动物学报 53(4):773 – 776, 2007
*Acta Zoologica Sinica*

# Molecular detection of a *Labeo calbasu* × *Labeo rohita* hybrid, from the river Ganges, India

Kuldeep K.LAL[1*], Rajeev K.SINGH[1], Vindhya MOHINDRA[1], Anup MANDAL[1], Peyush PUNIA[1], B.N.MISHRA[2], W.S.LAKRA[1]

1. National Bureau of Fish Genetic Resources (ICAR), Canal Ring Road, P.O. Dilkusha, Lucknow-226002, UP, India
2. Department of Biotechnology, Institute of Engineering & Technology, U.P. Technical University, Lucknow, UP, India

# 印度恒河蓝黑鲩与南亚黑鲩杂交个体的分子检测

Kuldeep K.LAL[1*] Rajeev K.SINGH[1] Vindhya MOHINDRA[1] Anup MANDAL[1] Peyush PUNIA[1] B.N.MISHRA[2] W.S.LAKRA[1]

1. National Bureau of Fish Genetic Resources (ICAR), Canal Ring Road, P.O. Dilkusha, Lucknow-226002, UP, India
2. Department of Biotechnology, Institute of Engineering & Technology, U.P. Technical University, Lucknow, UP, India

摘 要 利用鉴别性的同工酶位点从印度恒河水系的支流 Banganga 中检测到了蓝黑鲩(*Labeo calbasu*)与南亚黑鲩(*Labeo rohita*)的一个杂交个体。线粒体 DNA 的单倍型模式进一步确认这个杂交个体的母本是河蓝黑鲩[动物学报 53(4): 773 – 776, 2007]。

关键词 蓝黑鲩 南亚黑鲩 杂交 同工酶 mtDNA

**Key words** *Labeo calbasu*, *Labeo rohita*, Hybrid, Allozyme, mtDNA

Natural hybridization in freshwater fishes at intergeneric and interspecific levels is more common than in other groups of vertebrates (Hubbs, 1955). Among fishes, Cyprinids are more prone to such hybridization. Detection of natural hybrids in fishes is often complicated and spot identification during vigorous sampling is quite difficult, as they may closely resemble any one of their parents. Species-specific molecular markers provide precise evidence in detecting hybrids between different species. A single diagnostic locus is adequate to detect an $F_1$ hybrid, however, 5 – 6 loci can provide information on back crossed/post $F_1$ hybrids (Campton and Utter, 1985). Mitochondrial DNA is predominantly inherited maternally and reveals the immediate maternal parent. Diagnostic allozyme loci coupled with mtDNA profiles can provide effective molecular tools for the detection of hybrids (Avis and Sunders, 1984; Agnese et al., 1997, Strussmann et al., 1997).

The present study reports characterization of a hybrid *Labeo* specimen, using allozyme and mitochondrial markers. The study arose from routine genotyping of *L. rohita* where one sample exhibited the heterozygote pattern at several loci with alleles, that have not been observed in *L. rohita*. To explain the observed genotype profile, the study was conducted to determine if the particular specimen could be a hybrid and if so, what could be the parent species?

## 1 Materials and methods

The fish specimens were obtained from commercial catches from the Banganga River (latitude 29°46′ N, longitude 78°02′ E). The fish specimen in question (referred as test specimen, Acc.No.LR1373) was part of the collection of 44 specimens, obtained for population structure studies of *L. rohita* from the river Banganga, a tributary of Ganges. The accessions (no. given) available at the Tissue Repository of National Bureau Fish Genetic Resources, Lucknow were used in this study. The test specimen was not identified as hybrid during field sampling. In the initial analysis, the test specimen was examined for allozyme profile, generated on the same gel for 8 samples, 2 each for the four Indian major carps, viz., *Catla catla* (Hamilton, 1822; Acc. No. CC504, CC529), *Labeo rohita* (Hamilton, 1822; Acc. No.

