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A N I N T E R N A T I O N A L J O U R N A L

Bacterial Diversity in the Digestive Tracts of Four Indian Air-Breathing Fish Species Investigated by PCR Based Denaturing Gradient Gel Electrophoresis

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ABSTRACT

An investigation was conducted to identify the allochthonous microbiota (entire intestine) and the autochthonous microbiota in proximal intestine (PI) and distal intestine (DI) of four species of Indian air-breathing fish (climbing perch; Anabas testudineus, murrel; Channa punctatus, walking catfish; Clarias batrachus and stinging catfish; Heteropneustes fossilis) by PCR based denaturing gradient gel electrophoresis (DGGE). High similarities of the allochthonous microbiota were observed between climbing perch and murrel, walking catfish and stinging catfish, indicating similar food behavior. The autochthonous microbiota of PI and DI from climbing perch and murrel revealed more similarity, than the result obtained from walking catfish and stinging catfish. The autochthonous microbiota of climbing perch and murrel were similar with regard to the allochthonous microbiota, but no such similarity was observed in case of walking catfish and stinging catfish. The fish genotype and intestinal bacteria are well matched and show co-evolutionary relationship. Three fish species has its unique bacteria; autochthonous Enterobacter cloacae, Edwardsiella tarda and Sphingobium sp. in DI of climbing perch, Pseudomonas sp.; allochthonous and autochthonous in PI of walking catfish and uncultured bacterium (EU697160.1), uncultured bacterium (JF018065.1) and uncultured bacterium (EU697160.1) for stinging catfish. In murrel, no unique bacteria were detected.

Key words: Allochthonous and autochthonous gut microbiota, air-breathing fish, bacterial diversity, DGGE

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INTRODUCTION

The gastrointestinal (GI) tract of an animal consists of a very complex and dynamic microbial ecosystem which is very important from a nutritional, physiological and pathological point of view [1-3]. Being rich in nutrients, the digestive tract of fish, in comparison with the surrounding water, confers a favorable growth environment for microorganisms [4,5]. It is generally accepted that the GI tract microbiota of fish are defined as either autochthonous (indigenous) bacteria, able to colonize the epithelial surface of the host, or allochthonous (transient) bacteria [6]. In several studies, the culturable gut bacterial communities of various Indian craps have been reported [7-12]. The fish gut bacterial communities may contain pathogenic, symbiotic and commensal bacteria and they can exert great effect on the host welfare which may result in mucosal tolerance or inflammation [13,14]. Elucidation of the gut microbiota, both allochthonous and autochthonous of four Indian air-breathing fish species is an essential step in developing strategies and dietary applications to fortify and modulate these communities.

Historically, the general approach to study the gut microbiota of fish was by use of conventional culture methods [4,15]. However, conventional culture methods are often time consuming and lack accuracy and sensitivity in characterizing certain fastidious and obligate anaerobes that require special culture and growth conditions [16-20]. Therefore, culture based studies of the GI microbiota leads to an uncertain picture of the microbial community residing in the GI tract. Nowadays, several novel molecular technologies, such as genetic fingerprint method based on polymerase chain reaction (PCR) amplification of 16S rDNA and denaturing gradient gel electrophoresis (DGGE) have become a popular method to investigate the gut microbiota in fish [17-19, 21,22]. However, it's still limitation in the efficiency of DNA extraction and the PCR amplification process affecting the DGGE bands, and that the concentration of the microbial species detection limits ranging between 10^4 and 10^8 CFU mL^{-1} [23]. Although some information on the identification of autochthonous bacteria in the GI tract of Indian freshwater fish are available [2, 24, 25]. To our knowledge no information is available on the use of culture-independent methods to evaluate the gut microbiota of Indian freshwater fish. The aim of the present study was therefore to investigate the allochthonous; from the entire intestine and the autochthonous gut microbiota from proximal and distal intestine of four species of adult Indian air-breathing fish (climbing perch, murrel, walking catfish and stinging catfish) using the PCR-DGGE approach.

