

and *M. tomentosa*. This was mainly because of comparatively higher variations in the girth and height of sample trees.

Results presented are in conformity as some species showed good correlation with $\text{cbh}^2 \times \text{height}$, whereas other species with the log function. The degree of correlation varies from $R^2 = 0.94$ for *Z. xylopyra* to $R^2 = 0.99$ for *M. tomentosa*, with SE of 0.12 and 1.33 respectively. Further, the samples taken to generate these equations are from different girth classes, geographical locations and elevations, which ensures better representation of each tree species. The percentage error of biomass estimation for *B. racemosa*, *T. grandis* and *Z. xylopyra* is in the range -2.23 to $+2.87$, whereas for *M. tomentosa* and *L. coromandelica* it is 25.6 and 26.7 respectively. These species are found in abundance in central India. Hence these equations would be a valuable input in estimating species-specific bole biomass at the local and regional level.

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Phylogenetic analysis of bacterial endophytes showing antagonism against *Rhizoctonia solani*

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Rice genotypes Pant Dhan-6, Pusa Basmati-1 and UPRBS92-4 of agronomic significance were screened for the presence of endophytic bacteria. Limited morphological diversity was observed after dilution plating of surface sterile root and shoot tissues. This was confirmed by the Shannon diversity index, i.e. 1.34, 1.30 and 0.97 for Pant Dhan-6, Pusa Basmati-1 and UPRBS92-4 respectively. Seven bacterial isolates showing antagonism against *Rhizoctonia solani* were assessed for phylogenetic analysis using PCR-based techniques.

RICE (*Oryza sativa*) is arguably the most important cereal crop in the world, feeding more than 50% of the world's population^{1,2}. However, the population is growing at an alarming rate; therefore rice yields will need to be enhanced to match the increased consumption. Such higher yields by 2020 will require at least double the amount of N fertilizers currently being used, because after water, N is the most limiting nutrient for rice growth³. An alternative to the increased use of chemical fertilizers is to obtain nitrogen from biological N₂ fixation⁴.

Rice is grown in a diverse range of environments, which may be divided into two groups based on water-tolerance. The deep-water varieties survive prolonged periods of submergence under water, whereas irrigated varieties are less tolerant to submergence. For rice to adapt to such a wide range of environments, it has to compete with bacteria and fungi within these habitats. It has long been known that rice can form natural association with various bacteria, both phototrophs and heterotrophs⁵. It has also been suggested that bacteria colonizing the plant interior might interact more closely with the host, with less competition for carbon sources and a more protected environment for N₂ fixation^{6,7}, such as that occurring in the relatively efficient N₂-fixing symbioses between rhizobia and legumes⁸. It has further been proposed that in natural ecosystems, plant pathogens have evolved to exist in balance with their host plant as well as with other microorganisms. This suggests that there exists some kind of natural control of plant pathogens. The biological control strategy using endophytic microorganisms to control plant pathogens of rice has not been exploited. There are only a few reports on the biological control of rice pathogens using bacteria, and these involve those isolated from the

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rhizosphere. Recently, there were reports on the use of corn and cotton endophytes in biological control. Here we describe the phylogenetic relationships of the antagonistic isolates recovered as rice endophytes.

Three rice varieties, Pant Dhan-6, Pusa Basmati-1 and UPRBS 92-4, were subjected to isolation of endophytes. The agronomic importance of these varieties includes resistance against the fungal pathogen, *Rhizoctonia solani* and low N-requirement. The varieties are being cultivated at the Crop Research Centre, G.B. Pant University of Agriculture and Technology, Pantnagar for the past several years.

Sampling was done at 23 days after sowing (DAS). Plants were selected, uprooted, rinsed under running tap water and blot-dried on towel paper. Root and shoot tissues were excised, transferred into 500 ml conical flask containing 20 g sterile glass beads, and shaken vigorously for 35–45 min. They were rinsed with sterile distilled water (SDW) and exposed to 0.1% mercuric chloride for 3–5 s. Finally, they were given a treatment of 1% chloramine T for 1 h, with occasional shaking every 5–7 min. Tissue samples were washed again 5–6 times with SDW. Samples were now triturated in a sterile mortar and pestle containing 1 ml phosphate buffered saline (pH 7.0). Serial dilutions were prepared of this homogenized mixture and 1 ml of each dilution was plated on half strength tryptic soy agar (1/2 TSA). The diversity of the morphotypes obtained on 1/2 TSA was studied using Shannon and Weaver's diversity and evenness indices.

