1Detecting natural hybridization among Indian major carps through advance

2morphometric approach

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4Detect hybrids among Indian major carps through advance morphometry

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26Abstract

27 Interspecific natural hybridization is an indicator of altered ecosystem. Habitat destruction
28increases competition with fish species in close proximity for spawning habitat with
29overlapping reproductive activities, thereby causing natural hybridization. This study is first
30investigation on detecting hybrids among Indian major carps (*Labeo rohita*, *Cirrhinus*
31*mrigala* and *Gibelion catla*) from the Ganga River by applying a cost-effective method,
32“Geometric Morphometrics”. The relative warps (RW), principal component analysis (PCA)
33and canonical variate analysis (CVA) were employed on superimposed images to determine
34morphometric variations. Deformation grid of RW showed that *G. catla* and hybrid specimens
35have a deeper whereas *L. rohita* and *C. mrigala* specimens have slender body profile. The
36PCA showed separation among specimens of four groups (three species and one hybrid) with
37slight overlap between *G. catla* and hybrid. CVA extracted Mahalanobis and Procrustes
38distances among four non-overlapping groups found to be highly significant ($P < 0.0001$) with
39hybrid specimens lying between position of *L. rohita* and *G. catla* specimens in close
40proximity to *G. catla*, suggesting that hybrids are product of crossing between *L. rohita* and
41*G. catla*. The CVA separated four groups with 100.00% classification, indicating that all the
42three species and hybrid were clearly distinct from each other. In this study, all the four
43specimens of hybrid were caught from upstream of four barrages (Bijnor, Narora, Kanpur and
44Farakka) commissioned along the middle and lower stretch of the Ganga River. This suggests
45that, barrages obstruct upward movement of fish population and impact on reproductive
46activities, thereby increases possibilities of natural hybridization among these species.

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48Introduction

49 River Ganga, India’s National River is home to a wide variety of relic species, is
50fragmented by dams and barrages constructed along its entire stretch. Over the last few

51decades, wild capture fishery in Ganga River appears to be declined [1] and changes in fish
52distribution, phenotypic traits and biological characteristics have also been reported [2].
53Indian major carps (Order- Cypriniformes, Family- Cyprinidae) are economically important
54warm-water teleost, inhabitant of Indo-Gangetic riverine system spread across northern and
55central India, and the rivers of Pakistan, Bangladesh, Nepal and Myanmar [3, 4]. In the Ganga
56River, Indian major carps (IMCs) are distributed all along the middle (Bijnor to Patna) and
57lower stretch (Patna to Hooghly) [5]. They have been effectively transplanted out of its
58natural range within India and parts of Asia as well as Europe [6, 7]. The major source of seed
59in India is contributed from natural resources. Indian major carps (*Labeo rohita*, *Cirrhinus*
60*mrigala* and *Gibelion catla*) contribute nearly 4% to the global aquaculture [8]. Hybridization
61among these Indian major carps is most common in nature, especially between *L. rohita* and
62*G. catla* [9-12].

63 Cyprinid fishes are prone to interspecific hybridization in wild [13]. Several causes
64have been suggested to account for the occurrence of hybrids in nature. Hubbs [14] holds the
65view that hybridization in nature is facilitated when there is scarcity of one species and
66dominance of an allied species in close proximity. This phenomenon is particularly common
67when populations invade new environment and overlap temporally and spatially in spawning
68activities [15-18]. This tendency is further enhanced by anthropogenic factors, such as habitat
69destruction, which increases competition for spawning habitat [19, 20]. Many studies have
70stressed that interspecific hybrids exhibit significant reductions in viability, fertility, and
71fitness [21-22].

72 Misidentification of pure and hybrid fingerlings of Indian major carps from wild may
73lead to hybridization during aquaculture practices subsequently deteriorating the quality of
74seed produced [23-25]. During fish diversity assessment, misidentification of hybrids may
75lead to taxonomic ambiguities. Few studies have been conducted in recent past to differentiate

76hybrids from pure fingerlings employing various tools such as phenotypic traits, allozymes
77and mitochondrial DNA markers [25-27]. However, the phenotypic traits described in these
78studies are more or less complex and molecular techniques used are also very costly.
79Therefore, the present scenario demands a cost effective, reliable and user friendly method to
80detect hybrids among Indian major carps.

