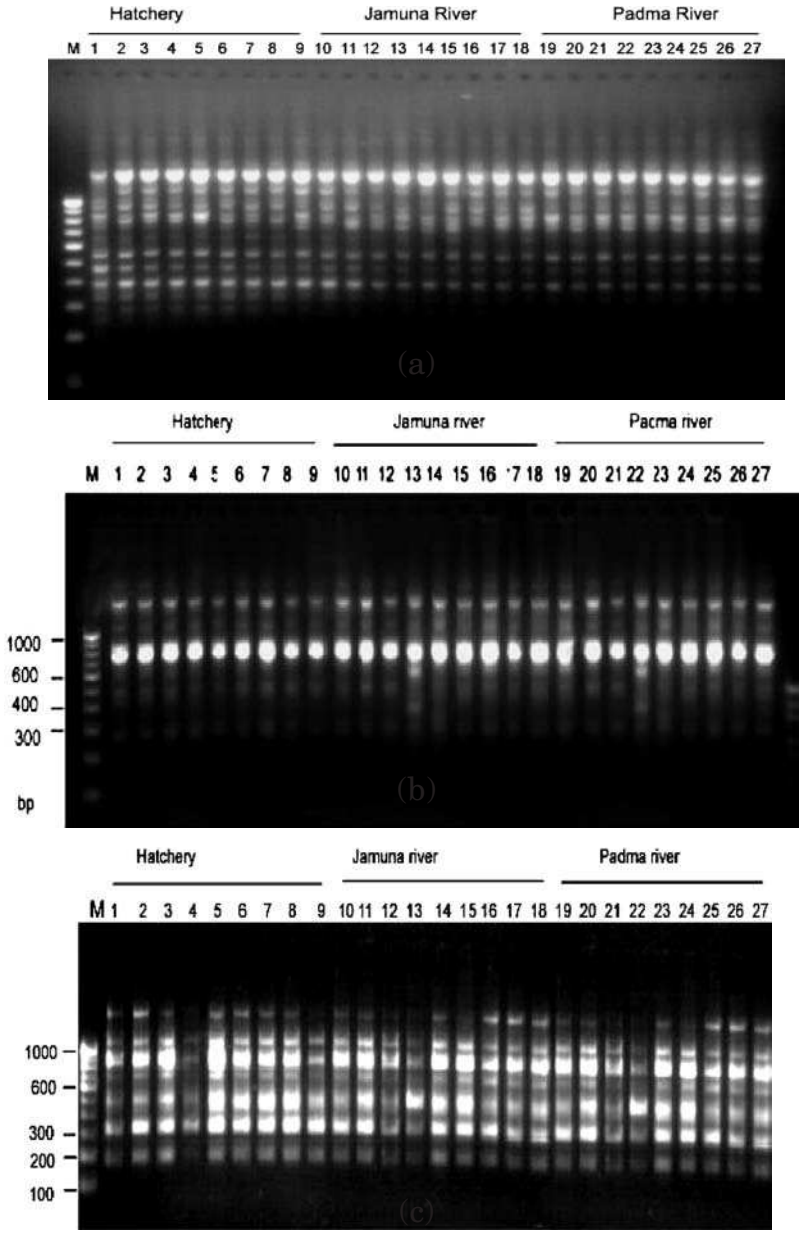

*Fig. 3. RAPD Profile of Kalibaus (*Labeo calbasu*, Hamilton, 1822) fish at three different habitat using primer OPA01 (a), OPB02 (b), OPC03 (c); Lane 1–9: Hatchery; Lane 10–18: Jamuna river; Lane 19–27: Padma river.*
*Slika 3. RAPD profil kalibausa (*Labeo calbasu*, Hamilton, 1822) s triju različitih staništa, koristeći primer OPA01 (a), OPB02 (b), OPC03 (c); trake 1–9: mrjestilište; trake 10–18: rijeka Jamuna; trake 19–27: rijeka Padma.*

Table 1. RAPD primers with corresponding bands scored
Tablica 1. RAPD primeri s odgovarajućim brojevima traka

Primer codes	Sequences (5'–3')	Total number of bands scored
OPA01	5'–CAGGCCCTTC–3'	12
OPB02	5'–TGATCCCTGG–3'	7
OPC03	5'–GGGGGTCTTT–3'	7
	Total ukupno =	26

Table 2. Number and proportion of polymorphic loci, gene diversity and Shannon's information index for the studied Labeo calbasu *population*
Tablica 2. Broj i udio polimorfičnih lokusa, genetska raznolikost i Shannonov indeks za proučavane populacije Labeo calbasu

Population	Number of polymorphic loci	Proportion of polymorphic loci	Gene diversity	Shannon's Information index
Hatchery	8	30.77%	0.1224	0.1779
Jamuna river	11	42.31%	0.1726	0.2506
Padma river	7	26.92%	0.1238	0.1756

(1999) also observed 47.4% polymorphic bands from eight random primers in striped mullet from Mediterranean Sea.

This may be attributed to the maintenance of a limited number of individuals sampled from the wild and their repeated propagation over a long period. Inbreeding may be another reason for reduced genetic variation in the hatchery population, as was also reported by Ekanth and Doyle (1990) on the basis of effective population size. All the river populations showed almost similar level of intra–population similarity indices in the present study implies that individuals within each population are genetically close to each other. The similar result was also established in different Indian major carps and other fin fishes like *L. calbasu* (93%) (Das et al., 2005); *L. rohita* (94.88%, 97.5%) (Islam and Alam, 2005; Barman et al., 2003); etc. This implies that individuals within each population are genetically more similar to each other, as was expected, than to individuals from all other populations (Macdonald, 1995). The pair–wise inter–population similarity indices between the Jamuna and hatchery was lower (88.73%) compared to the Padma and hatchery (90.70%). This is the cause that, as expected, broods are used in the Vai Vai hatchery might be collected from the Padma River.