Bacterial isolates were tested for antagonism against *R. solani*. The assay was done by spot-inoculating the bacterial isolate at one edge of the petri plate, and placing 3 mm³ fungal disk at the other end of the plate of 1 : 1 potato dextrose agar: nutrient agar (PDA : NA). Plates were incubated at 28°C for 72 h and inhibition, if any, of fungal growth was recorded.

Genomic DNA was extracted by a modified method of Bazzicalupo and Fani⁹.

Primers with the following sequence were used for PCR amplification of 16S rDNA:

GM3f, 5'AGAGTTGATCMTGG3'(8 to 23) and
GM4r, 5'TACCTTGTTACGACTT3'(1492 to 1507).

Reaction was set in 80 µL volume with the following concentration of reagents: 8 µl 10x assay buffer, 1.6 µl MgCl₂, 2.0 µl mix of dNTPs (1 : 1 : 1 : 1), 2.0 µl primer 1 (GM3f), 2.0 µl primer 2 (GM4r), 0.3 µl Taq DNA polymerase (5 U/µl), 60.1 µl TDH₂O, 5.0 µl template. Reaction conditions: Initial denaturation, 94°C, 3 min; denaturation, 94°C, 1 min; annealing, 54°C, 1 min; extension, 74°C, 1 min; 22 cycles from step-2 of denaturation, 94°C, 1 min; annealing, 52°C, 1 min; extension 74°C, 1 min. The reaction was performed in a PTC-100 thermal controller (M.J. Research). The PCR product was run on 0.8% agarose gel and visualized under UV.

The amplified 16S rDNA was digested with restriction endonucleases, viz. *RsaI*, *AluI* and *Sau3A*.

Each amplified product (25 µl) was set for restriction digestion in 30 µl of reaction volume by adding buffer (3 µl per reaction) and restriction enzyme (1U per reaction). The digested product was analysed in 2.5% agarose gel. DNA bands generated by digestion of PCR-amplified 16S rDNA with the restriction enzymes were used to construct a dendrogram using the unweighed pair groups mean average (UPGMA) method¹⁰. Bands were scored as molecular marker for presence and absence, ignoring their intensities.

For BOX-PCR fingerprinting, the primer sequence was BoxA1R, 5'GAT CGG CAA GGC GAC GCT GAC G3'.

The amplification was carried out using the following: template DNA, 5–10 ng; primer, 400 ng; dNTPs, 250 µM; taq DNA polymerase, 1U; total volume, 40 µl.

The reaction conditions were initial denaturation, 94°C, 3 min; denaturation, 94°C, 1 min; annealing 60°C, 1 min; extension, 74°C, 1 min; end filling, 74°C, 5 min; holding at 4°C till use. The amplified product was resolved on 2.5% agarose gel at 60V for 3 h and visualized after staining with ethidium bromide.

Intergenic spacer region between 16S and 23S ribosomal DNA was amplified with the following pair of primers: Phy, 5'TGCGGCTGGATCACCTCCTT3'; and P23SR01, 5'GGCTGCTTCTAAGCCASC3'.

The amplification was performed in 50 µl final reaction mixture containing 10X PC buffer (1X), 5.0 µl; MgCl₂, 1.0 µl; dNTP's, 1.25 µl; IGS1, 0.50 µl; IGS2, 0.50 µl; Taq (5 U/µl), 0.50 µl; TD sterile water, 41.25 µl. Initial denaturation, 95°C, 1 min; annealing, 50°C, 1.5 min; extension, 72°C, 2 min; final extension 72°C, 5 min; holding at 4°C till use. The amplified products were analysed on agarose gel (0.8%); 5 µl of the amplicon was loaded with 2 µl of dye and electrophoresed at 100V. PCR-amplified products were digested with tetracutter enzymes, *AluI* and *RsaI*. The amplified product was restriction digested in a total reaction volume of 30 µl containing 10X buffer, 21 µl; enzyme, 0.7 µl; template, 5.0 µl; TD sterile water, 3.3 µl. The digestion mixture was incubated at 37°C for 3 h and the product analysed on 2.5% agarose gel.

Cluster analysis was done by NTsys program using UPGMA, Jaccard similarity coefficient. Gel images were processed using Nikon cool-pix 995 Digital Imaging System (Biosystematica).

