For the present study, specimens of *Cyprinus carpio communis* L. and *rohita* (Ham.) were collected from the landing stations of the Gobindsagar (Himachal Pradesh), Ropar wetland (Punjab), Harike wetland (Punjab) and Ficchamkaur Sahib, Ropar (Punjab) during the period from 2011-2014 to study morphometry, length-weight relationship and regeneration pattern of the two fish species in question. Since the fishes are not available in large number throughout the year; trips to selected localities were planned keeping in mind the availability of fish in numbers. No collections were made during the months of May-August even because of closed season on account of breeding period. Random sampling was so as to include specimens of all age groups. Though, every effort had been made by us to include the specimens of all ranges, it was a difficult preconvincing the contractor at the site to get specimens of different weights as they employ the gill nets of same mesh size. In all, 100 specimens of each fish from each locality have been analyzed for studying the differences in the morphometric parameters.

### 5.1. MORPHOMETRIC AND MERISTIC ANALYSIS

#### 5.1.1. MORPHOMETRY

The morphometric measurements of all the 100 specimens from each locality were done in the field itself from the left side of the fish body by a single person, so as to minimize the errors (if any). The morphometric parameters were recorded with the help of vernier caliper (with an accuracy of ±0.1 cm), weighing balance (with an accuracy of ±10 gm) and morphometric scale. The morphometric readings were converted into percentages and been expressed with respect to total fish length and head length. These percentages were then used to compute mean, standard deviation and range. Actual values were used to calculate correlation coefficients, regression equations and other statistical calculations.
equations between independent and dependent parameters and for regression lines using SPSS (version 18).

Measurements were taken in two groups: In one group, the measurements were taken with respect to the total length and in the second group, measurements were taken with respect to the head length. All the measurements were taken in cm and the weight was recorded in gm. The morphometric measurements were recorded:

**Total length of fish (TL) vs.**

1. Standard length (SL)
2. Pre-dorsal distance (Pre D)
3. Post-dorsal distance (Post D)
4. Length of dorsal fin (LDF)
5. Depth of dorsal fin (DDF)
6. Length of anal fin (LAF)
7. Depth of anal fin (DAF)
8. Pre-anal distance (Pre A)
9. Length of pectoral fin (LPF)
10. Length of pelvic fin (LP’F)
11. Minimum body width (MBW)
12. Maximum body width (M’BW)
13. Distance between pectoral and ventral fins (DPP)
14. Distance between pelvic and anal fins (DPA)
15. Length of caudal fin (LCF)
16. Length of caudal peduncle (LCP)
17. Length of rostral barbel (RBL)
18. Length of maxillary barbel (MBL)
19. **Head length (HL) vs.**
20. Head depth (HD)
21. Pre-orbital distance (Pre Or)
22. Post-orbital distance (Post Or)
23. Eye diameter (ED)
24. Intra-orbital distance (IoD)
Figure 6. Various morphometric characters of *Cyprinus carpio comm*

The morphometric characters considered for the present study followed after Jayaram (2013) without any modifications, which are (Fig. 6):

- **a. Total Length (TL)**
  The greatest distance between the anterior projecting part of the posterior most tip of the caudal fin. The measurement is along and is not taken over the curves of the body.

- **b. Standard Length (SL)**
  The straight distance from the anterior part of the head to the vertebral column is taken as the standard length.

- **c. Pre-dorsal Distance (Pre D)**
  A straight measurement from the midpoint of snout or upper anterior most part of the head to the structural base of the anterior dorsal fin ray is the pre-dorsal distance.

- **d. Post-dorsal Distance (Post D)**
  A straight line measurement from the structural base of the dorsal fin to the flexure line of the body over the caudal peduncle or the vertebral column is post dorsal distance.
Materials and methods

e. Length of Dorsal Fin (LDF)
The greatest distance of the dorsal fin measured in a straight line from its anterior most to the posterior most junctions with the body.

f. Depth of Dorsal fin (DDF)
It is measured from the anterior point of junction of dorsal fin with the body to the anterior tip of the fin or spine even if other rays do not reach this point.

g. Length of Anal Fin (LAF)
The greatest distance of anal fin measured in the straight line between the anterior most and posterior most points of junction with the body.

h. Depth of Anal Fin (DAF)
It is measured from the anterior point of junction of anal fin with the body to the anterior tip of the spine or fin even if other rays do not reach this point.

i. Pre-anal Distance (Pre A)
A straight line distance from the base of anterior most fin ray of the anal fin to the anterior most point of the head.