81 It is also obvious that over the past few years, science of taxonomy has been suffering
82from dwindling number of experts [28]. Moreover, the pace of traditional taxonomy is also
83very slow. Taxonomists carefully count meristic characters, measure multiple morphometric
84variables of body shape and examine external body features to discriminate Indian major
85carps, still there is a risk of misidentification during visual assessment [29]. However, in the
86recent past, the pace of data gathering and analysis in taxonomy has been greatly increased by
87the development of information technology [30]. A family of software tools have been
88designed for gathering and analyzing data on shape variation from images of specimens [31].
89“Geometric Morphometric” (GM) method is currently considered to be the most rigorous,
90cost effective and user friendly morphometric technique in capturing information about the
91shape of an organism from digital images with more powerful statistical analyses for species
92differentiation [18, 32-34].

93 Till date, no study has examined shape variation among Indian major carps and
94detecting their hybrids using “Geometric Morphometrics” in nature. During fish diversity
95assessment of Ganga River, four individuals were encountered, which were suspected to be
96the hybrids of *L. rohita* and *G. catla*, having scale colour and pattern of *L. rohita* and body
97profile especially head region and body depth profile of *G. catla*. To resolve the taxonomic
98ambiguity, this study was conducted with the aim to evaluate morphometric variation among
99Indian major carps and detecting their hybrids using “Geometric Morphometric” method to
100provide new insights into features of shape change. By examining the external morphology

101 and distribution range of the Indian major carps and their hybrids in the Ganga River, shape
102 variations of the latter from parental species were explored along with anthropogenic factors
103 responsible for their successful adaptation.

104

105 **Materials and Methods**

106 **Study areas and sample collection**

107 A total of 96 specimens of Indian major carps including 4 individuals of hybrids were
108 collected with the help of local fishermen using different fish gears (gill net, drag net and cast
109 net) from ten locations along the middle and lower stretch of Ganga River between May 2017
110 and April 2018. The sample size and relative information for all the sampling sites are
111 presented in Table 1 and Fig 1.

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113 **Fig. 1.** Map of Ganga River showing collection area of Indian major carps and hybrids

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Table 1: Collection localities, sample size and size statistics (based on the Standard Length) of Indian major carps in Ganga River

Collection site	Site code	GPS location			Sample size			
		Latitude (N)	Longitude (E)	Altitude (m)	<i>L. rohita</i> (36)	<i>C. mrigala</i> (32)	<i>G. catla</i> (24)	Hybrid (4)
Upstream Bijnor Barrage	UBB	29°27'46"	78°02'51"	222	3	2	2	1
		"						
Downstream Bijnor Barrage	DBB	29°17'05"	78°06'07"	220	6	5	3	-
Upstream Narora Barrage	UNB	28°48'59"	78°08'46"	200	3	3	2	1
Downstream Narora Barrage	DNB	28°3'12"	78°30'57"	175	4	4	3	-
Upstream Kanpur Barrage	UKB	26°36'17"	80°16'48"	100	2	2	1	1
Downstream Kanpur Barrage	DKB	26°11'16"	80°36'58"	100	4	4	3	-
Varanasi	VAR	25°19'26"	83°08'15"	65	3	3	3	-
Patna	PAT	25°31'4"	85°18'10"	40	4	2	2	-
Upstream Farakka Barrage	UFB	24°54'10"	87°55'21"	25	2	3	2	1
Downstream Farakka	DFB	23°26'45"	88°22'21"	15	5	4	3	-
Range (mean ± SD) cm					24.54-57.79 (36.36±9.86)	24.13-59.67 (40.90±8.32)	30.75-71.30 (56.64±8.48)	40.73-50.70 (46.27±4.16)

127 Ethics statement

128 As per IUCN status, Indian major carps are under Least Concern (LC) category. Fish
129 samples were obtained from wild and kept live in water tanks. Sites from where fishes were
130 collected fell outside Protected Areas (PAs) and therefore no permits were required from
131 State Forest and Wildlife Department. After taking photographs, fishes were released back to
132 the river system. No fish samples were sacrificed or anesthetised. Therefore, ethics committee
133 approval not required.