A limited diversity was observed on 1/2 TSA. The number of colonies observed in each genotype varied from 30 to 120. However, the number of isolates recovered from root was greater than those from shoot. Maximum number was recovered from Pant Dhan-6. A total of 40 different morphotypes were randomly picked up. Their varietal and tissue based distribution is presented in Table 2.

Shannon and Weaver's diversity and evenness data are presented in Table 3, which suggest existence of limited diversity and only small difference on account of the varietal influence.

Table 4 shows that isolates PDR1, PBR2, PDR16, PBR19, UPS25, UPR36 and UPR40 were effective antagonists against *R. solani* that causes sheath blight disease of rice. Among these, three isolates were recovered from rice variety UPRBS92-4.

Molecular analysis of endophytic bacteria recovered from rice genotypes was carried out based on ARDRA.

For PCR-RFLP of 16S rDNA (16S-ARDRA), an amplicon of 1.3 kb was amplified, which on digestion with *Rsa*I, *Alu*I and *Sau*3A produced 2–3 bands. Isolate PDR1, PBR2 and PDR16 gave similar banding pattern when digested with *Rsa*I. UPR36 gave two bands of molecular sizes, 400–500 bp. As a result of digestion with *Alu*I, total number of bands (400–1000 bp) was 2–3 in each isolate. A similar banding pattern for isolates PDR1, PBR2, PDR16, PBR19, UPS25 and UPR36 was observed (Figure 1). They

all looked similar, except for PBR19 and UPR40. On digestion with *Sau*3A, different banding patterns were observed in isolates PBR19, UPR36 and UPR40. However, isolates PDR1 and PBR2 shared similarities with two other isolates, PDR16 and UPS25.

For PCR-RLFP of 16S–23S intergenic spacer (1GS-ARDRA), in most isolates, more than one amplicon was observed, whereas no amplification was obtained in isolate PDR1 (Figure 2). All the seven isolates could be

Table 1. Important properties of genotypes

Genotype	Property
Pusa Basmati-1	High yield, good kernel elongation.
Pant Dhan-6	Moderately resistant to leaf and neck blast, scold disease of leaf. Completely resistant to brown spot.
UPRBS92-4	Recently named as Pant Sugandh Dhan 15. Moderately resistant to leaf blast, neck blast and sheath rot diseases. Tolerant to stem borer and leaf folder.

Table 2. Endophytic bacterial isolates recovered from rice varieties used in this study

Variety	Total	Root	Shoot
Pant Dhan-6	15	8	7
Pusa Basmati-1	12	11	1
UPRBS92-4	13	9	4

Table 3. Shannon and Weaver's diversity and evenness indices

Genotype	Shannon and Weaver's diversity (H')	Evenness (D')
Pant Dhan-6	1.34	0.78
Pusa Basmati-1	1.30	0.82
UPRBS92-4	0.97	0.87

Table 4. Antagonistic action of endophytic bacterial isolates against *Rhizoctonia solani*

Isolate	Zone of inhibition (mm)
PDR1	5.0
PBR2	6.1
PDR16	3.2
PBR19	5.8
UPS25	6.0
UPR36	2.0
UPR40	2.1