j. Length of Pectoral Fin (LPF)
It is measured between the origin and place of insertion of pectoral fin into the body to its extreme tip.

k. Length of Pelvic Fin (LP’F)
It is measured between the origin and place of its insertion of pelvic fin into the body to its extreme tip of the fin or spine even if other rays do not reach this point.

l. Minimum Body Width (M’BW)
It is the shortest height of caudal peduncle between the end of the anal fin and the origin of the caudal fin.

m. Maximum Body Width (MBW)
It is the maximum perpendicular distance from the dorsal side to ventral side.

n. Length of Caudal Fin (LCF)
It is measured from the anterior point of junction of caudal fin with the
Materials and methods

body to the extreme tip of the fin or the spine even if the other rays do not reach this point.

o. Distance between pectoral and ventral fins (DPP)
   It is the distance between the anterior most point of the pectoral fin to the anterior most point of the ventral fin taken across the length of the fish.

p. Distance between pelvic and anal fins (DPA)
   It is the distance between the anterior most point of the pelvic fin to the anterior most point of the anal fin taken across the length of the fish.

q. Length of Caudal Peduncle (LCP)
   An oblique measurement from the last point of contact of anal fin posterior to the end of the vertebral column or the flexure of the body over the caudal peduncle.

r. Length of Rostral Barbel (RBL)
   It is the measurement from base to the tip of barbel extended from nostrils.

s. Length of Maxillary Barbel (MBL)
   It is the measurement from base to tip of barbel present on either side of the mouth.

t. Head Length (HL)
   A straight-line measurement of distance from the tip of the snout to the most distant point on the opercular membrane on the upper angle of the gill opening.

u. Head Depth (HD)
   The perpendicular distance measured from the midline at the occipital vertically downwards to the ventral contour of head or breast.

v. Preorbital Distance (Pre Or)
   The distance from the most anterior midpoint on the snout or upper tip to the front margin of the orbit.

w. Post-orbital Distance (Post Or)
   The greatest distance from the posterior edge of the orbit to the posterior tip of the fleshy operculum.
Materials and methods

x. Eye Diameter (ED)
   The maximum distance between margins of the cartilaginous eyeball across the cornea.

y. Intra-orbital Distance (Io D)
   Least distance of the orbit within the eye.

5.1.2. MERISTICS
Mainly two types of counts have been considered in describing the fishes under report viz., scale count and fin ray count.

i) LATERAL LINE SCALES
   It is the number of scales having perforations and present along the lateral line starting from the end of operculum edge up to the base of caudal fin or end of caudal peduncle. In case the lateral line is absent, then scales are counted in the lateral series along its flank in the same manner. There are several variations in this regard which are specie specific.

ii) FIN RAY COUNTS
   The method of counting the fin rays in all the fins (both paired and unpaired) is almost the same, hence individual fins are not discussed. All the fin rays of fins were counted namely:
   a. Dorsal fin rays (DFR)
   b. Pectoral fin rays (PFR)
   c. Pelvic fin rays (P’FR)
   d. Anal fin rays (AFR)
   e. Caudal fin rays (CFR)

5.2. STATISTICAL ANALYSIS (DFA and PCA)

Hypothesis: The hypothesis for the present study is that the stocks of *Cyprinus carpio communis* L. and *Labeo rohita* (Ham.) are different at different localities.

\[ H_0 = \text{There was no difference between the four stocks of } Cyprinus carpio communis \text{ L.} \]

\[ H_0 = \text{There was no difference between the four stocks of } Labeo rohita \text{ (Ham.)} \]

To determine intraspecific variations in *C. carpio communis* L. and *L. rohita* (Ham.), multivariate analysis were carried out using SPSS (version 18) separately for morphometric and meristic characters, since these variables are different statistically.
(meristics being discrete, morphometrics being continuous) and biologically (meristics being fixed earlier in development while morphometrics are more susceptible to the environment) (Erguden et al., 2009; Ihssen et al., 1981; Hurlbut and Clay, 1998).

