Taxonomic validation of five fish species of subfamily Barbinae from the Ganga river system of northern India using traditional and truss analyses

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Abstract

Morphometric differences were investigated among five fish species of subfamily Barbinae from the Ganga river system through traditional morphometrics and the truss network system. Species taken into account were Puntius chola (Hamilton 1822), Puntius sophore (Hamilton 1822), Pethia ticto (Hamilton 1822), Pethia conchonius (Hamilton 1822) and Systomus sarana (Hamilton 1822). Although, taxonomists carefully examine external body features to discriminate these species, there is still a risk of misidentification during a visual assessment. In the present study, the traditional morphological analysis included 22 morphometric measurements and 10 meristic counts. Truss network system of 14 landmarks was interconnected to yield 91 distance variables. The principal component analysis (PCA), discriminant function analysis (DFA) and cluster analysis (CA) were employed in order to determine morphometric variations. In traditional analysis, 29 characters out of 32 were found significant ($p < 0.05$). Eight principal components were extracted through PCA explaining 85.30% of the total variance in samples, DFA correctly classified 100.0% of original grouped cases and 100.0% of cross-validated grouped cases. Truss analysis showed that all the 90 characters were significant ($p < 0.05$). PCA extracted four principal components explaining 96.45% of the total variance. DFA correctly classified 96.1% of original grouped cases and 92.1% of cross-validated grouped cases. The results acquired from the traditional as well as truss analyses indicate significant morphometric heterogeneity. However, variations are not the same for the two different methods (traditional and truss) employed for the analyses. Shape differences among species were evident from relative warps (RW) supporting truss network analysis. Geometric morphometric methods (GMM), but limited use of Procrustes methods revealed even very small dissimilarity between groups. In spite of determining the morphometric differentiation among species, the present study also provides a useful insight on the application and complementary role of truss analysis with traditional morphometric analysis in the correct classification of the selected species.
Introduction

Barbinae is a taxonomic subfamily, within the family Cyprinidae, that belongs to order Cypriniformes. The Barbin fishes of genus *Puntius* are native to South Asia, Mainland Southeast Asia, and Taiwan [1]. *Puntius* has been familiar as a “catch-all” genus and encompasses more than 60 species found in India and new species are continuously being discovered [2–5]. The *Puntius* is an economically important ornamental, as well as a food fish locally sold fresh in markets. It is highly valued in recreational fisheries and constitutes a major component of the tropical fish trade. *Puntius sarana* (now allocated to *Systomus*) also plays a significant economic role in aquaculture [6–8]. In spite of its economic significance, comprehensive knowledge on systematic/taxonomy is still incomplete for this genus. Further, generic placement/status of particular species of *Puntius* genus remains questionable.

Many closely related *Puntius* species exhibit taxonomic indistinctness [9–10]. Therefore, interrelationships of this genus are not well inferred [2, 11–14]. Since the beginning, genus *Puntius* has undergone many serious nomenclature and systematic changes. Morphological characters have long been used to study the diversity and taxonomy of these cyprinids [15–16]. Unfortunately, taxonomic ambiguity in *Puntius* is yet to be resolved though several studies on systematic relationships have been done within the subfamily Barbinae [17–21]. Based on morphological characteristics, Rainboth [22, 23] classified *Puntius* into another three genera, *Systomus*, *Barbodes*, and *Hypsibarbus*. However, in contrast to Rainboth [22, 23], Champasri et al [24, 25] and Rajasekaran and Sivakumar [26] stated that *Puntius* should not be split into three genera as Rainboth [22, 23] failed to provide distinct special characters to differentiate three new genera within the *Puntius* genus. The division of *Puntius* into three genera by Rainboth [22, 23] has remained as a controversial issue among fish taxonomists. Later, on the basis of osteological studies, Shantakumar and Vishwanath [27] have documented new separate groups within the genus *Puntius*. Furthermore, Pethiyagoda et al [28] reported five well-supported clades as distinct genera within South Asian *Puntius* namely *Pethia*, *Dawkinsia*, *Dravidia*, *Systomus*, and *Puntius*. Their study was based on two mitochondrial DNA gene fragments, external-morphological, and osteological characters. On the basis of their study Pethiyagoda et al [28] split the *Puntius* genus and allocated *Puntius sarana* to *Systomus*, *Puntius conchonious* and *Puntius ticto* to *Pethia*. Many other species (*P. barbodes*, *P. desmopuntius*, *P. haludaria*, *P. oliotius*, *P. puntigrus* and *P. sahyadria*) which were primarily placed within the *Puntius* genus have also been shifted to other genera [29–31].