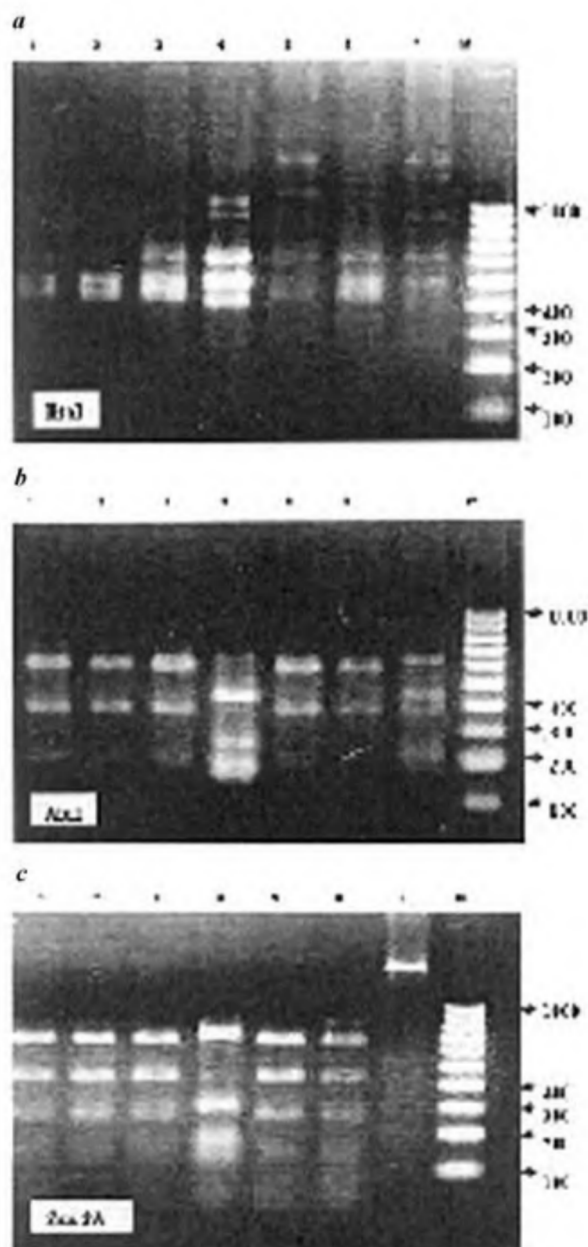


Figure 1. 16S RFLP of bacterial isolates PDR1, PBR2, PDR16, PBR19, UPS25, UPR36 and UPR40.

differentiated from each other with *RsaI*, since this profile was distinctly different from that of *AluI*.

Two to three bands were generated in each isolate when BOX-PCR amplification was performed (Figure 3). The amplicon obtained by BOX-PCR represents the whole genome and is considered suitable for revealing differences among the strains belonging to the same species.

Cultural analysis of endophytes showed existence of limited morphotypes, suggesting that the genetic structure in such bacterial populations is restricted on account of

the protection provided by the habitat and is highly conserved. Restriction pattern of 16S rDNA with *AluI*, *Sau3A* and *RsaI* showed that the seven isolates were represented by three ARDRA types. Isolates PDR1, PBR2 and PDR16 were placed in a single cluster irrespective of the restriction enzyme used. Isolate UPR36 shared more similarity to this cluster compared to other isolates. Restriction analysis based on IGS placed isolates UPS25 and UPR36 in a single cluster. IGS analysis however placed these isolates in 4–5 ARDRA types. BOX-PCR amplification