Because of the variation in size of fish from different areas, morphometric and meristic data was statistically adjusted to permit comparative analysis in terms of shape and counts independently of size (Costa et al., 2003; Thorpe, 1976). The multivariate techniques involved the use of Discriminant Function Analysis (DFA) and Principal Component Analysis (PCA). All morphometric analysis was transformed to common logarithms to obtain a better approximation to multivariate normality since linearity and normality are usually more closely approximated by logarithms rather than original variables (Hair et al., 1998). Detected outliers in the morphometric regression analysis would have subsequently been withdrawn from further consideration. This procedure did not find any outliers, thus no specimen was removed from further consideration. Morphometric character measurements were standardized to mean total length using the formula (Absunza et al., 2001):

$$\text{Log}_{10} M_{adj} = \text{Log}_{10} M_{obs} - b (\text{Log}_{10} L_0 - \text{Log}_{10} L_t)$$

Where

- $M_{adj}$ = standardized morphometric variable
- $M_{obs}$ = the uncorrected variable value (observed measurement)
- $L_t$ = mean total length considered for all samples (from all locations)
- $L_0$ = total length of each fish and
- $b$ = the allometric coefficient for the respective character (slope of the relationship between log $M_{obs}$ and log $L_0$).

This regression model was chosen because none of these variables could be considered either independent or explanatory (Murta, 2000) hence; it is an appropriate procedure for objective analysis of the data when there is a size overlap among the groups being examined. Correlation coefficients between each pair of characters were calculated using SPSS statistical software, to check if the data transformation (log transformation) was effective in reducing the influence of size in the measurements. The canonical variates 1 and 2 were regressed against total length and size effect was removed if the regressions show significance at $p<0.001$ (Claytor and MacCrimmon, 1987).
A chi-square transformation ($\chi^2$) of Wilk’s lambda ($\lambda$) was used to test equality among the group centroids and whether all canonical variables reflected population differences (Costa et al., 2003). The graphical display of canonical variates 1 and 2 was also useful for demonstrating group differences because fish belonging to same group appear closer together on the plot rather than fish from different groups (Schaefer, 1991a). The expected actual error rates of the classification functions were estimated using cross validation by leaving one-out-procedure (Costa et al., 2003). The proportion of individuals correctly classified was taken as a measurement of the integrity of that group. The degree of intermingling amongst the population was indicated by the number of misclassified individuals (Yakubu and Okunsebor, 2011). All mathematical procedures were performed using SPSS statistical package.

In the PCA, determinant of the correlation matrix was used to test for multicollinearity and singularity. Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy tests whether the partial correlation among variables is sufficiently high. KMO values lies between 0 and 1, with values above 0.5 as acceptable (Kaiser, 1974), between 0.5 and 0.7 as mediocre, 0.7 and 0.8 as good and between 0.8 and 0.9 as excellent (Field, 2000). Bartlett’s Test of Sphericity (BTS) tests the null hypothesis that the original correlation matrix is an identity matrix (small significance levels support the hypothesis that there are zero correlations between the variables) were computed to test the validity of the principal component factor analysis of the data set (Nimalathasan, 2009).

Cumulative proportion of variance criterion was employed in determining the number of factors to extract. Reproduced and residual correlations were used to test the appropriateness of the number of factors extracted. The varimax criterion of the orthogonal rotation method was employed in the rotation of the factor matrix to enhance the interpretability of the factor analysis. The factor loadings greater than 0.30 were considered to be significant; 0.40 as more important and 0.50 or greater as very significant (Nimalathasan, 2009).

DFA and PCA are particularly suitable for such a study, being most appropriate statistical technique when separation of more than two groups is required (Claytor and MacCrimmon, 1987; Schaefer, 1991 b).
5.3. SCANNING ELECTRON MICROSCOPY (SEM) FOR REGENERATED SCALE STUDIES

The scales of fish were removed from the left side of the body beneath the dorsal fin from second or third row. The regenerated scales were selected by observing under compound microscope. The scales had been washed thoroughly in tap water and cleaned gently by rubbing them between the tips of fingers. Scales were then subjected to sonication in triple distilled water twice for 10 min. each to remove mucous, organic particles, dust and extraneous matter. The scales were then dried between the folds of Whatman Filter Paper No. 1. Cleaned and dried scales were mounted on the aluminium stubs with the help of double adhesive tape in such a way that the dorsal surface is exposed and the ventral surface is adhered to the stub.

The mounted stubs had been coated with thin layer of gold palladium alloy of thickness 100Å. Coating of heavy metals like gold or palladium etc. made the specimen more conductive. The gold coating overcomes the problem of “charging” and “beam damage” as well as increases the strength of secondary electron signal from the surface. The scales were then viewed under vacuum in Leica Stereo Scan 360 Scanning Electron Microscope (Leica S360 SEM) at an accelerating voltage of 20 KV. The SEM facility was provided by CIL, Panjab University, Chandigarh.