134 Geometric morphometric analysis

135 Sampled specimens were uniformly placed on laminated graph sheets, body posture
136 and fins were teased into a natural position. Each individual was labelled with a specific code
137 for identification. A Cyber shot DSC-W300 digital camera (Sony, Japan) was used to capture
138 the digital lateral image of the left side of each individual.

139 TPSUTIL, version 1.52 was used to build a tps file from the photographs [35]. On
140 each specimen, 14 homologous landmarks were digitized using the software TPSDIG version
141 2.16 [36] (Fig 2). Scale factor using set scale option was performed on each specimen. 2D X-
142 Y coordinate data for 14 landmarks of all the 96 specimens were generated using software
143 ImageJ version 1.50i (<http://imagej.nih.gov/ij/>) and saved in tps format.

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145 **Fig. 2.** Location of 14 landmarks on Indian major carps a. *L. rohita*, b. *G. catla*, c *C. mrigala*.
146 Landmarks refer to 1. anterior tip of snout at upper jaw 2. most posterior aspect of
147 neurocranium (beginning of scaled nape) 3. origin of dorsal fin 4. end of dorsal fin 5. anterior
148 attachment of dorsal membrane from caudal fin 6. posterior end of vertebral column 7.
149 anterior attachment of ventral membrane from caudal fin 8. end of anal fin 9. origin of anal
150 fin 10. insertion of pelvic fin 11. insertion of pectoral fin 12. ventral edge of the operculum
151 13. posterior end of eye 14. anterior end of eye

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153 Landmarks were converted to shape coordinates by Procrustes superimposition [37],
 154 standardizing each specimen to unit centroid size, or an estimate of overall body size [38].
 155 The pattern of covariation between shape and size as well as shape and sex was analysed
 156 using Partial Least Square (PLS) analysis [39, 40]. Relative warp (RW) analysis was
 157 performed using TPSRELW [41] to synthesize and visualize the morphological variations.
 158 Wireframes connecting the digitized landmarks on a shape were generated to visualize and
 159 interpret shape changes between the two seasonal migrants. Principal Components Analysis
 160 (PCA) and Canonical Variate Analysis (CVA) were performed to assess the shape variation
 161 between the two seasonal migrants. Principal components analysis was applied to outline
 162 groups of samples and to identify influential variables [42]. Canonical variate analysis is a
 163 method of finding the set of axes that allows for the greatest possible ability to discriminate
 164 between two or more groups [43]. All statistical analyses were carried out with the software
 165 MorphoJ, version 1.06d [44].

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167 Results

168 Shape coordinates were successfully superimposed by Procrustes analysis (Procrustes
 169 sums of squares: 0.363 and Tangent sums of squares: 0.361). PLS revealed a non-significant
 170 covariance between shape and size ($R = 0.54$; $P > 0.001$). In this case, plot shows overlapping
 171 among groups (Fig 3).

172

173 **Fig. 3.** Scatter plot of the partial least square analysis in Indian major carps and hybrids
 174 computed on size (Block 1 PLS1) and shape (Block 2 PLS1) variables

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177 RW analysis showed deformation in shape from the reference that corresponds to
178selected positions in the ordination. As shown by the deformation grid, *L. rohita* and *C.*
179*mrigala* have slender and *G. catla* and hybrid have deeper body profile. Deformed wireframe
180was drawn on the shape of three species and hybrid to interpret shape changes which supports
181the relative warp analysis (Fig 4).

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183**Fig. 4.** Deformation grid of relative warps and wireframes between two groups in Indian
184major carps and hybrids

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186 The PCA extracted 24 components with 100.00% variance (Fig 5). The first two
187principal components (PC) account for 79.66% of the total variance (74.32% for PC1, 5.34%
188for PC2). Separation among four groups with slight overlap between *G. catla* and hybrid was
189clearly evident in the PCA plot of the first against the second PC axes (Fig 6). CVA based
190upon 14 landmarks showed four groups with no overlaps among groups with position of
191hybrid specimens lying between position of *L. rohita* and *G. catla* specimens in close
192proximity to *G. catla* (Fig 7). CVA plot suggested that hybrids are resultant due to crossing
193between *L. rohita* and *G. catla*. CVA extracted three CVs accounting for 100.00% variation
194(92.47% for CV1, 5.86% for CV2). CVA extracted Mahalanobis and Procrustes distances
195among four groups found to be highly significant ($P < 0.0001$), indicating that all the four
196groups were clearly distinct from each other (Table 2, 3). Classification results of CVA
197indicated that all the specimens of each group were assigned to their respective group with no
198misclassification rate (Table 4).