MATERIALS AND METHODS

Experimental fish and their maintenance

Four species of adult Indian air-breathing fish, the climbing perch (*Anabas testudineus*), murrel (*Channa punctatus*), walking catfish (*Clarias batrachus*) and stinging catfish (*Heteropneustes fossilis*) were used in the present study (Table 1). The fish were obtained from a local fish farm near Santiniketan, West Bengal, India (23˚41′30″ N latitude and 87˚41′20″ E longitude). Six individuals of each fish species were randomly selected and killed with sharp blow on the head. The ventral surface of the fish were carefully scrubbed with 1% iodine solution to remove unwanted material. The fish were dissected on ice and the entire intestinal tract was aseptically removed quickly inside the laminar air flow [26]. Intestinal contents from the entire intestine containing non-adherent (allochthonous) bacteria were immediately transferred to sterile Nunc tubes containing 96% ethanol according to Liu et al.[21]. For detection of the autochthonous gut microbiota, the digestive tracts were divided into proximal intestine (PI) and distal intestine (DI) according to He et al. [27]. Each sample, pooled from six fish were homogenized separately using automatic homogenizer (Omni homogenizer) and the samples were transferred to 1.5 mL Eppendorf tubes and stored at -20˚C until further use. Analysis of pooled samples from several individuals is a normal procedure for evaluating the gut microbiota by PCR-DGGE [28-30] to avoid individual variations in the gut microbiota [30-32].

Table 1- Food habits, average live weight, average fish length, relative intestinal length and average intestine weight of the air-breathing fish species examined.

Fish species	Feeding habit*	Average live weight (g) (SD)[1]	Relative intestinal length[2]	Average intestinal weight (g) (SD)[1]
Anabas testudineus	Insects, insect larvae, water fleas, smaller fish, vegetable debris etc.	65.2 (2.9)	0.43	7.2 (0.61)
Channa punctatus	Insects, zooplankton, insect larvae, small fish	86.1 (2.4)	0.51	2.89 (0.51)
Clarias batrachus	Insect larvae, shrimps, worms	94.1 (3.2)	0.50	9.2 (0.58)
Heteropneustes fossilis	Insect larvae, shrimps, worms, small fish, organic debris	70.8 (4.2)	0.53	3.11 (0.61)

* Jhingran (1997)[59]
[1] Standard deviation given between brackets, n=6.
[2] Relative intestinal length = length of intestine (cm)/total length of fish (cm).

DNA extraction

DNA was extracted from homogenized gut samples (Table 2) from the four fish species according to Yu and Morrison [33] with modification described by He et al. [27]. The quality of the extracted was checked on 0.8% agarose gels, stained with ethidium bromide, visualized on a UV transilluminator (Gel Doc 2000, BIO-RAD).

Table 2- Gut samples from four species of Indian air-breathing fish.

Sample no.	Fish species	Part of the intestine evaluated
1	*Anabas testudineus*	Entire intestine (allochthonous microbiota)
2	*Anabas testudineus*	Proximal intestine (autochthonous microbiota)
3	*Anabas testudineus*	Distal intestine (autochthonous microbiota)
4	*Channa punctatus*	Entire intestine (allochthonous microbiota)
5	*Channa punctatus*	Proximal intestine (autochthonous microbiota)
6	*Channa punctatus*	Distal intestine (autochthonous microbiota)
7	*Clarias batrachus*	Entire intestine (allochthonous microbiota)
8	*Clarias batrachus*	Proximal intestine (autochthonous microbiota)
9	*Clarias batrachus*	Distal intestine (autochthonous microbiota)
10	*Heteropneustes fossilis*	Entire intestine (allochthonous microbiota)
11	*Heteropneustes fossilis*	Proximal intestine (autochthonous microbiota)
12	*Heteropneustes fossilis*	Distal intestine (autochthonous microbiota)

PCR -DGGE

The V3 region of the 16S rrs gene was amplified with primers 338-GC-f (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCA-3′) and 519r (5′-ATTACCGCGGCTGCTGG-3′). The PCR reaction mixture (50 μL total volume) contained 5 μL of 10× PCR Buffer, 4 μL of 2.5 mM deoxynucleotide triphosphate mixture, 2.5 μL of 20 mg mL^{-1} bovine serum albumin, 1 μL of each primer (at 10 μM), 1 μL of 5 U μL^{-1} Taq polymerase, and 2 μL DNA template. The amplification condition was 94°C for 5 min (initial denaturation), followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C

for 30 sec, and extension at 72°C for 30 sec. A final extension step was carried out at 72°C for 5 min. The PCR products were confirmed by electrophoresis on 1.5% agrose gel.

