Methodology

3. Methodology

The morphometric analysis of leaves is a critical component in understanding the phenotypic diversity and taxonomic relationships within plant families. In this study, we focus on members of the Convolvulaceae family, a group known for its diverse leaf shapes and ecological adaptations. The methodology employed encompasses several steps, from the collection of plant specimens in their natural habitats to the detailed statistical analysis of leaf morphology. The following sections describe in detail the processes involved in plant collection, leaf sampling, herbarium preparation, image acquisition using a high-resolution scanner, and subsequent image and data analysis using SHAPE, PAST, and R software. Each step is meticulously designed to ensure the accuracy and reproducibility of the findings, contributing to a comprehensive understanding of leaf morphometry in the Convolvulaceae family.

3.1. Plant Collection

The collection of plant specimens was conducted across various ecologically diverse locations in western India, including regions with varying altitude, climate, and soil types. This diversity in collection sites ensured a broad representation of the Convolvulaceae family's morphological and ecological variability. Sites were chosen based on their known richness in Convolvulaceae species, as indicated by previous botanical surveys and literature. Specific criteria for site selection included:

- Sites spanning different biomes such as coastal areas, inland plains, and hilly regions.
- Locations that could be accessed with minimal disturbance to the ecosystem.

Specimens were collected during the growing season, ensuring that the plants were in their optimal growth state. GPS coordinates, altitude, and habitat details were recorded for each collection site. Collected samples were identified with the help of available taxonomic literature (**Cooke, 1908; Hooker, 1885; Shah, 1978**). To ensure the long-term preservation and availability of collected specimens for future reference, herbarium sheets were prepared following the standard methodology as described by **(Jain & Rao, 1976)** and deposited at the BARO herbarium of the Maharaja Sayajirao University of Baroda, Vadodara.

3.2. Leaf Sampling:

Once in the laboratory, leaf samples were selected from the collected specimens. This step was crucial for ensuring that the morphometric analysis would be based on representative and comparable leaf samples. The following criteria were used to select leaves.

- Fully expanded leaves from initial stage to maturity were chosen to include all the variations due to growth stages.
- Leaves with any signs of damage, disease, or pest infestation were excluded.
- A minimum of 50 leaves per individual plant were selected to account for intra-individual variation.

Selected leaves were carefully detached from the plant and prepared for further processing. Leaves were pressed between blotting paper sheets using plant presses to retain their shape and structure. Pressed leaves were dried in a well-ventilated area to prevent mould growth and to maintain their form (**Babu et al., 2018**).

3.3. Image Acquisition:

A Canon CanoScan LiDE 120 flatbed scanner was used to scan the leaf samples. Each leaf was placed abaxial surface faced down directly onto the scanner bed with a contrasting background to enhance edge visibility. A 30 x 30 mm calibration scales was included in each scan to allow for accurate measurements. The scanner was set to 600 DPI to capture fine details of leaf morphology, ensuring high-quality images suitable for precise morphometric analysis **(Iwata & Ukai, 2002).** Scanned images were saved in high-resolution formats (e.g., TIFF) to preserve detail and facilitate further analysis. Each leaf was scanned multiple times, and the best-quality image was selected for analysis. Images were inspected for any scanning artifacts, such as blurs or distortions, which were corrected or rescanned if necessary.

3.4. Image Analysis

The images obtained after scanning the leaf samples were converted into grayscale (.bmp) format by using MS Paint software to simplify edge detection. The bitmap images were then processed with SHAPE v.1.3d, image processing software **(Iwata & Ukai, 2002)** which provides

tools for detailed morphometric analysis. Each colour image was converted into a binary image, from which the outline was traced and then transcribed in chain-code using the ChainCoder software program.

Outlines were then reduced to the coefficients of Elliptic Fourier descriptors (EFDs) of 30 harmonics for simple leaves and 50 harmonics for palmate/finely dissected leaves using CHC2NEF software program. Elliptic Fourier descriptors (EFDs) were calculated to quantitatively describe the leaf shapes which capture the shape's geometry in a way that is invariant to size, orientation, and position. EFDs were normalized to remove variations due to size, rotation, and translation, ensuring that the shape comparisons were accurate. These coefficients effectively become shape variables. These coefficients are mathematical descriptors of forms that can be statistically analysed by routine methods **(Kuhl & Giardina, 1982).**

PrinComp software was used to perform principal component analysis (PCA) on the EFD data. PCA reduced the high-dimensional EFD data to a smaller set of principal components that capture the major sources of variation.

The principal components were plotted to visualize the morphological variation among the leaf samples using PrinPrint software. The resulting images included major variation depicted by effective PCs.

3.5. Data Analysis

The procedure for calculating EFDs from the original digitized image reconstructs the outlines to an approximate level and the decision to set the number of harmonics was arbitrary to some extent **(Rohlf & Archie, 1984).** In this aspect, the coefficients of the Elliptic Fourier descriptors were calculated so that the score on each principal component was equal to the mean with ±2 SD (standard deviation), and the scores of the remaining components were kept zero (**Iwata et al., 1998; Yoshioka et al., 2004**). The resulting data matrix of normalized EFDs was explored by principal component analysis (PCA) using a variance-covariance matrix described in the procedure of **Yoshioka et al. (2004).** The PCA of the coefficient matrices reduced the data dimensionality of uncorrelated shape descriptor variables to a smaller number.

The data generated by SHAPE software were subjected to further analysis using PAST (Hammer et al., 2001) and R software (2024). The PCA results were verified to ensure consistency with SHAPE outputs. Additional plots and graphs were generated to aid in the interpretation of the PCA results. Cluster analysis was performed on the EFD data using PAST (Hammer et al., 2001) and RStudio (2023) software. The PC scores extracted from the four effective principal components were utilized to perform cluster analysis. A similarity matrix using Euclidean distance was utilized in the principal component scores. The resulting matrix was employed to create a cluster analysis using single linkage, complete linkage or nearest neighbour clustering method. Dendrograms were generated to illustrate the relationships among the leaf samples, providing insights into the taxonomic and phylogenetic relationships within the Convolvulaceae family.

3.6. Data Interpretation

The results from the image and data analyses were interpreted in the context of taxonomic and ecological significance. The principal components were examined to understand the major trends in leaf shape variation. Key morphological traits contributing to the variation were identified. The ability of PCA to distinguish between different species based on leaf morphology was assessed. The dendrograms from the cluster analysis were analysed to understand the relationships between and among the species. Distinct clusters representing different species or groups of species were identified. The implications of these clusters for the taxonomy of the Convolvulaceae family were discussed. The findings were compared with existing taxonomic literature to validate the observed patterns. Consistency with previously reported morphological and taxonomic data was assessed. Any novel insights or discrepancies were highlighted and discussed in the context of their significance for understanding the morphological diversity and evolution of the Convolvulaceae family.