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Fig. 5. Scree plot of percentage variance and principal components for Indian major carps and hybrids

Fig. 6. Plot of principal component analysis for Indian major carps and hybrids showing loadings of each sample on the first two principal components

Fig. 7. Plot of canonical variate analysis for Indian major carps and hybrids showing frequency of specimen distribution in respective group on the first two axis

Table 2: Mahalanobis distances among groups (upper diagonal) and p value (lower diagonal) of canonical variate analysis

Groups	<i>L. rohita</i>	<i>C. mrigala</i>	<i>G. catla</i>	Hybrid
<i>L. rohita</i>		8.491	13.580	10.210
<i>C. mrigala</i>	<0.0001		20.350	16.947
<i>G. catla</i>	<0.0001	<0.0001		7.443
Hybrid	<0.0001	<0.0001	<0.0001	

Table 3: Procrustes distances among groups (upper diagonal) and p value (lower diagonal) of canonical variate analysis

Groups	<i>L. rohita</i>	<i>C. mrigala</i>	<i>G. catla</i>	Hybrid
<i>L. rohita</i>		0.055	0.085	0.067
<i>C. mrigala</i>	<0.0001		0.134	0.114
<i>G. catla</i>	<0.0001	<0.0001		0.050
Hybrid	<0.0001	<0.0001	<0.0001	

Table 4: Classification of individuals/percentage in respective group (in diagonal), misclassification of individuals (upper diagonal) and their percentage (lower diagonal)

Groups	<i>L. rohita</i>	<i>C. mrigala</i>	<i>G. catla</i>	Hybrid
<i>L. rohita</i>	36/100.00	0	0	0
<i>C. mrigala</i>	0.00	32/100.00	0	0
<i>G. catla</i>	0.00	0.00	24/100.00	0
Hybrid	0.00	0.00	0.00	4/100.00

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218 Discussion

219 Natural hybridization has received much attention in animal evolution in recent past
220 [18, 21, 22, 45, 46]. Historically, majority of hybrids were assumed to be inherently unfit with
221 reduced fertility and referred as hybrid inferiority [47, 28]. However, recent studies have
222 provided evidence for hybrid fitness when compared to parental species [18, 45, 49-52].
223 Frequent interspecific hybridization encourages risk of declining natural population of
224 indigenous fish species and thereby hamper conservation efforts [53]. Anthropogenic
225 activities such as aquacultural activities, decline in natural population, species introductions,
226 and loss or alteration of habitats have been frequently cited as most common contributing
227 factors for natural hybridization [14-20, 53]. Several studies have highlighted the importance
228 of identification, extant and causes of natural hybridization and loss of fisheries biodiversity
229 [18, 53].

230 The present study focused on detecting hybrids among Indian major carps (*L. rohita*,
231 *C. mrigala* and *G. catla*) collected from Ganga River along their distribution range using
232 “Geometric Morphometric” showed four morphologically distinct groups. Size-related
233 characters play a major job in morphometric assessment and the results may be flawed if not
234 adjusted prior to statistical analyses of data, which were effectively removed here by
235 Procrustes analysis [37]. “Geometric Morphometric” could be a useful method to distinguish
236 different fish species [30, 32, 33, 54, 55]. CVA showed no overlapping between groups with
237 100.00% correct classification in respective group. This differentiation was solely related to
238 body depth of the fish. Samples of *G. catla* and hybrid have relatively deeper-body compared
239 to samples of *L. rohita* and *C. mrigala*. CVA depicted hybrids position between groups of *L.*
240 *rohita* and *G. catla* indicating that hybrids resultant from crossing between two species.