Afterwards, Saroniya et al [32] studied the meristic characters of some of *Puntius* species of central India and revealed deviations from the earlier studies of different workers. Saroniya et al [33] on the basis of 18S rDNA sequences, disagree over the polyphyletic origin of genus *Puntius* and revealed closeness among *Puntius chola*, *Puntius sophore*, *Puntius ticto* and *Puntius conchonious*. Furthermore, as per IUCN Red List 2017 ver. 3.1, the generic status of *Puntius sarana* is still uncertain and continually shifting between *Barbodes* and *Puntius*. Although FishBase considered *Pethia ticto*, *Pethia conchonious*, *Systomus sarana* ‘valid’, still investigators are continuing with previously valid names [34–36].

Taxonomic tribulations within the *Puntius* group have been confirmed by many previously reported studies [37]. Morphometric characters have been efficiently employed for taxonomic related problems [38, 39]. The traditional morphometric method involves direct quantification of a range of morphological characters that are subsequently analyzed via multivariate methods [40–41]. This method is remarkably useful in identification of closely related species and provides preliminary insights on fish taxonomy [42]. Meristic characters were also found to be valid in the race and species identification for adequate aqua-management and fisheries statistics [43–45]. The traditional morphometric methods are coupled with some limitations in
describing the fish shape, therefore this has been criticized [46, 47]. Consequently, a strong landmark-supported tool based on statistical analysis called ‘truss network technique’ is used for distinguishing species and this technique was extensively used by many workers in discrimination of species [48–53]. Geometric morphometrics analysis of landmarks from digital images is highly effective in capturing information about the shape of an organism [54, 55].

In this context, the present study aims to examine the morphometric variations among fishes of subfamily Barbinae available in the Ganga river system of northern India. The examined species are *Puntius chola* (Hamilton 1822) known as ‘swamp barb’, *Puntius sophore* (Hamilton 1822), known as ‘pool barb’, *Systomus sarana* (Hamilton 1822) known as ‘olive barb’, *Pethia ticto* (Hamilton 1822) known as ‘two spot barb’ and *Pethia conchonius* (Hamilton 1822), known as ‘rosy barb’. The study is based on traditional and truss network analyses with the objective to employ useful insights into morphometric characters responsible for morphometric and shape variations among the five selected fish species.

### Materials and methods

#### Sample collection

Ninety fish samples of five species were collected from different fishing sites of Ganga river system in northern India (Fig 1), with the assistance of local fishermen during August, 2016 to December, 2016. Information about the number of samples collected, sampling sites, and geographical coordinates of locations for Barbinae species studied in the present study is provided in Table 1. Each fish specimen was immediately preserved in a 10% formalin solution and subsequently subjected for identification according to Talwar and Jhingran [56], Jayaram [57], Pethiyagoda and Kottelat [58]. Representative photographs of each species are showed in Fig 2.

#### Traditional morphometrics

**Data collection.** In the laboratory, a total of 23 morphometric measurements (S1 Table) were recorded for each specimen (Fig 3). Measurements were made with vernier caliper closest to 0.1 mm. Counts and measurements were taken as far as possible on the left side of fish specimens following standard methods for cyprinid taxonomy [58] with some modifications.

**Meristic characteristics.** Ten meristic characteristics were counted. A comparison of meristic characters of five species is showed in Table 2. Meristic characters were counted twice by the same observer. Radiographs were taken using digital X-ray machine. The total number of vertebrae was counted from the radiographic images. The initial four fused vertebrae (weberian apparatus) were not incorporated in the vertebral counts.

#### Landmark-based morphometrics

**Data collection.** Sampled specimens were placed on a flat surface on a plastic-coated graph paper, which was used for standardizing the coordinates of the digital images. Each fish sample was given a specific code for identification. A digital camera (Canon IXUS 145) was used to capture the digital images. All fish specimens were positioned laterally on their right side, with their body posture and fins teased into a natural position (Fig 4). Images of fish specimens were captured and were transferred to the computer for further analysis.

#### Traditional morphometric analysis

Each of fourteen morphometric characters was divided by standard length (SL) and remaining 8 characters were divided by head length (HL) to eliminate the size effect (correlation < 0.5 for
all variables). All the morphometric values were log-transformed prior to analysis using computer software PAST 1.47 (PAleontological Statistics) [59]. Multivariate statistical techniques, ANOVA, principal component analysis (PCA), discriminate function analysis (DFA) and cluster analysis (CA) were performed on log$_{10}$-transformed measurements. PCA is an effective method for morphometric data reduction and extracting independent variables. DFA is a predictive model for group membership. The source for the discrimination among samples was based on the percentage of correctly and incorrectly classified fish. Eigenvalues, a percentage of variance, cumulative percentage and canonical correlation were acquired using correlation matrix from PCA. Statistical analyses were performed with the computer software programs SPSS 16.0 and PAST 1.47. A hierarchical cluster analysis based on UPGMA (Unweighted Pair Group Method with Arithmetic mean) was carried out on Mahalanobis distances. Software STATISTICA was used to test the significance of Mahalanobis distance between species.