PCR-DGGE was performed according to Liu et al. [21] and Zhou et al.[34] using a DCode universal Mutation System (Bio-Rad Laboratories, Hercules, CA). About 800 ng PCR products were separated on 10% (wt/vol) polyacrylamide gels (40% acrylamide/bis solution, 37.5:1; Bio-Rad Laboratories, Hercules, CA) in 1× TAE (40 mM Tris, 20 mM Acetate, 1.0 mM Na2 -EDTA) using denaturing gradient ranges of 40 to 60% urea–formamide denaturing gradient. Electrophoresis was performed at 60 V, 60°C for 12 hr. Gels were then stained using ethidium bromide solution (0.5 mg mL^{-1} in Tris-acetate-EDTA buffer for 20 min), visualized on a UV transilluminator, and photographed. The appropriate bands were excised, re-amplified, and purified (TIAN quick Midi purification Kit, Tiangen, China), then sequenced.

Sequence and data analysis

Selected DGGE bands were excised from the gel with a sterile scalpel blade and incubated overnight at 4°C with 50 μL of 0·5× TE buffer to allow diffusion of the DNA. The PCR amplification was performed as described earlier with the same primers, adding 2 μL of the solution of extracted DNA. The purified product were cloned to pEasy- T vector (Transgen, Beijing, China), and the right clone were sent to Sanbo Biotech (Beijing, China) for sequence. Based on the presence or absence of bands a matrix character was elaborated, from which Nei-Li/Dice's coefficient of similarity was calculated and used to construct an UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

RESULTS

DGGE profile

The 16S rDNA V3 region fingerprints of the allochthonous and autochthonous gut microbiota of four species of Indian air-breathing fish are shown in Figure 1, and eighteen distinct bands were revealed in the DGGE gels. The band distribution of 16S rDNA V3 fragments from the different fish species, varied from 2 bands; autochthonous bacteria in PI of climbing perch to 11 bands; allochthonous bacteria of stinging catfish. The similarity coefficients (Cs) of the band patterns are displayed in Figure 2 and Table 3. The intestinal microbiota derived from these four fish species could be divided into four groups based on the cluster analysis results (Fig. 2), cluster 1, 1(entire intestine for climbing perch), 4 (entire intestine for murrel) and 3 (DI for climbing perch); cluster 2, 2 (PI for climbing perch), 5 (PI for murrel), 6 (DI for murrel) and 8 (PI for walking catfish); cluster 3, 9 (DI for walking catfish), 11 (PI for stinging catfish) and 12 (DI for stinging catfish); cluster 4, 7 (entire intestine for walking catfish) and 10 (entire intestine for stinging catfish).

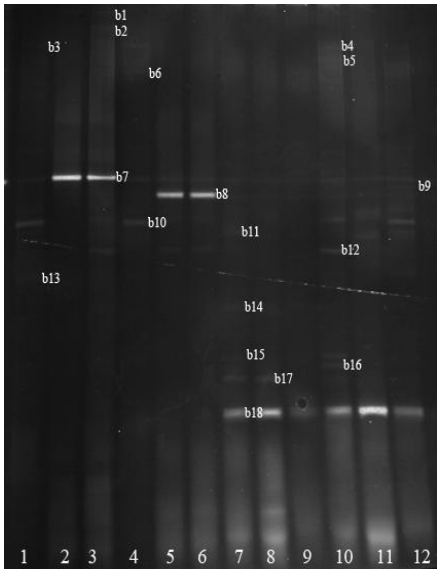

Figure 1- Denaturing gradient gel electrophoresis fingerprints of the 16S rDNA V3 region of the adhesive gut

bacterial communities from four species of Indian air-breathing fish.1,2,3:climbing perch (*Anabas testudineus*);4,5,6: murrel (*Channa punctatus*);7,8,9: walking catfish (*Clarias batrachus*) and 10,11,12: stinging catfish(*Heteropneustes fossilis*). Total 18 bands have been detected and identified by sequencing analysis.

