Materials and Methods
3. MATERIALS AND METHODS

3.1. Study area
The river Ganga originates in the Garhwal Himalayas (30° 55' N; 70° 7' E) at an elevation of 4100 m above mean sea level from Gaumukh glacier in the western Himalayas in the Uttarakhand state of India. It flows about 2525 km before falling into the sea (Kamyotra, 2009) and finally drains into the Sundarban delta in the Bay of Bengal. Yamuna River originates from the Yamunotri glacier (Saptrishi Kund) near Bander punch peaks (380°59' N, 780°27' E) at an elevation of circa 6320 m above mean sea level in the Mussoorie range of the lower Himalayas in Uttarkashi district of Uttarakhand, India and traverses some 1336 km through five states before finally merging into the river Ganga in Allahabad, Uttar Pradesh, India (Sengupta, 2006).

The Gomti river is the tributary of the river Ganga. The river Gomti, originates from a natural lake (28° 34' N; 80° 07' E) Gomat Taal near Madho Tanda, Pilibhit town in Uttar Pradesh, India about 50 km south of the Himalayan foothills (Sarkar et al., 2010). It travels about 750 Km to finally merge into the river Ganga near Saidpur Kaithi in Ghazipur district bordering Varanasi district in Uttar Pradesh, India (Sarkar et al., 2010).

3.2. Sampling
Fish samples were collected from Narora (27° 30' N; 78° 25' E) and Kanpur sites (26° 28' N; 80° 24' E) on the river Ganga, Firozabad site (27° 09' N; 78° 24' E) on the river Yamuna and Lucknow site (26° 55' N; 80° 59' E) on the river Gomti (Figure 1). Morphological identification of the fish was based on the descriptions of Taiwar and Jhingaran (1991); Jayaram (1999) and Froese and Pauly (2008). The collected fish samples were comprised of *Heteropneustes fossilis*, *Channa punctata*, *Clarias batrachus*, *Wallago attu* and *Sperata seenghala*. The details of fish samples collected are given in Table 1-5.

3.3. Truss morphometric measurements
The truss network was constructed to form a network on fish body for morphometrics as described by Strauss and Bookstein (1982). Truss analysis quantifies the multidimensional shape of the fish. In this technique, the whole body of fish is
covered by distances between pairs of landmarks across the body, thus forming a sequential series of connected polygons called truss box. It systematically detects shape differences in oblique as well as horizontal and vertical directions (Strauss and Bookstein, 1982).

*Heteropneustes fossilis* and *Channa punctata*

Fish were placed on water resistant sheet and the measurements were taken by piercing the needle to paper corresponding to the anatomical landmarks. Eleven morphometric landmarks were chosen in *H. fossilis* which determined the 23 interlandmark distances as shown in Figure 2. Ten landmarks were chosen which determined the 21 interlandmark distances as shown in Figure 3. The measurements were taken immediately after the collection of fish samples. Additional data such as total length (TL), standard length (SL), head length (HL), post orbital length (POL), eye diameter (ED), body depth (BD), pectoral fin length (PFL) and ventral fin length (VFL) were also recorded.

*Clarias batrachus, Wallago attu and Sperata seenghala*

Fish were photographed after cleaning and placing each specimen separately on a flat platform with dorsal and anal fins held erect by pins. A digital camera (Sony, Japan) was used to capture the digital images. The images were saved in JPEG format for further analysis. The images provided a complete archive of body shape and allowed a repeat of the measurements when necessary (Cadrain and Friedland, 1999). Additional data such as total length (TL), standard length (SL), head length (HL), post orbital length (POL), eye diameter (ED), body depth (BD), pectoral fin length (PFL) and ventral fin length (VFL) were also recorded. A total of 23 distance measurements were taken in *C. batrachus* and *W. attu* using the 11 morphometric landmarks to construct the truss network on the fish body (Fig. 4 and 5). A total of 28 distance measurements were taken using the 13 morphometric landmarks to construct the truss network in *Sperata seenghala* (Fig. 6). Apart from TL, SL, BD, ED, POL, PFL, VFL and HL, head depth (HD) and Interorbital distance (ID) were also recorded for *Sperata seenghala*. The truss distances were extracted from the digital images of the specimens using tpsutil, tpsDig2
v2.1 (Rohlf, 2006) and Paleontological Statistics (PAST) (Hammer et al., 2001) softwares.

3.4. Removal of otoliths
The sagittal otoliths of *H. fossilis*, *C. punctata*, *C. batrachus*, *W. attu* and *S. seenghala* were removed from otic capsules by opening the otic bulla after morphometric measurements. The otoliths were collected in plastic vials, brought to the laboratory and stored in paper envelope. Otoliths were taken from the fishes of similar lengths to include the fishes of same cohort.