**Truss-based morphometric analysis**

The extraction of the truss distances from the digital images of specimens was done using a combination of the software platforms, tpsUtil, tpsDig 2 v2.1 and PAST [59–61]. Software
tpsUtil converts JPEG image into tps format. For covering entire shape of fish specimen, two-dimensional Cartesian coordinates of 14 landmarks were recorded on the lateral view of each specimen (Fig 5). The locations of the landmarks were selected according to the following two criteria: reliability in terms of correspondence between specimens, and the ability to best describe the geometry of the form under study. All the landmarks were digitized and truss networks were constructed by interconnecting the landmarks using software tpsDig. Using the computerized Pythagorean theorem in software PAST, X–Y coordinate data was transformed into linear distances for subsequent analysis. Altogether, 91 morphometric characters were attained connecting these landmarks [60]. The truss data generated by PAST were log-transformed to conserve allometries and to standardize variances [62].

To eliminate size effect data were M-transformed by employing formula given below [63].

$$M - \text{trans} = \log M - b (\log \text{SL} - \log \text{SL mean})$$

Table 1. Sampling sites, their locations with latitude and longitude, sample size and size range of five species of Barbinae used in the present study.

| Species                  | Sample size | River | Sampling sites                     | Latitude and Longitude | Standard Length (SL) range (cm) | Mean SL (cm) ±SD (CV) | Mean Total Weight (TW) (g) ±SD |
|--------------------------|-------------|-------|------------------------------------|------------------------|---------------------------------|-----------------------|-------------------------------|
| *Systomus sarana*        | 24          | Ganga | Ganga barrage, Kanpur              | 26.50°N, 80.31°E       | 10.4–23.7                      | 16.82 ±3.04 (18.10)   | 21.2 ±3.21                    |
|                          |             | Gomti | Pakka pul, Lucknow                 | 26.87°N, 80.91°E       |                                 |                       |                               |
|                          |             | Betwa | Sahurapur daria, Hamirpur          | 25.89°N, 80.04°E       |                                 |                       |                               |
|                          |             | Yamuna| Mutthi ganj, Allahabad             | 25.42°N, 81.84°E       |                                 |                       |                               |
| *Pethia ticto*           | 20          | Ganga | Ganga barrage, Kanpur              | 26.50°N, 80.31°E       | 4.8–6.0                         | 5.45 ±0.32 (5.91)     | 4.56 ±1.78                    |
|                          |             | Gomti | Gomti barrage, Lucknow             | 26.85°N, 80.96°E       |                                 |                       |                               |
|                          |             | Sai   | Takia kalan, Raebareli             | 26.23°N, 81.21°E       |                                 |                       |                               |
|                          |             | Betwa | Sahurapur daria, Hamirpur          | 25.89°N, 80.04°E       |                                 |                       |                               |
| *Pethia conchonius*      | 08          | Yamuna| Mutthi ganj, Allahabad             | 25.42°N, 81.84°E       | 5.0–6.2                         | 5.75 ±0.45 (7.83)     | 5.54 ±2.89                    |
|                          |             | Ganga | Dadri Ghat, Gazipur                | 25.57°N, 83.57°E       |                                 |                       |                               |
|                          |             | Gomti | Pipe wala pul, Lucknow             | 26.87°N, 80.91°E       |                                 |                       |                               |
| *Puntius sophore*        | 06          | Gomti | Gomti barrage, Lucknow             | 26.85°N, 80.96°E       | 6.0–7.8                         | 7.13 ±0.76 (10.63)    | 11.5 ±3.02                    |
|                          |             | Ganga | Ganga barrage, Kanpur              | 26.50°N, 80.31°E       |                                 |                       |                               |
|                          |             | Betwa | Sahurapur daria, Hamirpur          | 25.89°N, 80.04°E       |                                 |                       |                               |
| *Puntius chola*          | 12          | Ganga | Dadri Ghat, Gazipur                | 25.57°N, 83.57°E       | 7.6–8.6                         | 8.18 ±0.32 (3.96)     | 17.2 ±3.21                    |
|                          |             | Gomti | Saeed smarak, Lucknow              | 26.86°N, 80.92°E       |                                 |                       |                               |
|                          |             | Betwa | Sahurapur daria, Hamirpur          | 25.89°N, 80.04°E       |                                 |                       |                               |
|                          |             | Yamuna| Mutthi ganj, Allahabad             | 25.42°N, 81.84°E       |                                 |                       |                               |

https://doi.org/10.1371/journal.pone.0206031.t001
Where, $M_{\text{trans}}$ is the transformed measurement, $M$ is the original measurement, $b$ is the within-group slope regression of the log $M$ versus log $SL$, $SL$ is the standard length of the fish and $SL_{\text{mean}}$ is the overall mean of the standard length (correlation $< 0.5$ for all variables).