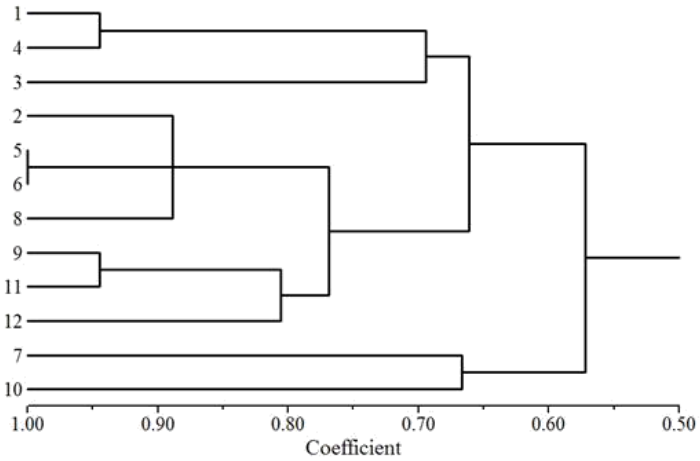

Figure 2- Cluster analysis of the adhesive gut bacterial communities of four species of Indian air-breathing fish based on the 16S rDNA V3 region using denaturing gradient gel electrophoresis fingerprints.

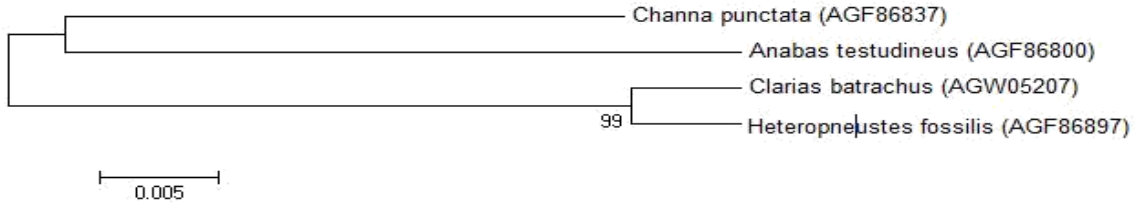

Figure 3- Neighbour-joining tree of the COI sequences in the four fish species and their closest relative sequences deposited in the NCBI database. Data represented two major cluster formed by *C. punctatus-A. testudineus* and *C. batrachus-H fossilis.*

Table 3- Pair-wise similarity coefficients (Cs) matrix for gut bacterial community.

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.0											
2	0.72	1.0										
3	0.72	0.67	1.0									
4	0.94	0.78	0.67	1.0								
5	0.72	0.89	0.78	0.72	1.0							
6	0.72	0.89	0.78	0.72	1.0	1.0						
7	0.44	0.61	0.39	0.50	0.61	0.61	1.0					
8	0.44	0.89	0.67	0.61	0.89	0.89	0.72	1.0				
9	0.72	0.89	0.56	0.61	0.78	0.78	0.72	0.89	1.0			
10	0.44	0.50	0.39	0.56	0.61	0.61	0.67	0.61	0.61	1.0		
11	0.56	0.83	0.50	0.61	0.72	0.72	0.67	0.83	0.94	0.56	1.0	
12	0.50	0.67	0.44	0.72	0.67	0.67	0.61	0.78	0.78	0.72	0.83	1.0

Regarding the allochthonous gut microbiota, a Cs value of 0.94 between allochthonous microbiota of climbing perch (1; entire intestine) and murrel (4; entire intestine), while for allochthonous microbiota of walking catfish (7; entire intestine) and stinging catfish (10; entire intestine) a Cs value of only 0.67 was detected. The highest Cs value, high degree of similarity (1.0) was detected between the autochthonous microbiota in PI and DI of murrel, in contrast to a Cs value of 0.67 for the autochthonous microbiota in PI and DI of climbing perch. The autochthonous microbiota in PI for climbing perch and murrel was 0.89, while the Cs value in DI of climbing perch and murrel was 0.78. A more closed cluster relationship between walking catfish

and stinging catfish was observed (Cs value results of PI = 0.83 and for DI = 0.78).

Different intestinal contents and gut sections (PI and DI) of the four fish species had only one common band, band 8 (Table 4). Furthermore, PCR-DGGE analysis of murrel revealed one common band; band 11, while in samples from walking catfish and stinging catfish, band 9, 12 and 18 were common. Some bands were unique for one fish species. Climbing perch; band 1 - 3 (autochthonous in DI), band 14 (allochthonous), band 17 (allochthonous and autochthonous in PI) in walking catfish and in stinging catfish; band 5 and 6 (allochthonous and autochthonous in DI) and band 10 (autochthonous in PI and DI).

