CHAPTER 3

3. MATERIALS AND METHODS

3.1 Study area

The study area of the present research work is the entire places falling under present political boundary of Assam (Fig 1). Assam is situated within the northeastern region of India, spans from approximately 24˚10'N to 27˚58'N latitude and 89˚49'E to 97˚26'E longitude. Covering a geographical area of 78,438 sq. km, the state is divided into 31 districts. Assam is surrounded by Bhutan kingdom and Arunachal Pradesh to the North, Nagaland, Manipur and Arunachal Pradesh to the East, Mizoram and Tripura to the south and the country Bangladesh and states Meghalaya and West Bengal to the West. Brahmaputra valley, Barak Valley and the North Cachar hills and Karbi Anglong hills divides the Assam into the three physiographic domains. The state is comprised of approximately 70% Brahmaputra Valley, with the Barak Valley encompassing 8.85%, while the hills of Karbi Anglong and North Cachar districts make up the remaining 21.15% of the total area. Assam hails with the tropical monsoon climate with maximum 35–38°C in summer season and minimum 6–8°C in winter season.The protected areas comprising of Wildlife Sanctuaries and National Park comprise of 3925 sq.km. constitutes about 5% of total geographical areas of Assam. It includes 7 National Parks, 18 Wildlife Sanctuaries and 2 Biosphere Reserves. The primary forest types found in the state include, Tropical Semi Evergreen, Tropical Moist Deciduous, Tropical Wet Evergreen, Subtropical Pine Forests and Tropical Dry Deciduous.

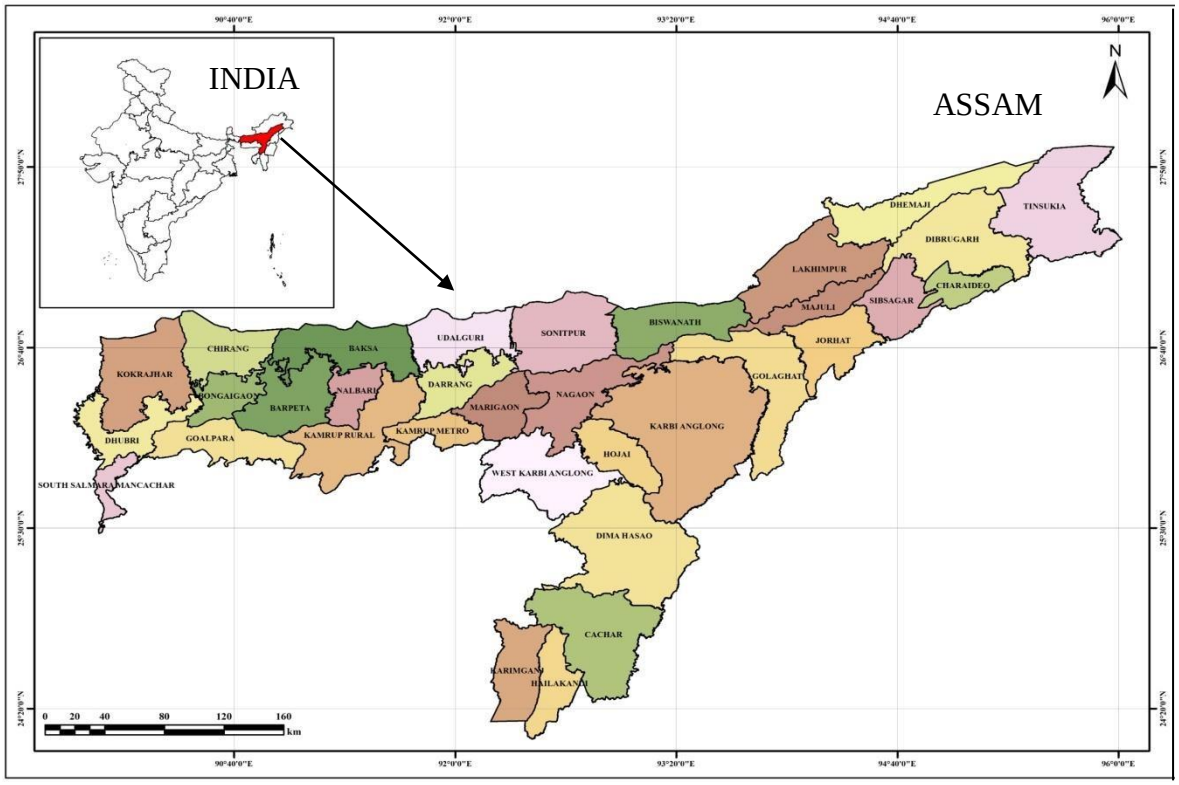

Fig 1 Map showing study area Assam

The taxonomic examination of the genus *Ophiorrhiza* L. in Assam relies on conducting surveys, collecting fresh specimens, and studying them. Furthermore, consultation and analysis of herbarium specimens from various collections, including scanned images and physically deposited specimens, along with referencing all relevant literature and protologues related to the genus were undertaken. The study stick to standardized methodologies for the comprehensive investigation of the genus. The methodologies include the following:

3.2 Exploration and Collection of fresh specimens

The present study deals with field survey which includes collection of live plant material from wild sources from different regions of Assam. Field surveys were conducted from the year 2019 to 2021 in the different locations of Assam. Fresh specimens were collected to study their morphology, anatomy and to also assess present distribution status in the state Assam. Utmost care was taken while collecting the specimens from their natural habitat without disturbing its natural populations and other associated species. Field observations like habit, habitat, color of flowers, flowering and fruiting time were recorded along with G.P.S. data, date of collection to draw a

conclusion about the distribution and locations of species in the study area. Specimens were gathered and preserved in accordance with the established herbarium methodologies followed by Jain & Rao (1977) and Das (2021). Flowers and fruits were carefully collected and stored in 4% formaldehyde solution for morpho-reproductive analysis.

3.3 Consultation of literatures and herbarium

Consultation of protologues and type specimens is crucial for establishing the accurate identity of a species. The protologues were critically studied and referenced from various digital institutions and libraries. Additionally, relevant literature from various journals and online sources focusing on taxonomy and distribution aided in easy species' identification process. The type specimens, deposited in various foreign and national herbaria, were consulted using virtual herbarium and scanned photographs obtained from the respective herbaria. The herbarium specimens were primarily referenced from the following foreign herbaria like Royal Botanic Garden Edinburgh Herbarium (E), Herbarium Royal Botanic Gardens, Kew (K), Meise Botanic Garden Herbarium (BR), Museo Nacional de Historia Natural, Cuba (MNHN), University of Michigan, U.S.A. Michigan Ann Arbor (MICH), Naturhistorisches Museum Wien, Austria (W), Naturalis Biodiversity Centre, The Netherlands, Leiden (L), The New York Botanical Garden, U.S.A. New York (NY), The herbarium of the Botanische Staatssammlung Munchen (M) in the form of digital images and in Indian herbarium like Central National Herbarium (CAL), Eastern Regional Centre Herbarium, Shillong (ASSAM), Arunachal Pradesh Regional Centre, Itanagar (ARUN), Central Ayurveda Research Institute, Guwahati (NEHAR). The herbarium specimens were examined through in-person visits to the respective herbaria. For foreign herbaria, the specimens were accessed via images obtained on loan through direct communication with the curators and staff of various herbaria, or accessed through digital images available in virtual herbaria.

3.4 Preparation of distribution map

Study area map, distribution map of collected species and probable habitat distribution map was prepared using GIS software QGIS 3.4.

3.5 Macromorphological study

3.5.1 Taxonomic enumeration

The collected specimens were studied in the laboratory for detailed morphotaxonomic descriptions and flowers were dissected and studied under microscope for preparation of photo plates, line drawing and descriptions. A comprehensive macro-morphological analysis of the fresh specimens was conducted utilizing a light microscope and a measuring scale. Photographic documentation of the species, including both vegetative and reproductive components was prepared using Adobe Photoshop 7.0. All the taxonomic terminologies used in the descriptions were consulted from the Kew Plant Glossary (Beentje 2010).

The taxonomic enumeration of the taxa includes the detailed descriptions made from fresh specimens along with original citations of accepted names, synonyms, author citation. The enumeration of the species includes vegetative and reproductive descriptions of the taxa which begin with habit followed by nature of stem, description of leaves, stipules. The description of the vegetative parts is followed by the description of reproductive parts which begins with inflorescence type, nature of bracts and bracteoles, nature of corolla, hypanthium, nature of stamen (homostylous or heterostylous), style, nature of fruits. Following the completion of documenting a taxonomic account, species' phenology, distribution, habitat, ecology, and a comprehensive list of examined specimens along with critical notes was mentioned. The herbarium details were followed using the herbarium acronyms listed in the 'Index Herbariorum' website (http://sweetgum.nybg.org/ih/). Scientific names and nomenclature were referenced following 'Authors of Plant Names' by Brummitt and Powell (1992) and the International Plant Name Index (IPNI) website (www.ipni.org). Flowering and fruiting data and specific taxonomic notes were included with field observations conducted during surveys. GPS coordinates and collection locations were documented for each taxon. Species distribution information was recorded from field collections, published literature, herbarium records, and online sources such as Global Biodiversity Information Facility (https://www.gbif.org). Elaborate dichotomous keys were developed for all examined taxa, utilizing morphological characteristics to facilitate in the identification process.