Received Dec. 29, 2006; accepted May 02, 2007
* Corresponding author. E-mail: kulvin100@yahoo.co.in

LR1152, LR1461), *L. calbasu* (Hamilton, 1822; Acc. No. LK157, LK201) and *Cirrhinus mrigala* (Hamilton, 1822; Acc. No. CM526, CM721). Thereafter, the test sample was re-examined with putative parent species, *L. rohita* and *L. calbasu*. Ten samples each of *L. rohita* (Acc.No.LR1152, 1154, 1155, 1362, 1364, 1461, 1528, 1702, 1704 and 1705) and *L. calbasu* (Acc. No. LK157, 201, 203, 253, 262, 277, 279, 280, 500, 507), collected from three distant rivers (Satluj, 31°09′ N, 74°56′ E; Banganga, 29°46′ N, 78°02′ E; and Brahmaputra, 26°16′ N, 91°46′ E) were again examined electrophoretically with the test sample.

The liver samples were dissected at the sampling site and immediately immersed in liquid nitrogen (−196℃) and stored at −80℃ (until analysis). Liver tissue (approx. 100 mg) from each sample was mildly crushed in cooled 1.5 ml micro centrifuge tubes with four volumes of extraction buffer (0.17 mol/L sucrose, 0.2 mol/L EDTA, 0.2 mol/L Tris-HCl, pH 7.0) for allozyme analysis. Homogenized samples were centrifuged for one hour at 10 000 r/min at 4℃ and 200 μl of supernatant was recentrifuged for 20 minutes. Allozyme variation was determined by vertical polyacrylamide gel (7%) electrophoresis (Gopalakrishnan et al., 1997). Electrophoresis was carried out at constant voltage (150 V) in cooling chamber (6℃) in Tris-Borate Buffer (500 mmol/L Tris, 650 mmol/L Boric acid, 16 mmol/L EDTA pH 8.0). Allozyme profiles were visualized using histochemical staining methods (Whitmore, 1990) at 37℃. For nomenclature of loci and alleles, recommendations of Shaklee et al.(1990) were followed. The most frequent allele in *Labeo rohita* was assigned as 100. Alternate alleles were designated as per their mobility relative to the most common allele. Multifactorial analysis (She et al., 1987) was performed using AFC2D option in Genetix 4.02 (Belkhir et al., 1997). The individual genotype data of each individual is represented by the score derived from each variable (alleles at different loci), for the absence of an allele (0), for the presence of the allele to the state heterozygote (1) and the state homozygote (2).

For mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis, total DNA was extracted from frozen liver samples (stored at −80℃) using the method of Stevens et al.(1993). The ND1 and 16 S mitochondrial rRNA genes of mtDNA were amplified using the primers (Forward 5′ ACCCCGCCTGTTTACCAAAAACAT 3′ Reverse 5′ CGTATGAGCCCGATAGCTTA 3′) described by Cronin et al.(1993). Total genomic DNA (approximately 25 − 100 nanogram) was used as template for amplification. PCR reaction was set up in a volume of 50 μl and consisted of 0.05 μmol/L − 0.2 μmol/L primer, 0.2 mmol/L of dNTPs mix, 1× assay buffer (10× = 100 μmol/L TAPS, pH 8.8; 500 μmol/L potassium chloride, 0.1% gelatine) and 1 − 3 U of *Taq* DNA polymerase. *Taq* polymerase was added after the initial denaturation step during PCR. Amplification cycle conditions were (1) 95℃ for 5 min for initial denaturation (1 cycle); (2) 94℃ for 30 s, 50℃ for 1 min 72℃ for 1 min 30 s (30 cycles); (3) 72℃ for 10 min; (4) soak at 4℃ in a Perkin Elmer CETUS thermal cycler 480. The PCR amplified products were digested with three restriction enzymes, viz. *Mbo* Ⅰ, *Mva* Ⅰ and *Hin*f Ⅰ according to the conditions specified by the manufacturer (MBI Fermentas Inc., U.S.A.). Purified restricted DNA was loaded along with undigested PCR sample and three markers (λDNA *Eco*R Ⅰ / *Hin*d Ⅲ double digest, øX174 *Hae* Ⅲ digest and 100 bp ladder) in 6% polyacrylamide (29∶1) gel at 150 V for 3 h for electrophoresis. After electrophoresis, the restriction patterns were visualized by silver staining (Silver plus one, Amersham Pharmacia, U.S.A.). Each individual was scored for sizes of its restriction fragments and a haplotype was assigned to it for all the restriction enzymes tested.