From the final analysis, Standard length ($SL$) was excluded, since $SL$ was used as a basis for transformation [64, 65]. All statistical analyses were performed for combined sexes as there were no significant differences of tested variables between the sexes ($p > 0.05$). One-way analysis of variance (ANOVA) was performed for each character between the species and significant variables were retained [66, 67]. Tukey’s-b significance difference test was executed as a post-hoc multiple-comparisons test. Subsequently, significant variables were subjected to PCA, DFA and CA. The holdout leave-one-out cross-validation procedures proposed by Lachnerbruch [68], were also carried out to calculate misclassification rate of DFA. Average shape of all specimens of each species were computed and aligned using tpsRelw software to perform an analysis of relative warps (RW), i.e., a principal components analysis of shape variation relative to spatial scale [69–71]. Each spline is a visualization of the group mean relative to the grand mean. Statistical analyses were performed with the computer software programs SPSS.
A hierarchical cluster analysis based on UPGMA was carried out on Mahalanobis distances. Software STATISTICA was used to test the significance of Mahalanobis distance between species.

**Ethics-statement**

Fish samples were obtained from the wild, directly from the commercial catches. Samples of all fish species were procured from local fish markets after commercial consignment with the fish vendor. Sites from where fishes were collected fell outside Protected Areas (PAs) and therefore no permits were required from the State Forest and Wildlife Department. Fish were captured by gill nets. For morphometric and meristic study, fish, if alive were euthanized with MS222 (Sigma) to ameliorate suffering and transported to the laboratory on ice to avoid

**Fig 3.** Boxplots of selected species based on morphometric characters: PCH (*P. chola*), PCO (*P. conchonius*), PSO (*P. sophore*), PTI (*P. ticto*), SSR (*S. sarana*) {SL: Standard length; MBD: Maximum body depth; MBW: Maximum body width; PDL: Pre-dorsal length; PVL: Pre-ventral length; PAL: Pre-anal length; PANSL: Pre-anus length; LCPD: Length of caudal peduncle; DCPD: Depth of caudal peduncle; LDF: Length of dorsal fin; LPF: Length of pectoral fin; LVF: Length of ventral fin; LAF: Length of anal fin; LPA: Length of pelvic axial; HL: Head length; ED: Eye diameter; PROL: Pre-orbital length; POOL: Post-orbital length; IOD: Inter-orbital distance; IND: Inter-narial distance; MHW: Maximum head width; HDE: Head depth at eye; HDN: Head depth at nape}. Fourteen characters were divided by SL, eight by HL, SL not shown.

https://doi.org/10.1371/journal.pone.0206031.g003

**Table 2.** Meristic characters of five selected species of Barbinae with code, mean±SD and their significance level.

| S. No. | Meristic character               | Code | *S. sarana* | *P. sophore* | *P. chola* | *P. ticto* | *P. conchonius* | P-values |
|--------|----------------------------------|------|-------------|-------------|------------|------------|----------------|----------|
| 1      | Scales in Lateral Series        | SLL  | 31.708±1.083| 24.000±0.894| 26.417±1.379| 24.200±0.894| 25.125±0.991   | 0.000*   |
| 2      | Scales in Transverse Series     | STR  | 10.208±0.388| 8.000±0.000 | 9.167±0.389| 8.700±0.571 | 9.250±0.463    | 0.000*   |
| 3      | Pre-dorsal Scales               | PDS  | 10.958±0.204| 8.667±0.516 | 9.083±0.793| 10.500±0.513| 8.875±0.641    | 0.000*   |
| 4      | Caudal Circumferential Scales   | CCS  | 14.971±1.018| 12.000±0.632| 12.167±0.835| 11.150±0.366| 11.875±0.354   | 0.000*   |
| 5      | Dorsal Fin Rays                 | DFR  | 12.208±0.451| 9.000±0.000 | 10.000±0.000| 9.000±0.000  | 11.000±0.000   | 0.000*   |
| 6      | Pectoral Fin Rays               | PFR  | 14.250±0.532| 15.000±0.000| 14.833±0.389| 11.700±0.470| 12.625±0.744   | 0.000*   |
| 7      | Pelvic Fin Rays                 | PFR  | 9.000±0.000 | 8.000±0.000 | 7.333±0.492 | 9.00±0.000   | 7.750±0.463    | 0.000*   |
| 8      | Anal Fin Rays                   | AFN  | 9.125±0.448 | 9.000±0.000 | 9.000±0.000 | 6.000±0.000  | 9.000±0.000    | 0.040*   |
| 9      | Caudal Fin Rays                 | CFR  | 19.208±0.415| 19.000±0.000| 19.000±0.000| 19.000±0.000| 19.000±0.000   | 0.032*   |
| 10     | Vertebral column count          | VCC  | 32.333±0.637| 26.000±0.000| 27.583±0.793| 25.450±0.887 | 25.000±0.000   | 0.000*   |