Table 4- Identification of 18 sequenced bands from 4 Indian air-breathing fish species.

Phylum	No.	Closest relative (obtained from BLAST search)	similarity (%)	1	2	3	4	5	6	7	8	9	10	11	12
Proteobacteria	b1	*Enterobacter cloacae* (HQ697282.1)	99	-	-	+	-	-	-	-	-	-	-	-	-
	b2	*Edwardsiella tarda* (HM003641.1)	100	-	-	+	-	-	-	-	-	-	-	-	-
	b3	*Sphingobium* sp. (HM005244.1)	100	-	-	+	-	-	-	-	-	-	-	-	-
	b4	Uncultured *Aeromonas* sp. (HQ658850.1)	99	+	-	+	+	-	-	-	-	-	-	-	-
	b9	*Stenotrophomonas* sp. (AB560630.1)	100	-	+	+	-	+	+	+	+	+	+	+	+
	b11	*Acinetobacter* sp. (GU290322.1)	99	+	-	+	+	+	+	+	-	-	+	-	+
	b12	*Acinetobacter lwoffii* (DQ341260.1)	100	-	-	-	-	-	-	+	+	+	+	+	+
	b14	*Stenotrophomonas* sp. (AB560630.1)	99	-	-	-	-	-	-	+	-	-	-	-	-
	b17	*Pseudomonas* sp. (HM468095.1)	100	-	-	-	-	-	-	+	+	-	-	-	-
Uncultured	b5	Uncultured bacterium (EU697160.1)	100	-	-	-	-	-	-	-	-	-	+	-	+
	b6	Uncultured bacterium (JF018065.1)	100	-	-	-	-	-	-	-	-	-	+	-	+
	b7	Uncultured bacterium (JF011316.1)	100	+	-	-	+	-	-	-	-	-	+	-	-
	b8	Uncultured bacterium (JF016709.1)	95	+	+	+	+	+	+	+	+	+	+	+	+
	b10	Uncultured bacterium (EU697160.1)	100	-	-	-	-	-	-	-	-	-	-	+	+
	b13	Uncultured bacterium (GQ360025.1)	96	-	-	+	-	+	+	-	-	-	+	-	-
	b15	Uncultured bacterium (JF016747.1)	100	-	-	-	-	-	-	+	-	-	+	-	-
	b16	Uncultured bacterium (JF018065.1)	100	-	-	-	-	-	-	+	-	-	+	-	-
	b18	Uncultured bacterium (EU697160.1)	99	-	-	-	-	-	-	+	+	+	+	+	+

Sequences from DNA bands in DGGE gel

Eighteen bands were successfully sequenced and the sequences of approximately 200 bp were compared using a classifier tool in RDP (Ribosomal Database Project) are revealed in Table 4. The only bacterial species common in the GI tract of the four species of Indian air-breathing fish was identified as an uncultured bacterium (band no. 8) revealing 95% similarity to accession no. JF016709, which showed relatively low sequence similarities with the reference strains examined. Table 4 also revealed that band 1, 2 and 3were most closely related to *Enterobacter cloacae* (similarity = 99%), *Edwardsiella tarda* (100% similarity) and *Sphingobium* sp. (100% similarity), respectively. Band no. 4 was identified to uncultured *Aeromonas* sp. (similarity = 99%) and was detected in entire intestine (allochthonous microbiota) and DI (autochthonous microbiota) of climbing perch, and

as allochthonous in the entire intestine of murrel. Band no. 14 showed 99% similarity to *Stenotrophomonas* sp. but was only detected in allochthonous microbiota of walking catfish. Meanwhile, band no. 17and 12 were assigned to *Pseudomonas* sp. a111-5 (100% similarity) and *Acinetobacter lwoffii* strain F78, respectively.

The presences of the uncultured bacterium clones were different between the fish species and they were dominant as 10 out of 18 bands were identified as uncultured bacterium clones (Table 4). The uncultured bacterium clones; band no. 5 and 10 (both displaying 100% similarity to uncultured DGGE bacterium), band no. 6 (uncultured bacterium clone ncd320g09c1) and the uncultured bacteria clone ncd112e08c1; band no. 7 were detected.