The percentage of polymorphic loci was found to be higher in the Jamuna population is indicative of relatively high level of genetic variation, as well as

Table 3. Genetic identity and genetic distance between three populations of Labeo calbasu *(Nei's Original Measure of Genetic Identity and Genetic Distance, 1972)*
Tablica 3. Genetski identitet i genetska udaljenost među trima populacijama Labeo calbasu *(Nei's Original Measure of Genetic Identity and Genetic Distance, 1972)*

Population ID	Hatchery	Jamuna river	Padma river
Hatchery	***	0.2028	0.1355
Jamuna river	0.8165	***	0.1429
Padma river	0.8733	0.8668	***

Note: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).
Opaska: Nei's genetic identity (gore dijagonalno) i genetic distance (dolje dijagonalno).

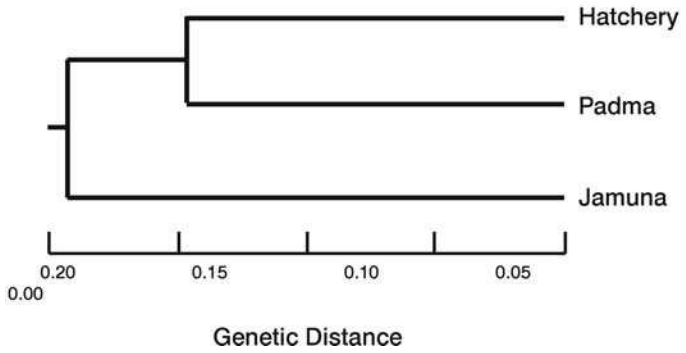

Fig. 4. UPGMA dendrogram based on Nei's (1972) original measures of genetic distance, summarizing the data on differentiation between L. calbasu *populations according to RAPD analysis*
Slika 4. UPGMA dendrogram baziran na Nei (1972) metodi mjerenja genetičke udaljenosti, sumirajući podatke o raznolikosti među populacijama L. calbasu *prema RAPD analizi*

lower level of percentage of polymorphic loci was in hatchery population. Like percentage of polymorphic loci, other measurements such as gene diversity and Shannon's information index were also higher in the Jamuna River population than other individuals of studied populations but comparatively lower in the hatchery population. The higher value of the percentage of polymorphic loci and gene diversity in the individuals of the Jamuna River are usually expected, because it is well known to all that the Indian major carps are the natural inhabitants of the Jamuna River. Therefore, in Bangladesh, genetically more diversified *L. calbasu* individuals can only be found in the Jamuna River.

Significant departure from homogenity was found at 15 loci out of 26 scorable bands among the studied populations of *L. calbasu*. Islam and Alam (2005) detected four loci (of 43) in rohu, *L. rohita*, which were significant departure from homogeneity, as the overall gene flow among the populations of the present study was lower (4.589) than their findings (9.340).

Nei's original measures of genetic distance (D) were also used to evaluate the genetic variability and relatedness among the *L. calbasu* populations. The highest pair–wise genetic distance was between the Jamuna and hatchery populations and lowest in the Padma–hatchery populations. On the geographical point of view, though the hatchery located near to Jamuna River and far distant from Padma River, it shows close similarity with the population of Padma River. It may be assumed that the samples of hatchery might possible be collected from the Padma River population or the brood fish in that hatchery might be from the Padma river.

RAPD markers have been proved as effective tools to monitor the genetic variation in different populations of organisms. Using only three primers and nine samples from each population, the present study revealed a remarkable level of intra– and inter–population genetic variation of *L. calbasu*. Although no specific markers were detected to discriminate studied *L. calbasu* populations, the fact that 57.69% of the bands were found to be polymorphic (much closer to that obtained by Islam and Alam (2005) in another important Indian major carp, *L. rohita*) indicating that the RAPD marker system may be more useful to generate molecular markers for genetic characterization in this species.

Sažetak

GENETSKA RAZNOLIKOST DIVLJIH I UZGAJANIH RIBA (*Labeo calbasu*, Hamilton, 1822) S RAPD ANALIZOM GENOMSKE DNA

M. G. Mostafa[1], A. S. Ishtiaq Ahmed[2*], M. G. Mustafa[1], M. G. Rabbane [1], M. N. Islam[3], S. M. Rafiquzzaman[4]

Genetska raznolikost dvaju divljih kalibausa, *Labeo calbasu*, i jedne mrjestilišne populacije proučavana je pomoću *random amplified polymorphic* DNA

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(RAPD) metode. Tri 10–mer nasumična primera (OPA01, OPB02 and OPC03) postigla su ukupno 26 ponovljivih i dosljedno prebrojivih RAPD traka, od kojih je 15 (57,69%) bilo polimorfično (P_{95}), upućujući na visoku razinu genetske varijacije u svim proučavanim populacijama. Od triju populacija, Padma populacija je pokazala relativno nižu razinu genetske raznolikosti (0,1238), što bi moglo biti uzrokovano degradacijom staništa. UPGMA dendrogram baziran na N e i e v o j (1972.) metodi mjerenja genetičke udaljenosti (D) uputio je na segregaciju dviju divljih i jedne mrjestilišne populacije na dva jasna klastera: *Mrjestilište* i *Padma* činile su jedan, a *Jamuna* drugi klaster, što upućuje na podrijetlo mrjestilišnog matičnog jata iz rijeke Padma. Istraživanje je pokazalo i da RAPD može biti učinkovito oruđe u utvrđivanju genetske strukture populacija kalibausa.

Ključne riječi: *Labeo calbasu*, genetska raznolikost, RAPD markeri, genomska DNA

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