*Significant at p<0.05
(P-values were calculated using SPSS)

https://doi.org/10.1371/journal.pone.0206031.t002

16.0 and PAST 1.47. A hierarchical cluster analysis based on UPGMA was carried out on Mahalanobis distances. Software STATISTICA was used to test the significance of Mahalanobis distance between species.
damage to its morphological characters that are crucial for taxonomic investigations. The Ethical committee of Lucknow University, Lucknow India has approved the design and implementation of the study.

Results

Traditional morphometric analysis

In one way ANOVA, 29 characters were significant out of 32 characters ($p<0.05$). These were then subjected to principle component analysis (PCA). PCA extracted 8 principal components at Jolliffe’s rule with eigenvalues of at least 0.7 responsible for 85.30% variations in which PC1 accounts for maximum variation in the samples which is 39.78%, and PC2 contributes 18.65%. Significant variables were subjected to DFA and a resultant 100.0% of original grouped cases correctly classified. Also, 100.0% of cross-validated grouped cases were correctly categorized (Table 3). The DFA plot is shown in Fig 6.
DFA extracted 8 discriminant variables (dorsal and anal fin length, head depth at the eye, number of pre-dorsal scales, number of caudal circumferential scales, vertebrae counts, dorsal and pectoral fin rays, S2 Table). Combination of these characters is responsible for variations among species. Mahalanobis distances from traditional morphometric data suggested that the five species are at significant distance from each other (Table 4).

On the basis of morphometric and meristic data, a dendrogram of the species was derived by the unweighted pair group (UPGMA) cluster analysis. The UPGMA cluster analysis based on the Mahalanobis distance between group centroids showed that the five species produced two major clusters. *P. chola*, *P. sophore* and *P. conchonius* belong to one cluster and *P. ticto* belongs to sub-branch of the same cluster while *S. sarana* is most distinctly placed (Fig 7).

**Landmark-based analysis**

In one way ANOVA, all the 90 characters were found significant ($p<0.05$). Tukey’s-b post hoc test revealed that 13 characters grouped 5 supposed species into 5 groups. Although all the 90
characters grouped five species into more than single homogenous subsets therefore all the characters were retained for further analysis. These characters were subjected to PCA, at Jolliiffe’s rule with eigenvalues of at least 0.7. In total, 4 principal components were extracted through PCA responsible for 96.45% variation. The first two components extracted, accounts for a total variance of 94.26%, in which the first principal component (PC1) accounts for 92.38% while second PC2 contributes 1.89%.

In discriminant function analysis (DFA), 96.1% of original grouped cases were correctly classified and 92.1% of cross-validated grouped cases correctly classified (Table 5). Based on

![Fig 6. Discriminant function plot from traditional morphological variables. (Group Centroids: 1: P. chola; 2: P. conchonious; 3: S. sarana; 4: P. sophore; 5: P. ticto).](https://doi.org/10.1371/journal.pone.0206031.g006)

Table 3. Discriminant function analysis on traditional characters among five fish species (100.0% of original grouped cases correctly classified and 100.0% of cross-validated grouped cases correctly classified).

| Original percentage (%) | Species | P. chola | P. conchonious | S. sarana | P. sophore | P. ticto | Total |
|-------------------------|---------|----------|----------------|-----------|------------|----------|-------|
|                         | P. chola | 100.0    | .0             | .0        | .0         | .0       | 100.0 |
|                         | P. conchonious | .0     | 100.0          | .0        | .0         | .0       | 100.0 |
|                         | S. sarana | .0       | .0             | 100.0     | .0         | .0       | 100.0 |
|                         | P. sophore | .0      | .0             | .0        | 100.0      | .0       | 100.0 |
|                         | P. ticto  | .0       | .0             | .0        | .0         | 100.0    | 100.0 |