Phylogenetic analysis

All four sequences for each species were included in the phylogenetic analysis of COI genes. The NJ trees (Fig. 3) revealed identical phylogenetic relationship among the species. Two major clusters were obtained with the first cluster formed by the climbing perch (*A. testudineus*) and murrel (*C. punctatus*). The second cluster was formed by the species walking catfish (*C. batrachus*) and stinging catfish (*H. fossilis*).

DISCUSSION

The aquaculture is the fastest growing food sector in all over the world. The economy of different countries like China, India, Norway, Malaysia, Indonesia, Brazil, Canada and USA are highly dependent on aquaculture production. The production cost of cultured fish are high due to high cost of feed preparation. The GI tract bacteria play a critical role in fish nutrition and diseases prevention. The knowledge on composition of fish GI tract bacteria is thus very important to optimize the feed efficiency which will ultimately reduce the production cost in aquaculture sectors. In this present study, four Indian air-breathing fish species have been selected to analyze the bacterial diversity in their GI tract using the PCR-DGGE approach. Air-breathing fish are known to generally inhabit the freshwater swamps and other water bodies in the tropical regions and are able to survive for long time outside water due to the presence of their specially developed air-breathing organs [35]. Due to the presence of air-breathing organs, these fish species are suitable for culture in such water bodies where gill-breathing species fail to survive. The four air-breathing fish species used in the present study; climbing perch, murrel, walking catfish and stinging catfish differ in their external features and by having different types of air- breathing organs. These fishes are suitable for culture in any type of water bodies where the culture of carps is not possible. The air-breathing fishes are generally carnivorous in feeding habit. Some reports are available on the culture dependent bacterial communities in the GI tract of air-breathing fish [2,24,25], and these investigations revealed that the cultivable GI microbiota of both murrel and stinging catfish contained phytase, cellulase, protease, lipase and amylase-producing bacteria in PI and DI and they were identified as *Bacillus licheniformis* and *Bacillus* sp[12]. However, as conventional culture technique has limitation, further information is needed using a culture-independent approach.

The intestinal microbiota derived of the four fish species investigated in the present study could be divided into four groups based on the cluster analysis results and the relationship between intestinal microbiota and fish genotype by sequencing the fish cytochrome oxidase subunit 1(COI) gene. According to Hubert et al.[36] sequencing the fish cytochrome oxidase subunit 1 (COI) gene is an efficient DNA "*barcoding*" technique for identifying freshwater fish species and creating a phylogenetic tree. In the present study, the phylogenetic relationship reflected a similar dendogram tree of the intestinal microbiota, suggesting that fish genotype and intestinal bacteria showed co-evolution relationship. This result was similar to the results of Zoetendal et al.[37], Kovacs et al.[38] and Spor et al.[39] revealing that genotype had a significant effect on gut microbiota in humans and mice. DGGE profiles of fecal bacterial 16S rDNA amplicons from adult humans with varying degrees of monozygotic twins were compared by determining the similarity indices of the profiles of unrelated individuals. The similarity between fecal DGGE profiles of monozygotic twins were significantly higher than those for unrelated individuals (ts = 2.73, p1-tail = 0.0063, df=21). In addition, a positive relationship ($F1, 30 = 8.63$, $p = 0.0063$) between the similarity indices and the genetic relatedness of the hosts was observed [37]. The autochthonous DI microbiota of walking catfish was not strict cluster together with stinging catfish. This may be attributed to the

environmental factors interact to control the acquisition and to maintain gut microbiota. For example it is well established that dietary manipulations modulate the microbiota composition [27,34,40,41]. As several DGGE bands were retrieved in the present study that have either rarely or never been reported previously as a part of the gut microbial community of the four Indian freshwater fish investigated, some general information is therefore presented here.