5.4. LENGTH WEIGHT RELATIONSHIP

Length-weight relationship of fish population is a powerful tool for characterizing strains of same species, which involves detection of subtle variation of shape independent of size (Johal, 1994). A graph was plotted based on the regression and correlation of each morphometric character. Regression equation is given by the formula:

\[ Y = a + b(X) \]

Where;
\[ Y = \text{Dependent variable} \]
\[ a = \text{Constant} \]
\[ b = \text{Slope of line} \]
\[ X = \text{Independent variable} \]
The parameters of the length-weight relationships were calculated following equation:

\[ W = aL^b \] (Le Cren, 1951; Ricker, 1975; Pauly, 1984).

Where

- \( W \) = Weight of the fish in grams (gm)
- \( L \) = Total length of the fish (cm)
- \( a \) = Constant (intercept)
- \( b \) = the length exponent (slope)

The length-weight pairs were plotted initially in order to delete the possible outliers. The “\( b \)” is an exponent with value between 2 and 3 demonstrating normal growth dimensions or the interpretation of \( b \) being (Bagenal and Tesch, 1978; King, 1996 a, b). The transformation to linear transformation was made using the natural logarithms of the observed lengths and weights proposed by Zar (1984). This reduction of data into smaller values.

The expression of the equation is represented by the following

\[ \log W = b \log L + \log a \]

A graph of the log \( W \) against log \( L \) forms a straight line with and a Y-axis (log w) intercept of log a. In much of the fishery literature regression constant is represented by “\( c \)” rather than “\( a \)” and the regression coefficient is represented by “\( n \)” rather than “\( b \)”. Equations in the form logarithms (base e) and power functions are commonly used instead (Schneider et al., 2000). The fishes were weighed in grams on a weigh scale and length (cm) was measured with measuring scale. All the above calculations were done using the software SPSS (Version 18) and graphs were plotted using the observed values and log of observed body proportion between independent (X) and dependent (Y) characters calculated in order to determine the genetically and environmental characters.

5.5. MOLECULAR ANALYSIS

The muscle tissues were taken near dorsal fin and preserved in and stored in -4°C until further processed. The tissues were then:
Materials and methods

Chromous Biotech, Bangalore for the Col and Cyt b analysis.

5.5.1. GENOMIC DNA ISOLATION

Genomic DNA was isolated from the tissue sample using Chromous genomic DNA isolation kit Genomic DNA spin 50

PROTOCOL:
1. Take 100mg of muscle tissue
2. Add 750 ml of 1X suspension buffer; crush the tissue, until it forms a fine paste. Pipette this into a 2ml vial (using a tip that is cut at the bottom). To the above add 5 ml of the RNAase solution. Mix 5-6 times by inverting the vial. Place it at 65°C for 10 min with intermittent mixing.
3. To the above add 1ml of lysis buffer provided. Mix 5-6 times. Keep the mixture at 65°C for 15 min.
4. Spin at 13,000g for 1min at RT. Collect the clear supernatant in a 2ml vial.
5. Load the supernatant on the spin column (600 ml each time).
6. Spin at 13,000g for 1min at RT. Discard the contents of the collection tube. Place the spin column back in the same collection tube.
7. Add 500 ml of 1X Wash Buffer to the column. Spin at 13,000g for 1min at room temperature. Discard the contents of the collection tube. Place the spin column back in the same collection tube.
8. Repeat step 7. Spin the empty column with the collection tube at 13,000g for 3 min at RT.
9. Place the spin column in a fresh 1.5 ml vial.
10. Add 50 ml of warm Elution Buffer (already kept at 65°C) into the spin column.
11. Keep the vial along with the spin column at 65°C for 1 min. Spin at 13,000g for 1 min at room temperature.
12. Repeat step 12. Elute and collect in the same vial.

DNA obtained from the method is ready to use for any application without any further precipitation step.

49
5.5.2. PCR AMPLIFICATION

A. PCR AMPLIFICATION CONDITIONS

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>1 ml</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>400ng</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>400ng</td>
</tr>
<tr>
<td>dNTPs (2.5 mM each)</td>
<td>4 ml</td>
</tr>
<tr>
<td>10X Taq DNA Polymerase Assay Buffer</td>
<td>10 ml</td>
</tr>
<tr>
<td>Taq DNA Polymerase Enzyme (3 U/ml)</td>
<td>1 ml</td>
</tr>
<tr>
<td>Water</td>
<td>X ml</td>
</tr>
</tbody>
</table>