| Cross validated percentage (%) | Species | P. chola | P. conchonious | S. sarana | P. sophore | P. ticto | Total |
|---------------------------------|---------|----------|----------------|-----------|------------|----------|-------|
|                                 | P. chola | 100.0    | .0             | .0        | .0         | .0       | 100.0 |
|                                 | P. conchonious | .0     | 100.0          | .0        | .0         | .0       | 100.0 |
|                                 | S. sarana | .0       | .0             | 100.0     | .0         | .0       | 100.0 |
|                                 | P. sophore | .0      | .0             | .0        | 100.0      | .0       | 100.0 |
|                                 | P. ticto  | .0       | .0             | .0        | .0         | 100.0    | 100.0 |

https://doi.org/10.1371/journal.pone.0206031.t003
the discriminant function analysis, combined group plots of the five groups showing the differences among the groups and illustrated that there was little overlapping among the groups (Fig 8).

Eight discriminant variables were extracted (distance between origin of dorsal fin to end of dorsal fin and insertion of pelvic fin, distance between end of dorsal fin to origin of anal fin, distance between anterior attachment of ventral membrane from caudal fin to insertion of pectoral fin, head length, distance between origin of dorsal fin and anterior attachment of dorsal membrane from caudal fin, distance between origin of anal fin to posterior end of eye and distance between insertion of pelvic fin to insertion of pectoral fin), which were responsible for variation among species. Mahalanobis distances from truss morphometric data suggested that the five species are at a significant distance from each other (Table 6). Relative warps (RW) of each species' shape variation were easier to interpret through localization of fourteen landmarks on the entire body form on diagram grids (Fig 9). Apparently, shape variations are captured due to the relative positions of the following set of landmarks 3, 4, 5 and 8.

A dendrogram of the species based on the landmark-distances data was derived by the unweighted pair group (UPGMA) cluster analysis. The UPGMA cluster analysis based on the Mahalanobis distance between group centroids showed that the five species on the basis of similarity in body shape produced two major clusters.

S. sarana and P. sophore belong to the first cluster (cluster I) and P. chola placed as sub-branch while in another branch P. conchonius and P. ticto grouped together (cluster II) (Fig 10).

### Discussion

The morphometric variations based on traditional (body measurements and meristic characters) and truss network analysis of five species of Barbinae from Ganga river system of

|          | P. chola | P. conchonius | S. sarana | P. sophore | P. ticto |
|----------|---------|---------------|-----------|------------|---------|
| P. chola | 185.2358| 576.396       | 119.0583  | 688.970    |         |
| P. conchonius | 1.63593E-06* | 850.503 | 278.1810  | 468.848    |         |
| S. sarana | 2.13803E-21* | 1.39695E-18* | 879.3172  | 1315.182   |         |
| P. sophore | 0.00563457* | 0.371188 | 1.1027E-17* | 745.952    |         |
| P. ticto | 1.03384E-15* | 2.50182E-07* | 2.86447E-33* | 2.40004E-09* | |

(*p < 0.001)

https://doi.org/10.1371/journal.pone.0206031.t004

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Fig 7. Dendrogram derived from UPGMA cluster analysis based on the Mahalanobis distance between the species centroids from traditional morphological variables. (1: P. chola; 2: P. conchonius; 3: S. sarana; 4: P. sophore 5: P. ticto).

https://doi.org/10.1371/journal.pone.0206031.g007
northern India using multivariate analysis (PCA, DFA, RW and CA) are found to be valid for
discrimination among five species with varying in the degree of differentiation. The dissimilar-
ity of the confirmation for divergence among species probably reflects our use of a different set
of characters/landmarks in the traditional and truss methods, which incorporated a different
set of measurements. Furthermore, all the analyses applied in the present study, have sufficient
statistical capacity to discriminate among P. sophore, P. chola, P. ticto, P. conchonious and S. sar-
ana. It is encouraging and suggests that previous morphometric studies using traditional mea-
sures can be reliable.

Table 5. Discriminant function analysis on truss characters among five fish species (96.1% of original grouped cases correctly classified and 92.1% of cross-validated grouped cases correctly classified).

| Species      | P. chola | P. conchonious | S. sarana | P. sophore | P. ticto | Total |
|--------------|----------|----------------|-----------|------------|----------|-------|
| P. chola     | 100.0    | 0.0            | 0.0       | 0.0        | 0.0      | 100.0 |
| P. conchonious| 0.0      | 90.9           | 0.0       | 0.0        | 9.1      | 100.0 |
| S. sarana    | 5.6      | 0.0            | 94.4      | 0.0        | 0.0      | 100.0 |
| P. sophore   | 0.0      | 0.0            | 5.0       | 95.0       | 0.0      | 100.0 |
| P. ticto     | 0.0      | 0.0            | 0.0       | 0.0        | 100.0    | 100.0 |