*H. fossilis*
For elemental analysis of otoliths, a sub-sample of twenty otoliths were taken from the fish collected from each of the sites Narora and Kanpur of the river Ganga, as well as Lucknow site of the river Gomti. However, a sub-sample of only fifteen otoliths were taken for analysis from the fish collected from Firozabad site of the river Yamuna.

*C. punctata*, *C. batrachus*, *W. attu* and *S. seenghala*
For each species, twenty five otoliths were taken from the fish collected from each of the sites Narora and Kanpur on the river Ganga, Firozabad site on the river Yamuna as well as Lucknow site on the river Gomti.

3.5. Sample preparation
Otoliths (sagittae) from each fish were cleaned of surface contaminants. All implements and glassware were cleaned with analytical grade 1% nitric acid (HNO₃) before decontamination. Left sagittal otoliths were taken for elemental analysis whereas right sagittal otoliths were used for age estimation of the fish. Otoliths were soaked in ultra-pure water to remove all the adhering biological residues. To dissolve the remaining biological residues, otoliths were soaked in 3% hydrogen peroxide for 5 minutes and immersed for 5 minutes in 1% HNO₃ to remove surface contamination. To remove the acid, otoliths were then flooded with ultra-pure water for 5 min. Finally, the otoliths were dried under a laminar flow hood and weighed to the nearest
0.1 mg. The decontaminated otoliths were stored dry in sealed, acid-washed polypropylene vials until analysis (Turan, 2006; Khan et al., 2012).

3.6. Elemental analysis of otoliths
The decontaminated otoliths of *H. fossilis*, *C. punctata*, *C. batarchus* and *S. seenghala* were dissolved in 10 ml of 37% HNO₃ and the volume was brought up to 25 ml with Milli Q water. Inductively coupled plasma-atomic emission spectrometry (ICP–AES) (Thermo Electron IRIS Intrepid II XSP DUO) was used to analyze the elements (Ca, Na, Mg, Sr, Ba, Mn, Fe, Cu, Pb, Ni, Zn and K) in the otoliths. However in *C. punctata*, the elements Mn, Fe, Ni and Zn were not analyzed. Blank samples were used to correct for background noise in readings and to calculate detection limits. Internal standards Ga and In were added in samples and blanks which were used to correct for the remaining matrix effect and to compensate instrument drift. Different elements were determined at different spectral lines. For external calibration, multi elemental standards were prepared with high purity ICP multi-element standard solution IV certiPUR (NIST SRM) obtained from Merck (Germany) using ultra pure Milli Q water and 2% w/v HNO₃ analytical grade. A calibration blank was also prepared in similar manner. The calibration curve was obtained for five points. The concentration of elements in the sample and blank were calculated and expressed as μg/g on dry weight basis. To minimize the possibility of contamination, all samples, standards and blanks, were prepared in a laminar flow hood (Turan, 2006; Khan et al., 2012).

*W. attu*
Decontaminated otoliths were dissolved in 1ml ultrapure HNO₃ and diluted with Milli-Q water to a total of 5 ml. Blank samples were prepared similarly, without dissolving otoliths. These samples were used to correct for background noise in readings and calculate detection limits for elements. All samples and blanks contained the internal standards Ga and In at 100 ppb/l. The internal standards were used to correct for the remaining matrix effect not corrected for by the standard calibration and instrumental drift. To minimize the possibility of contamination, all standards, blanks and samples were prepared in a laminar flow hood. Calibration of The ICP-MS
was done at the start of each sample processing session or when showing signs of significant instrumental drift using high purity ICP multi-element standard solution IV certiPUR (NIST SRM) obtained from Merck (Germany). Sample blanks were analysed at the beginning of each ICPMS processing session and after every 30 otolith samples. Twelve elements were measured (Ba, Cr, Cu, Mg, Mn, Ni, Pb, Ca, Sr, Na, K, and Zn) using inductively coupled plasma mass spectrometry (ICP-MS; ELAN DRC-e, Perkin Elmer). Detection limits were calculated as three times the standard deviation of the blank. The concentration of elements in the sample and blank were calculated and expressed as µg/g on dry weight basis (Campana et al., 2000; Khan et al., 2012).

3.7. Data Analysis

3.7.1. Truss morphometry

Any heterogeneity in the size across the samples will result in heterogeneity in shape without providing information on differences in body proportions among populations as most shape measurements are in some way related to size (Reist, 1985). To remove the size effect of samples, several univariate and multivariate analyses can be used such as regression analysis, allometric methods, multiple group principal component analysis etc. The allometric methods are helpful to significantly achieve the size and shape separation and reasonably meet the statistical assumption. Adjustment of size using allometric transformation to a standard size was most suitable because it completely remove the size variation and the adverse effect were also minimal (Reist, 1985). Significant correlations were observed between size and morphometric characters of the samples. All the measurements were standardized following Elliot et al. (1995), to eliminate any variation resulting from allometric growth.