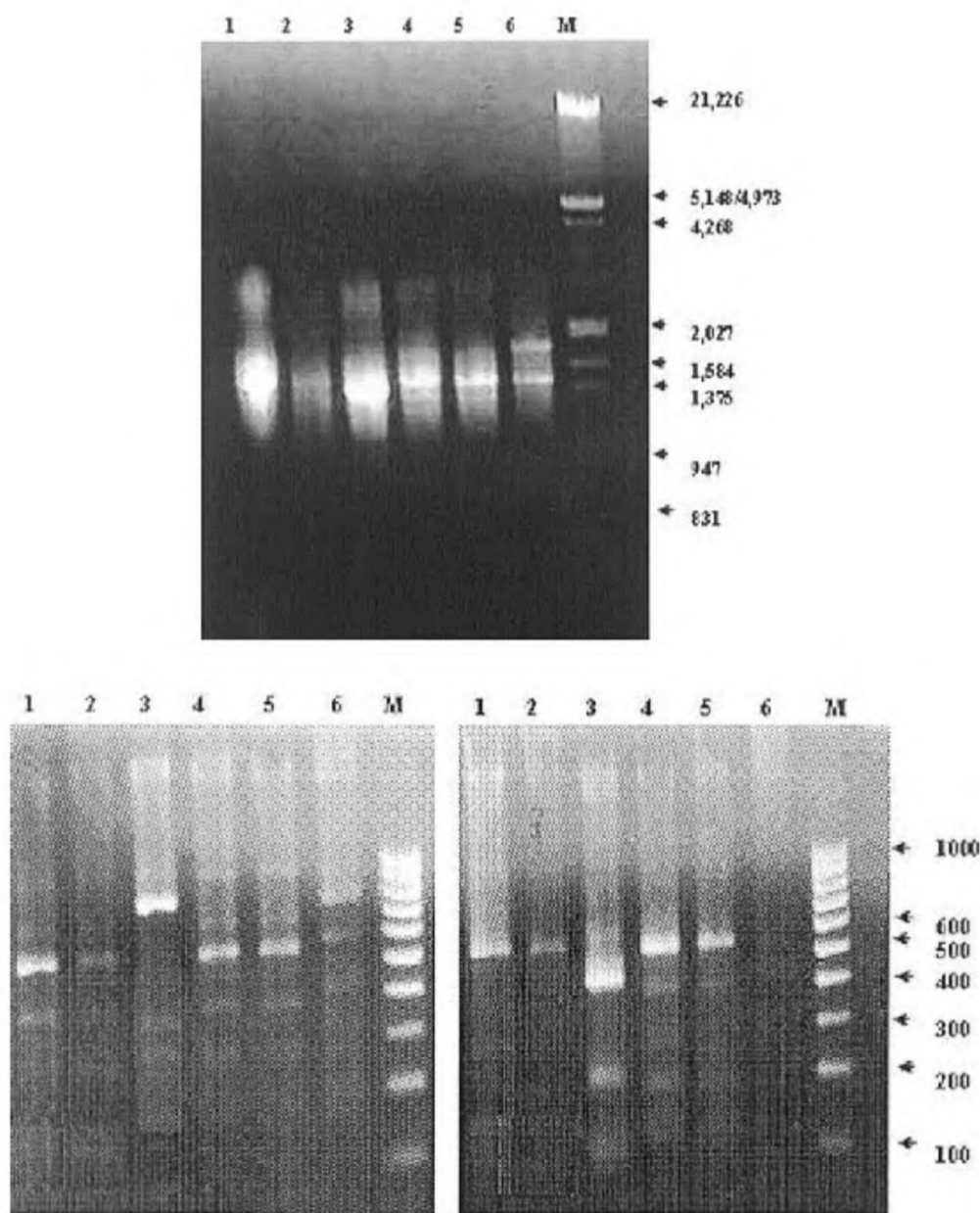


Figure 2. PCR-RFLP of 16S–23S intergenic spacer of bacterial isolates PBR2, PDR16, PBR19, UPS25, UPR36 and UPR40.

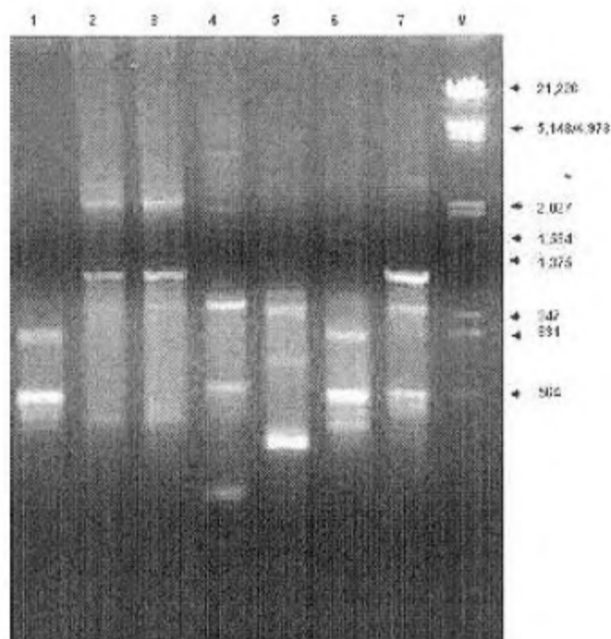


Figure 3. BOX-PCR fingerprints of bacterial isolates PDR1, PBR2, PDR16, PBR19, UPS25, and UPR34 and UPR40.

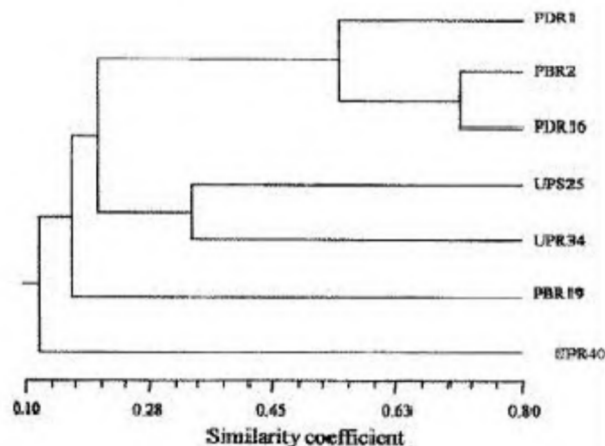


Figure 4. Composite dendrogram based on 16S RFLP, 16S-23S IGS RFLP and BOX-PCR amplification.

placed these isolates into five different groups. However, composite UPGMA dendrogram derived from the use of the three primers provided an altogether different picture (Figure 4).