Cross validated percentage (%)

| Species      | P. chola | P. conchonious | S. sarana | P. sophore | P. ticto | Total |
|--------------|----------|----------------|-----------|------------|----------|-------|
| P. chola     | 100.0    | 0.0            | 0.0       | 0.0        | 0.0      | 100.0 |
| P. conchonious| 9.1      | 81.8           | 0.0       | 0.0        | 9.1      | 100.0 |
| S. sarana    | 11.1     | 0.0            | 88.9      | 0.0        | 0.0      | 100.0 |
| P. sophore   | 0.0      | 5.0            | 5.0       | 90.0       | 0.0      | 100.0 |
| P. ticto     | 0.0      | 0.0            | 0.0       | 0.0        | 100.0    | 100.0 |

Fig 8. Discriminant function plot from truss morphometric variables. (Group Centroids: 1: P. chola; 2: P. conchonious; 3: S. sarana; 4: P. sophore 5: P. ticto).

https://doi.org/10.1371/journal.pone.0206031.g008
Table 6. Pair wise matrix of Mahalanobis distances between the centroids of the clusters (above diagonal) and \( p \) value (below diagonal) of discriminant truss morphometric characters among five fish species.

|          | \( P. \) cholal | \( P. \) conchonius | \( S. \) sarana | \( P. \) sophore | \( P. \) ticto |
|----------|-----------------|---------------------|-----------------|-----------------|--------------|
| \( P. \) cholal | 3727.456        | 2135.690            | 3030.644        | 3332.055        |              |
| \( P. \) conchonius | 0.00097405\(^*\) | 6499.911            | 7519.836        | 817.258         |              |
| \( S. \) sarana | 0.00411839\(^*\) | 8.45015E-09\(^*\)   | 750.174         | 4348.666        |              |
| \( P. \) sophore | 4.14166E-05\(^*\) | 4.70306E-05\(^*\)   | 1.65E-07\(^*\) | 5038.670        |              |
| \( P. \) ticto | 5.74079E-08\(^*\) | 0.000295858\(^*\)   | 2.4811E-15\(^*\) | 4.20184E-14\(^*\) |              |

\( (^* p < 0.001) \)

Fig 9. Landmark based geometric morphometric analysis showing the variation of the body shapes among species.

https://doi.org/10.1371/journal.pone.0206031.g009
In the present study, the most significant measures taken into account for discrimination through traditional analysis were the length of the dorsal fin, length of the anal fin, head depth at the eye, number of pre-dorsal scales, number of caudal circumferential scales, dorsal fin rays, pectoral fin rays, and vertebrae count. Discriminating characters extracted through truss analysis were related to anterior, posterior, dorsal, lateral and ventral distances. Geometric morphometry based relative warps of average shape also provide evidence on the distinctness of species.

Meristic characters like pre-dorsal scales, caudal circumferential scales, vertebrae count, number of dorsal and pectoral fin rays were found to be helpful in discriminating these species. De Silva and Liyanage [72] opined on the basis of their study that meristic characters are more effective than morphometric characters for discriminating the *Puntius* genus. Kotalawala and Jinadasa [73] also reported that meristic characters (counts of lateral line scales, gill rakers, pectoral rays, and vertebrae) were helpful for differentiating species of *Puntius*. Other remaining meristic characters showed a moderate degree of overlap among selected species. Caudal fin rays were found to be a common and non-variable character in all the species. Dorsal fin rays counts are in agreement with Saroniya et al [32] though incongruent with the findings of Day [74], Srivastava [75], Hamilton [76], Datta Munshi and Srivastava [77], Talwar and Jhingran [56]. No changes were reported in meristic counts with the increase in fish body length. The similar observation was reported by Rajasekaran and Sivakumar [26], Saroniya et al [32], Vladykov [78], Talwar and Jhingran [79] and Muhammad Zafar et al [80]. Variation in vertebrae counts among the *Puntius* species were found to be one of the discriminating variables though, less precisely contributing in the differentiation of species, as this character was found overlapping among the species *P. ticto* (vertebrae count 25–28), *P. sophore* (26), *P. chola* (27–29), *P. conchonius* (25), but not *S. sarana* (32–34). Shantakumar and Vishwanath [27] reported similar trends in vertebrae counts of *Puntius* species in consideration. Though, Weitzman and Cobb [81], Jenkins and Lachner [82] opined that vertebrae counts could be employed in the discrimination of genera and species. A single pair maxillary barbels present in *P. chola*, a pair of maxillary and a pair of rostral barbels are present in *S. sarana* while other species lacking barbels. Notably, the presence of barbels in every individual of a species increases the systemic importance of this characteristic [82], usage of barbels at the generic level has been corroborated [83].