\[ \text{Madj} = M(Ls/Lo)^b \]

where, \( M \) is the original measurement, \( \text{Madj} \) is the size adjusted measurement, \( Lo \) is the standard length (SL) of the fish, and \( Ls \) is the overall mean of the SL for all fish from all samples in each analysis. Parameter \( b \) was estimated for each character from the observed data as the slope of the regression of log \( M \) on log \( Lo \). The transformed data were checked for efficiency by testing the significance of the correlation between the transformed variables and standard length. Standard length was excluded from the final analysis.
The transformed data were subjected to univariate analysis of variance to evaluate the significant differences among four sites of the three rivers. The data were employed to principal component analysis (PCA) and discriminant function analysis (DFA) to examine any phenotypic differences among the populations. PCA helps in morphometric data reduction in decreasing redundancy among the variables and in extracting a number of independent variables for population differentiation (Samaee et al., 2009). The eigenvectors and eigenvalues were obtained from the covariance matrix in PCA which allowed the largest part of the variance of original variables in a low number of factors. This analysis enabled the evaluation of the relation between the populations by means of proximity in the space defined by components. The Bartlett's Test of sphericity and the Kaiser-Meyer-Olkin (KMO) measure was performed to examine the suitability of the data for PCA. The Bartlett's Test of sphericity tests the hypothesis that the values of the correlation matrix equal to zero (small significance levels support the hypothesis that there are real correlations between the variables). The KMO measure of sampling adequacy test is used whether the partial correlation among variables is sufficiently high (Nimalathasan, 2009). The KMO statistics varies between 0 and 1. Kaiser (1974) recommends that values greater than 0.5 are acceptable. Between 0.5 and 0.7 are mediocre, between 0.7 and 0.8 are good, and between 0.8 and 0.9 are superb (Field, 2000). Scree test is applied to reduce the number of factors to something below that found by using the eigenvalue greater than unity’ rule (Cattell, 1966). Eigenvalues are plotted against the factors arranged in descending order along the X-axis. The number of factors that correspond to the point at which the function so produced appears to change slope is deemed to be the number of useful factors extracted. This is a somewhat arbitrary procedure. It is worth mentioning that factor loading greater than 0.30 is considered significant, 0.40 more important, and 0.50 or greater is very significant (Nimalathasan, 2009). For parsimony, in this study for all fishes, only those factors with loadings above 0.50 were considered significant.

The Wilks' lambda criterion was used to compare the differences between and among all groups. DFA was done to assign the individuals into their original groups. The ability of the phenotypes to discriminate between the populations was assessed by a cross-validation test. The basis for differentiation among samples was based on the percentage of correctly and incorrectly classified fish. The measure of the
morphological distinctness of the population was given by percentage of correctly classified individuals. The degree of intermingling between the populations was shown by the number of misclassified individuals. The scatter plot of first two discriminant scores was drawn to visualize the separation of stocks on the graph.
3.7.2. Otolith chemistry

Correlation was carried out between elemental concentration and standard length (LS) of fish to check the effects of LS on elemental concentrations. Significant correlation was observed between fish size and elemental concentrations of the samples. The data were standardized according to Bergenius et al. (2005) to remove the effect of LS from each elemental concentration:

\[ C_{ij\text{adj}} = C_{ij} \pm b \left( L_{Si} - L_{Sj} \right) \]

where \( C_{ij\text{adj}} \) is the sample concentration of fish i adjusted to mean standard length of group j, \( C_{ij} \) is the sample concentration for fish i from group j, b is the slope of the relationship of \( C_{ij} \) to \( L_{Si} \) common to all groups, \( L_{Si} \) the standard length of fish i in group j and \( L_{Sj} \) the average standard length within group j.

The adjusted concentrations for all elements of interest were then analyzed by multivariate analysis of variance (MANOVA) to test for spatial differences in the multivariate elemental signatures. The assumption of homogeneity of variance for each dependent variable was examined using Levene's test and homogeneity of the group covariance matrix by Box's M test (MANOVA). Data were tested for normality using the Shapiro-Wilk test. A univariate ANOVA was used to explore patterns for each of the elements separately when significant effects were indicated in the MANOVA. Wilk's Lambda criterion was used to test for group differences in the MANOVAs. A stepwise DFA was used to examine the elemental signatures in discriminating the population among the sites and to investigate whether signatures could be used to classify samples to their original group. Leave-one out classification with cross-validation was carried out to assign the individuals into their original group. The scatter plots of first two discriminant scores were drawn to visualize the separation on the graph.

To compare the relative stock discriminating efficiency of the two methods used in the study (namely truss morphometry and otolith chemistry), the percentage classification accuracy of the two methods for the selected sites of each fish species were subjected to students t-test (Zar, 1996).

All the statistical analyses were carried out using MS-EXCEL (Microsoft Corporation, Redmond, WA, USA), SPSS vers. 16 (SPSS, Chicago, IL, USA) and Paleontological Statistics (PAST) (Hammer et al., 2001).