Enterobacter are Gram-negative, non-spore forming, facultative rod-shaped bacteria which can utilize lactose in presence of bile salt and detergent [42]. Trust and Sparrow [26] reported *Enterobacter* sp. in the GI tract of freshwater salmon. In the present study, one DGGE band, no. 1 displayed high similarity (99%) to *E. cloaceae* strain P 42 described by Moerschbacher and El Gueddari (unpublished data, National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/). As *E. cloacae* cause high mortality in flathead mullet (*Mugil cephalus*) [43], further studies are warranted evaluating whether *E. cloaceae* detected in the present study has any negative effect on health of Indian air- breathing fish species. Similarly, the genus *Edwardsiella* is a rod-shaped, Gram-negative, fermentative bacteria belongs to the family Enterobacteriaceae. *E. tarda* is an important member of this family and is distributed in fish where it can cause septicemia [44]. Band no. 2 showed 100% similarity to *E. tarda* strain GD080715-1 reported by Chen et al. (unpublished data, NCBI). Whereas, *Sphingobium* is Gram negative, aerobic, non-sporulating, rod shaped, non-motile bacteria which are widely distributed in contaminated soil, mainly clinical wastes [45]. From the biotechnological point of view, genus *Sphingobium* is very important as it is involved in different biodegradation pathway, such as degradation of methane and phenol by *Sphingobium yanoikuyae* strain FM-2 and *Sphingobium fuliginis*, respectively [46,47].To our knowledge; *Sphingobium* sp. has not been reported in Indian freshwater fish. *Sphingobacterium* spp. Commonly isolated from the GI tract of rainbow trout (*Oncorhynchus mykiss*) [48]. However, in the present study, DGGE band no. 3 revealed 100% similarity to *Sphingobium* sp. CO132 reported in a study evaluating anti- protease and immunomodulatory activities of bacteria associated with Caribbean sponges [49].

In a study on microbial community analysis of three municipal wastewater treatments plants in winter and spring by culture-dependent and culture-independent methods, Cui et al.[50] reported information on uncultured *Aeromonas* sp. clone hrb-449, accession no. HQ658850. Band no. 4 in the present study revealed high similarity to this accession number. *Stenotrophomonas* are Gram-negative, aerobic, motile, non-fermentative bacteria which were previously grouped in the genus *Xanthomonas* [51]. *S. maltophilia*is the most important member of this group[52] and is considered to be a potent human pathogen [53,54]. Furushita et al.[55] isolated and characterized *S. maltophilia* from the cultured marine fish, *Seriola quinqueradiata*. DGGE band no. 9 and 14 revealed high similarities to *Stenotrophomonas* sp. TeRB010 previously reported by

Someya et al.[56] in a study have also investigated the diversity of culturable chitinolytic bacteria from rhizospheres of agronomic plants in Japan. Whether, *Stenotrophomonas* detected in the present study can contribute to nutrition of the fish species investigated in the present study has not been elucidated and merits further investigations.

Acinetobacter are Gram-negative, oxidase-negative, catalase-positive and rod-shaped bacteria which have been isolated from the digestive tract of finfish [57,58]. However, to our knowledge the genus has not previously been reported in India freshwater fish. In the present study, DGGE band no. 12, revealed 100% similarity to *Acinetobacter lwoffii* strain F78 reported by Hanuszkiewicz et al. (unpublished data, NCBI) and band no. 11 displaying 99% similarity to *Acinetobacter* sp. 200915 described by Duan et al. (unpublished data, NCBI). Of the 18 bands reported in the present study, 11 bands revealed high similarities to uncultured bacterium isolates or uncultured clones illustrating that the gut microbiota of Indian freshwater fish is highly diverse.

CONCLUSION

The present investigation demonstrated the composition of dominant intestinal bacterial flora (both autochthonous and allochthonous) of four Indian air-breathing fish species namely *A. testudineus*, *C. punctatus*, *C. batrachus* and *H. fossilis*. To the authors' knowledge, it is the first report of both autochthonous and allochthonous bacterial flora in the GI tract of these fish species.

Previous studies have reported the bacterial composition in these air-breathing fish using culture dependent techniques, which is not sufficient to draw the exact picture of the microbial communities. Most of the bacterial species in the GI tract are non-culturable and thus culture independent techniques such as DEGE, TGGE and Next Generation Sequencing (NGS) are important to explore the microbial ecosystem of gut. The difference in bacterial community in GI tract of these four fish species is due to different feeding habit. Despite their differences in the intestinal microbiota, our data also indicated that factors related to the host genotype have an important effect on the bacterial community in the GI tract. The present study clearly demonstrated that the GI tract of these fish species is dominated by non-cultural bacterial species. This discrepancy highlights that further studies are needed to evaluate the gut microbiota of air-breathing fish. In addition, determination of the physiological role of the intestinal microbiota merits further investigations.

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