The results of the traditional morphometric study revealed that there were significant variations in the morphometric characters and also significant differences were detected in the meristic counts among selected species. These results confirmed that the differences among the species reflected the varied quantity of differences as depicted by truss analysis. Similar
findings were observed in *Labeo* genus by Lal et al [53]. DFA highlighted that investigated species can be precisely differentiated, distinctly clustered with only a partial overlap among them with applied truss analysis, but interestingly in traditional analysis, no overlap was visible.

To show hierarchical similarity clusters were built from traditional and truss morphometric data, resultant cluster topologies were not similar. Cluster based on traditional analysis shows that *P. chola*, *P. sophore*, *P. conchonius* and *P. ticto* belongs to one major group and *S. sarana* in another group. Traditional-based results are in partial congruence with that of earlier reports based on morphometric characteristics and meristic counts [see 79, 84, 85, 86]. In the present study, cluster drawn through the truss analysis showing a close relationship between *P. chola* and *P. sophore* and also between *P. conchonius* and *P. ticto*. Truss-based results were broadly congruent with previously proposed hypotheses of species relationships based on molecular phylogenetic studies [10, 28, 87] By contrast, sequences amplified through CO1 showed *P. ticto* highly resembled to *P. sarana* [88].

Utilizing standard methods, morphometric trees can simply be compared to molecular phylogenies trees to come up with a conclusion. Not to mention there is disagreement to the use of morphometric data in systematic contexts [89], although both morphometric and phylogeny contribute to a common fascination in the examination of morphological variation [89, 90]. Separation of species mostly reveals evolutionary relatedness if variables from different morphometric characters are utilized in a particular analysis [91]. Morphological differences are supposed to be characterized by gaps among taxonomic group, therefore morphological data are significant in biological systematics. These gaps may occur as a result of a number of evolutionary processes [89, 92]. On the whole, morphometric data consist of more than one species with different morphometric characters probably have a phylogenetic element responsible for variation in shape.

Although, molecular genetics techniques have been frequently employed to identify the distinctness among species, the feasibility and importance of classical techniques cannot be denied. Further, morphometric methods have been found to be powerful and feasible for investigating taxonomic problems with advances in improved data collection, a better description of shape, and the potential of new analytical techniques. In different biological contexts, the geometric method, provides shape-related additional information present in the relative locations of landmarks [93]. Geometric morphometrics (GM) is used as a potent tool to analyze body form. Most of the software used in GM is freely available, user-friendly and offers an integrated approach that explains its utility as a better resource, such that Geometric-based RW method acquires enhanced discriminating power [94–97]. Subsequently, in the present study an effort was made to incorporate this. RW visualized differences in the body form are highly effective for discriminating selected *Puntius* species. Apparently, these differences are attributed mainly to the curvature of the body. However, results acquired were initial and analytic but able to supplement present study.

Fish show more noteworthy variation in morphometric attributes both intraspecific and between species when compared to other vertebrates and are more disposed to ecological changes [98, 99]. Mallet [100] describes species as identifiable ‘morphological and genotypic clusters’. Morphometric characters are developed from the combination of genotypic and environmental factors, and they are governed by natural selection [101]. Therefore to authenticate the morphometric differences and for an enhanced perceptive about these examined species, genetic-level studies can be performed.

In the present study, both analyses independently discriminated selected species into their groups. This indicated that the traditional system, as well as a truss, could be effectively used for morphological differentiation of these species. Geometric-morphometric-based relative warps provide additional information about the change in body form of these species. To
summarize, findings of the present study suggest that truss technique is helpful in solving taxonomic ambiguity through quantifying shape variation. Questionable genera, here *Puntius*, could be differentiated, even with low sample sizes, provided they are used in combination with traditional morphological analysis. Thus, the present study offers the useful insight on the application and complementary role of truss analysis with traditional morphometrics.

**Supporting information**

S1 Table. Description of traditional morphometric measurements taken on the body of five selected species of *Puntius*.

(DOCX)

S2 Table. Standardized canonical discriminant function coefficients. Showing eight discriminative variables generated through traditional analysis.

(DOCX)

S1 File. Raw dataset of the traditional morphometric analyses.

(XLSX)

S2 File. Raw dataset of the truss morphometric analyses.

(XLSX)

**Acknowledgments**

The authors are grateful to the Head, Department of Zoology, University of Lucknow and authors would also like to thanks to the fishermen for their cooperation.

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