241 Geometric Morphometrics is related to change in the shape and is concerned to
242genetics and ecology [56]. Obtaining traditional morphometric measurements is laborious and
243slow process as taxonomists have to manually generate data from numerous specimens [30].
244The power of landmark based morphometry to separate fish based on varying body form
245supports the enhancement of this technique for field based diagnosis [57]. Cadrin and
246Friedland [58] assessed that image processing techniques have enhanced but not been
247frequently applied to morphometric analyses in fisheries research.

248 The results obtained from the “Geometric Morphometrics” indicated that the Indian
249major carps showed significant phenotypic heterogeneity among the three species with clear
250distinctness from hybrids of *L. rohita* and *G. catla*. Cavalcanti et al [32] studied
251morphometric variations among six species of serranid fishes and observed non-uniform
252changes in body shape. Parsons et al [33] revealed high morphometric variations in two
253cichlid fish species using CVA. Katselis et al [59] studied morphometric variation among four
254grey mullet species of Mediterranean and results of DFA revealed high classification of
255species into their respective groups. Sakai et al [60] studied the morphological variation
256among three crucian carps from the Ob River system, Kazakhstan and revealed high
257morphometric variation among species based on landmark based morphometry. Jacquemin
258and Pyron [61] studied morphological variation in Cyprinidae fishes from lentic and lotic
259systems to gain insight into long term patterns in morphology. Reist et al [62] detected
260hybrids among coregonid fishes based on morphological variations. Toscano et al [18]
261revealed morphometric variations between the hybrids of roach (*Rutilus rutilus* L.) and bream
262(*Abramis brama* L.), and their parental species inhabiting an Irish lake using “Geometric
263Morphometrics”.

264 In the present study, causes of natural hybridization among Indian major carps were
265also recognized. It is observed that all the four individuals of hybrids were encountered

upstream of barrages (Bijnor, Narora, Kanpur and Farakka) commissioned along the middle and lower stretch of Ganga River. Out of four barrages, three (Bijnor, Narora and Kanpur) are located in Uttar Pradesh and Farakka barrage in West Bengal. Starting from upstream to downstream, Bijnor barrage (1978), followed by Narora barrage (1966) and Kanpur barrage (2000) are located in middle stretch and Farraka barrage (1975) is situated in lower stretch of Ganga River.

Physical barriers commissioned along the Ganga River might have caused ecological imbalance which is evident from natural hybridization among Indian major carps. Habitat alterations are important contributing factor to the natural hybridization by facilitating geographic range expansion, creating dispersal corridors that allow movement of one species into the range of another, or reduce spawning habitat and thus limiting spawning activities [53, 63]. Physical barriers in form of barrages convert free flowing river into reservoir like habitat, affecting the ecosystem. These physical barriers restrict upward movement of fish resulting in an ecological trap for fish that ascend [64]. In the downstream area of barrages, most of the impacts are negative, while above the dam the effects are not much different [65]. Studies on loss of fish stocks by construction of physical barriers are also documented in past [65-67].

The goal of this study was to detect hybrids and their parental species among Indian major Carps (*L. rohita*, *C. mrigala* and *G. catla*) based on increased pace of data gathering and analysis from digital image of fish form. The results of the study showed potential of software tools in separating the parental species of Indian major carps from their hybrids using only a few body shape features, without application of DNA analysis. Therefore, this study provides a powerful tool in diagnosis of species and increasing the pace of taxonomic research.

290 The present study also recognizes the probable causes of natural hybridization among
291 the Indian major carps. Habitat alteration by barrages commissioned along the distribution
292 range of these species restricts the movement of declined fish population, thereby increasing
293 competition with species in close proximity for spawning habitat with overlapping
294 reproductive activities. Ranching of Indian major carps at various sites including upstream of
295 all the barrages along the Ganga River may resolve the existing problem of natural
296 hybridization.

297

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300

301 **References**

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Figure

Figure

Figure

L. rohita

C. mrigala-L. rohita

C. mrigala

C. mrigala-G. catla

C. mrigala-Hybrid

G. catla-L. rohita

Hybrid-*L. rohita*

G. catla

G. catla-Hybrid

Hybrid

Figure

Figure

Figure