## 2 Results

Comparative studies of allozyme patterns of the four Indian major carp species viz. *L. rohita*, *L. calbasu*, *Catla catla* and *C. mrigala* revealed that the test sample shared alleles with *L. rohita* and *L. calbasu*. To discriminate the two species, only loci yielding scorable activity in both species were considered. This provided 17 loci expressed for 10 enzyme systems for further analysis. Species-specific diagnostic differences between *L. rohita* and *L. calbasu* were observed at four loci, viz., *GPI*-3*, *AAT**, *SOD*-3* and *EST*-4* (Fig.1). Different loci and alleles observed in the two *Labeo* species are given in Table 1. The loci with only allele *100 in both the species are excluded. The test sample exhibited heterozygote genotype for these loci and banding patterns conformed to that expected as per structure of the enzymes. This confirmed that the test sample was a $F_1$ hybrid between *L. rohita* and *L. calbasu* (Fig.1). The multifactorial analysis depicted distinct groups for *L. rohita*, *L. calbasu* and the test specimen could be identified, since it occupies the intermediate position.

To determine parentage, the sample was further analysed with mtDNA RFLP. Six haplotypes were identified in the two *Labeo* species. Three haplotypes were characterized in *L. rohita* that differed from the three observed for *L. calbasu* as presented in Table 2. The test specimen had haplotypes similar to *L. calbasu* for the three markers used. This indicated that the maternal parent of the test specimen was *L. calbasu*.

## 3 Discussion

Sufficient evidence was available to conclude that the test specimen was a $F_1$ hybrid produced from two sympatric species, a male *L. rohita* and a female

**Table 1　Loci scored and alleles observed for different enzymes in *Labeo rohita* and *L. calbasu***

| Enzyme | Enzyme Commission number | Locus | Alleles | | |
|---|---|---|---|---|---|
| | | | *Labeo rohita* | *Labeo calbasu* | Test specimen |
| Aspartate amino transferase | 2.6.1.1 | *AAT** | 100(0.095), 110(0.05) | 071(0.05), 087(0.95) | 87, 100 |
| Esterase | 3.1.1. 1 | *EST*-1* | 083(0.05), 100(0.95) | 100(0.90), 116(0.05), 126(0.05) | 100 |
| | | *EST*-4* | 100(1.00) | 086(0.72), 094(0.28) | 86, 100 |
| Glucose-6-phosphate dehydrogenase | 1.1.1.49 | *G6PD** | 100(0.95), 115(0.05) | 100(0.95), 115(0.05) | 100, 115 |
| Glucose phosphate isomerase | 5.3.1.9 | *GPI*-3* | 100(0.80), 115(0.20) | 084(1.00) | 84, 100 |
| α-glycerophosphate dehydrognase | 1.1.1.8 | *GPDH** | 086(0.05), 100(0.95) | 086(0.20), 100(0.80) | 100 |
| Phosphogluconate dehydrogenase | 1.1.1.44 | *PGDH** | 100(1.00) | 092(1.00) | 92, 100 |
| Octanol dehydrogease | 1.1.1.73 | *ODH*-1* | 100(1.00) | 130(0.11), 165(0.89) | 100, 165 |
| Phosphogluco mutase | 5.4.2.2 | *PGM*-1* | 100(1.00) | 131(1.00) | 131 |
| | | *PGM*-2* | 100(1.00) | 128(1.00) | 100, 128 |
| Superoxide dismutase | 1.15.1.1 | *SOD*-3* | 100(1.00) | 171(1.00) | 100, 171 |

Allele frequencies for *L. rohita* and *L. calbasu* are given in parenthesis. For test specimen, the frequencies of the two alleles were same.