Analyses using more discriminating PCR-based methods are useful in subdivision of 16S rDNA genotypes. For instance, sequences of intergenic spacers between 16S and 23S rDNA genes are known to be variable between and within species¹¹. PCR-based methods involving random primers or consensus primers derived from repetitive sequences have been found to be efficient for subtyping the genomic groups¹². Other PCR-based strategies have been developed to rapidly classify bacterial strains at the species or genus level, such as tDNA-PCR fingerprinting¹³ or sequencing of a relatively small variable portion of a gene¹⁴. Genomic fingerprints with REP, ERIC and BOX primers have mostly been used for intra-specific comparisons of bacteria^{15,16}. BOX-PCR amplification on the other hand, can detect variation in major bands permitting analysis of strain differentiation. BOX-PCR also allows differentiation at the species level, since PCR profiles show several prominent bands of equal mobility, which are specific for each species.

A combination of PCR-based techniques allowed differentiation of bacterial endophytes of rice at the species level. As a result, seven apparently similar bacterial isolates were found to be distinct based on RFLP, IGS and BOX-PCR. Relative distinction of the seven isolates antagonistic towards *R. solani* showed that several bacterial

genera may help contribute towards biological control against the pathogens of rice genotypes. Considering the protection provided by the endophytic habitat, such isolates might produce novel secondary metabolites as well.

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Species specificity as evidenced by scanning electron microscopy of fish scales

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Features engraved on the surface of fish scales (normal, regenerated and lateral line) have been elaborated using the magnifying potentials of scanning electron microscopy. An attempt has been made to define structures that can contribute towards species discrimination among four species of *Labeo* (*L. calbasu*, *L. rohita*, *L. gonius* and *L. bata*). Normal scale showed distinctness with regard to focus, region below focus, circuli, lepidonts and chromatophores. Distinct regeneration pattern has been observed for all considered species. Lateral line scales with central canal and associated features have also proved their worth for species characterization.

SCALES, the dermal derivatives of fish body, are important structures used as versatile research material in fisheries. They exhibit distinct pattern of dark and light bands corresponding to closely and widely spaced circuli that

form annular zones depicting the age of fish in years. The engraved pattern of circuli on scale serves as a blueprint for the physiological epochs of fish life, narrating its growth history. Besides this established role of scales in fish biology, these have numerous hidden details in their sculptural design that contribute effectively to fish identification and classification. Earlier workers studied fish body scales to strengthen their role in fish taxonomy. Circuli, radii, ctenii, lateral line canal and other structures associated with scales have been used authentically for classification^{1–3}. More precision has been achieved through the application of scanning electron microscopy (SEM) on scale morphology to strengthen its utility in fish classification^{3–9}.

Various researches strongly suggest the utility of scales in distinguishing the taxonomic groups over a continuum ranging from higher taxa to species. The present communication is an attempt to throw light on the structural features engraved on the scales that can contribute towards identification among the species belonging to the genus *Labeo*. Four species of *Labeo*, viz. *L. calbasu*, *L. rohita*, *L. gonius* and *L. bata* have been considered for the analysis of scales (normal, regenerated and lateral line scale) through SEM.

Fish specimens were collected from Harike wetland, India (31°13'N, 75°12'E). Normal scales were removed from 2nd/3rd row of scales above the lateral line, below the dorsal fin. Lateral line scales were selected from the middle of the line, while regenerated scales were from appropriate regions of the body. All types of scales were washed, cleaned thoroughly and air-dried. These were coated with 100 Å thick gold in JEOL sputter ion coater and observed under JEOL-JSM 6100 SEM at 20 kV.

The scales of the four species of *Labeo* are cycloid in nature, with rough dorsal and smooth ventral surface. The surface of scale is divisible into anterior, lateral and posterior regions. Its centre is regarded as focus, around which the circuli are laid in a regular manner. These circuli are interrupted at regular intervals by transverse radii. The posterior, exposed part of the scale is equipped with colour-bearing chromatophores. Though similar in the basic structural layout, there is specificity with regard to the nature, arrangement, orientation, abundance and distribution of the features associated with scales of the considered species. These specificities thus confer uniqueness to each species. The structural variations of normal scale with regard to size, shape, location of focus, relative proportion of different fields, distribution of radii in respective fields and shape of circuli are obvious. These gross morphological features have been given profound weightage in classification of fishes^{5,10}.

Focus is the first part of the scale to be formed and has different location in the four species. SEM study showed the presence of distinct larval mark in *L. calbasu* and *L. rohita*. The mark however differs in the two species with respect to size, completeness and relative spacing of cir-

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