Total reaction volume: 100 ml

B. PRIMERS

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>C. carpio</td>
<td>5'-TGTAAAACGACGGCCAGTCAACCAACCACAAAGACA-3'</td>
<td>5'-CAGGAAACACGCTATGACACTTCAGGGTGACCCAAGA-3'</td>
</tr>
<tr>
<td></td>
<td>L. rohita</td>
<td>5'-GTTT GAT CCCGTTT CGT GA-3'</td>
<td>5'-AATGACTTGAAGAACCACCGT-3'</td>
</tr>
<tr>
<td>Cyt b</td>
<td>C. carpio</td>
<td>5'-GTGTGATCCCGTTTCGTGA-3'</td>
<td>5'-AATGACTTGAAGAACCACCGT-3'</td>
</tr>
<tr>
<td></td>
<td>L. rohita</td>
<td>5'-AAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'</td>
<td>5'-AAACTGCAGCCCCTCAGGAATATTTGTCCTCA-3'</td>
</tr>
</tbody>
</table>

C. THERMAL PROFILE

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Initial Denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoI</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>30 sec</td>
<td>30 sec</td>
<td>1 min</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35 cycles</td>
<td></td>
</tr>
<tr>
<td>Cyt b</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>4 min</td>
<td>1 min</td>
<td>1.5 min</td>
<td>2 min</td>
<td>7 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35 cycles</td>
<td></td>
</tr>
</tbody>
</table>

5.5.3. EXTRACTION OF PCR PRODUCTS:

- PCR products obtained were Gel eluted using Chromous Gel extraction kit gel extraction spin – 50.

PROTOCOL:

1. Cut the DNA fragment from the Agarose gel with a clean, sharp cutter
Materials and methods

2. Weigh the gel slice in a 2ml microcentrifuge tube.
3. Add 3 volumes of Gel extraction buffer to 1 volume of gel (100mg~100 ml)
4. Incubate the tube at 55°C for 5-10min (or until the gel piece has completely dissolved). Mix the sample by inverting the tube every 2-3 min during the incubation to solublize agarose completely.
5. After the gel piece has dissolved completely, add 1 gel volume of isopropanol to the tube and mix (If the Agarose gel piece is 100mg, add 100ul isopropanol).
6. Place the spin column in a 2ml collection tube provided.
7. Load the gel extracted solution mixed with isopropanol on to the spin column (600 ml each time).
8. Spin at 13,000g for 1min at room temperature. Discard the contents of the collection tube. Place the spin column back in the same collection tube.
9. Add 500 ml of wash buffer to the column. Spin at 13,000g for 1min at room temperature. Discard the contents of the collection tube. Place the spin column back in the same collection tube.
11. Spin the empty column with the collection tube at 13,000g for 3 min at RT.
12. Place the spin column in a fresh 1.5ml micro centrifuge tube.
13. Add 15 ml of Elution Buffer on to the spin column.
14. Keep the vial along with the spin column at Room temperature for 2min. Spin at 13,000g for 1 min at room temperature.
15. Again add 15 ml of Elution Buffer on to the spin column
16. Keep the vial along with the spin column at Room temperature for 2min. Spin at 13,000g for 1 min at room temperature.
17. Purified DNA is collected in the tube

Determination of Yield:

DNA concentration was determined by both UV spectrophotometer and quantitative analysis on agarose gel.
5.5.4. SEQUENCING

The Sequencing mix Composition is as follows (10μl Sequencing Reaction)

- Big Dye Terminator Ready Reaction Mix : 4μl
- Template (100ng/μl) : 1μl
- Primer (10pmol/μl) : 2μl
- Milli Q Water : 3μl

PCR Conditions: (25 cycles)

Initial Denaturation : 96°C for 1min
Denaturation : 96°C for 10 sec
Hybridization : 50 °C for 5 sec
Elongation : 60 °C for 4 min

Instrument and Chemistry Details

Sequencing Machine : ABI 3500 XL Genetic Analyzer
Polymer & Capillary Array : POP_7 polymer 50 cm Capillary Array.
Analysis protocol : BDTv3-KB-Denovo_v 5.2
Data Analysis Software : Seq Scape_v 5.2
Reaction Plate : Applied Biosystem Micro Amp Optical 96-Well Reaction plate

DATA ANALYSIS

The mtDNA sequences were analyzed for estimating the number of base substitutions per site between sequences, using Maximum Composite Likelihood model (Tamura et al., 1993). Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model. Relative values of instantaneous $r$ should be considered when evaluating them. For simplicity, sum of $r$ values is made equal to 100. For estimating ML values, a tree topology was automatically computed. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software; Tamura et al., 2013). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.