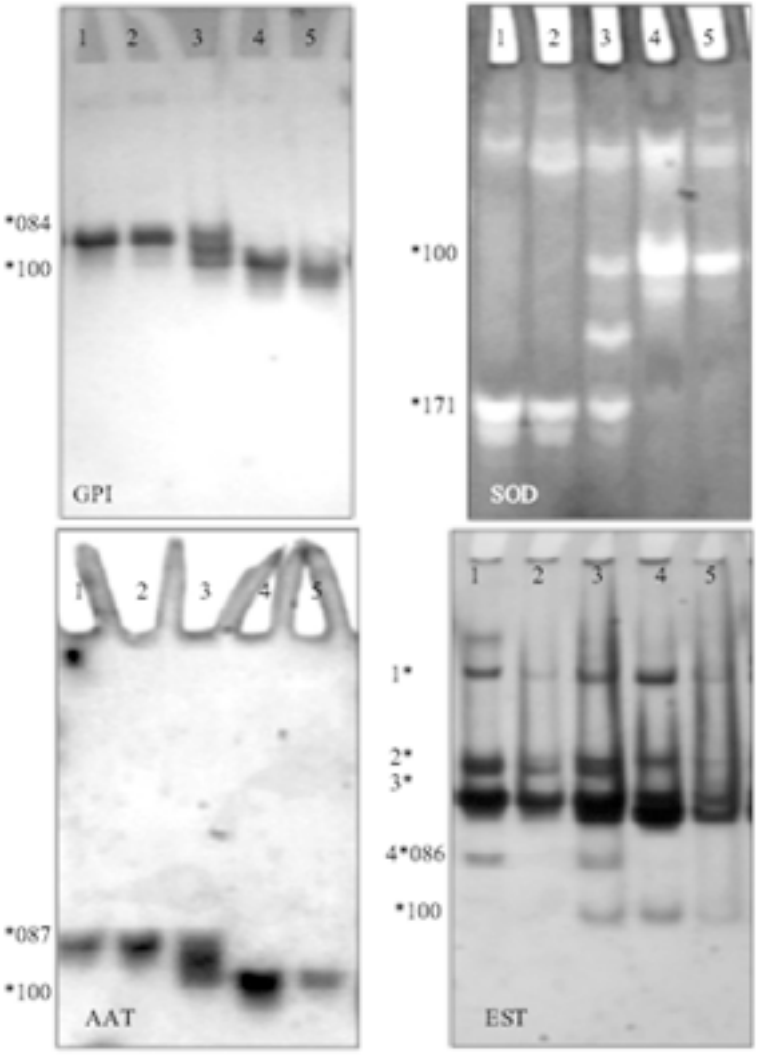


**Fig.1　Allozyme patterns of four diagnostic loci: *AAT**, *GPI*-3*, *SOD*-3 and *EST*-4*, for the *Labeo calbasu* (1, 2) and *L. rohita* (4, 5)**

Note the heterozgote pattern for individual 3, confirming it as $F_1$ hybrid between the two *Labeo* species

**Table 2 mtDNA restriction pattern and haplotype of *Labeo rohita*, Labeo calbasu and their putative hybrid (test specimen)**

| Restriction enzyme | Restriction pattern (bp) | | |
|---|---|---|---|
| | *Labeo rohita* | *Labeo calbasu* | Putative hybrid |
| *Mbo* Ⅰ | 746, 303, 226, 220, 215, 165, 156 | 684, 560, 229, 218, 166, 156 | 684, 560, 229, 218, 166, 156 |
| Haplotype | A | B | B |
| *Hin*f Ⅰ | 746, 310, 290, 238, 220, 120, 113, 102 | 728, 639, 305, 229 | 728, 639, 305, 229 |
| Haplotype | A | B | B |
| *Mva* Ⅰ | 829, 689, 596 | 1469, 734 | 1469, 734 |
| Haplotype | A | B | B |
| Composite haplotype (Sequence of enzyme: *Mbo* Ⅰ, *Hin*f Ⅰ, *Mva* Ⅰ) | AAA | BBB | BBB |

*L. calbasu*. The identified diagnostic allozyme loci and mtDNA haplotypes are useful tools for screening of hybrids between the two species.

Hybrids between the *L. rohita* and *L. calbasu* have been produced experimentally through reciprocal crosses in captivity (Talwar and Jhingran, 1991). The occurrence of *L. rohita* and *L. calbasu* hybrid in natural habitats has not been documented previously although inter-generic hybrids between other carps have been observed in reservoirs and bundhs in India (Prasad, 1976; Chondar, 1999). Hybrids found in natural waters can be due to escaped farmed fish or natural breeding at overlapping breeding grounds. Circumstantial evidence can be used to examine whether the observed hybrid was a result of natural breeding or farm escape. At present, *L. calbasu* is not used in aquaculture and therefore commercial level seed production does not exist in India. The areas adjoining the Banganga River (foothill region) do not have significant fish farming activity. The collection site is an inundated plain of the Banganga River and that could be a typical breeding ground for Indian major carps as described by Talwar and Jhingran (1991). In view of the fact that *L. calbasu* is not widely stocked for culture through hatchery-bred seed, the observed hybrid is likely to be the outcome of natural breeding between a *L. rohita* (male) × *L. calbasu* (female).

**Acknowledgements** The excellent technical assistance provided by Sh. R. S. SAH, Sh. Akhilesh MISHRA, Sh. Rajesh KUMAR and Sree RAM is duly acknowledged.

## References

Agnese JF, Teugels GG, Galbusera P, Guyomard R, Volckaert F, 1997. Morphometric and genetic characterization of sympatric populations of *Clarias gariepinus* and *Clarias anguillaris* from Senegal. J. Fish Biol. 50: 1 143 – 1 157.

Avise JC, Sunders NC, 1984. Hybridization and introgression among species of sunfish *Lepomis*: analysis by mtDNA and allozyme markers. Genetics 108: 237 – 255.

Belkhir K, Borsa P, Goudet J, Chikhi L, Bonhomme F, 1997. GENETIX ver. 4.02, Genetique logiciel sous windows pour Ia ge' ne' Tique des populations, http: //www. univ-montp2. fr/ ~ genetix/genetix. htm

Campton DE, Utter FM, 1985. Natural hybridization between steelhead trout *Salmo gairdneri* and coastal cutthroat trout *Salmo clarki clarki* in two Puget Sound streams. Can. J. Fish. Aquat. Sci. 42: 110 – 119.

Chondar SL, 1999. Biology of Finfish and Shellfish, 1st edn. Howrah, West Bengal (India): SCSC Publication (India), 34 – 47.

Cronin MA, Spearman WJ, Wilmont RL, Patton JC, Bickham JW, 1993. Mitochondrial DNA variation in chinook *Oncorhynchus tshawytscha* and chum salmon *O. keta* detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. Can. J. Fish. Aquat. Sci. 50: 708 – 715.

Gopalakrishnan A, Lal KK, Ponniah AG, 1997. Esterases in Indian major carps——"Rohu" *Labeo rohita* and "Mrigal" *Cirrhinus mrigala* (Teleostei, Cyprinidae). Indian J. Fish. 44 (4): 361 – 368.

Hubbs CL, 1955. Hybridization between fish species in nature. Syst. Zool. 4: 1 – 20.

Prasad R, 1976. On the occurrence of the hybrids *Labeo fimbriatus* (BL) × *Labeo gonius* (Ham.) and *Labeo calbasu* (Ham.) × *Catla catla* (Ham.) in Rangawan reservoir (Uttar Pradesh). J. Inland Fish. Soc. India 8: 107 – 108.

Shaklee JB, Phelps SR, Salini J, 1990. Analysis of fish stock structure and mixed-stock fisheries by the electrophoretic characterization of allelic allozymes. In: Whitmore DH ed. Electrophoretic and Isoelectric Focussing Techniques in Fisheries Management. Boca Raton, FL: CRC Press, 173 – 196.

She JX, Autem M, Kotoulas G, Pasteur N, Bonhomme F, 1987. Multivariate analysis of genetic exchanges between *Solea aegyptiaca* and *Solea senegalensis* (Teleosts, Soleidae). Biol. J. Linn. Soc. 32: 357 – 371.

Stevens TA, Withler RE, Goh SH, Beacham TD, 1993. A new multilocus probe for DNA fingerprinting in chinook salmon *Oncorhynchus tshawytscha*, and comparisons with a single-locus probe. Can. J. Fish. Aquat. Sci. 50: 1 559 – 1 567.

Strussmann CA, Akaba T, Ijima K, Yamagnehi K, Yoshizaki G, Takashima F, 1997. Spontaneous hybridization in laboratory and genetic markers for identification of hybrids between two Atherinid species *Odotesthes bonariensis* (Valenciennes, 1835) and *Patagonina latcheri* (Eigenmann, 1909). Aqua. Res. 28: 291 – 300.

Talwar PK, Jhingran AG, 1991. Inland Fisheries of India and Adjacent Countries. Vols. Ⅰ and Ⅱ. New Delhi: Oxford & IBH Publishing Co. Ltd.

Whitmore DH, 1990. Electrophoretic and Isoelectric Focussing Techniques in Fisheries Management. Boca Raton, FL: CRC Press.