Additionally, distinct identification keys were developed for the taxa studied based on micro-morphological and anatomical traits to further aid in accurate identification of a taxon.

3.6 Micromorphological study

3.6.1 Foliar epidermal study

3.6.1 (i) Foliar epidermal analysis by light microscope

For light microscope study, the methodology of Boulos & Beakbane (1971) and Ahmed *et al.* (2010) was adopted. The leaves will be selected from collected fresh and herbarium specimens. Both upper and lower epidermal peelings were extracted using a 10% aqueous solution of nitric acid, followed by staining with safranin solution. About 5 to 7 slides were created from leaf surfaces for microscopic study. Samples were studied under compound microscope (Labomed & Leica) and microphotographs were captured using a Samsung Galaxy F12 mobile camera. Microphotographs were utilized to examine the characteristics and distribution of stomatal guard cells, subsidiary cells, and the nature of epidermal cell walls, as well as to identify stomatal types and trichome characteristics. Terminology for describing stomatal types and epidermal cell characteristics were followed according to Stace (1984), Prabhakar (2004), Soh & Parnell (2011).

3.6.1 (ii) Foliar epidermal analysis by SEM

For scanning electron microscopic (SEM) analysis of the species, both lower surface and upper surface of leaves were carried out, a middle section of lamina were cut into square size into smaller pieces. Two parts of the leaf, one from the upper surface and one from the lower surface, were affixed to stubs using double adhesive tape and then coated with gold prior to analysis under electron microscope. The leaf samples were analysed under Field Emission Scanning Electron Microscope (Ziers Sigma 300) and microphotographs were captured. This method was adopted by the Department of Chemistry, SAIF, Gauhati University Assam and SAIC, Institute of Advanced Study in Science and Technology, Guwahati.

3.6.1 (iii) Quantitative analysis

Different quantitative measures, i.e. stomatal length, number of epidermal cells, epidermal cell size (l × b), stomata size (l × b), subsidiary cell size (l × b), stomatal pore

size (l × b), presence of trichomes and its types and stomatal index were analysed. Cells on both adaxial and abaxial surfaces were measured. The length and width of stomatal pores, stomatal gaurd cell, epidermal cells and subsidiary cells were also measured.The stomatal index (S.I.) value has been calculated with the following formula:

S.I. (%) = (S/S+E) × 100

Where, S= Number of stomata in microscopic field of view.

E= Number of epidermalcells in microscopic field of view.

3.6.2 Test for significance difference of quantitative data

In the present work to determine the significance level of the entire numerical values, one way ANOVA test was done using the software PAST 4.06. For the test 10 taxa of the genus *Ophiorrhiza* were used as treatments and epidermal and stomatal size, stomatal pore size, subsidiary cell size all these parameters were used as variables.

3.6.3 Leaf architecture study

For leaf architecture study using the methodology of Ahmed *et al.* (2010) fresh leaves were soaked in 30% nitric acid, boil with 2.0 gm of potassium chloride in a test tube for 1-2 minutes. These treated leaves were then washed with distilled water twice. Then the epidermis is peeled off and kept in 60% of potassium hydroxide solution for 1 to 2 hrs. Then thin sections of epidermis were suspended in lactic acid on glass slide for light microscope study. About 5–7 slides were made from leaf surfaces. Samples were studied under light microscope and photographs were captured using mobile camera (Samsung Galaxy F12). The terminology used to describe the venation pattern was followed according to terms proposed by Hicky (1973), Melvile (1976), and Amanda *et al.* (1999).

3.7 Anatomical study

Plant materials collected from the studied area were preserved in 70% alcohol for anatomical studies, and free hand sections were utilized for the anatomical analyses.

Preparations of slides were done by following the methods suggested by Radford *et al*. (1974). The hand sections were stained with safranin and fast green, followed by dehydration through an ethanol gradient (30% to 90%). Subsequently, the samples were treated with 100% ethanol, followed by xylol, and mounted with DPX for examination under a microscope. Photographs were captured using a mobile camera (Samsung Galaxy F12).

3.8 Assessment of habitat status and identification of areas for reintroduction by ENM

For the identification of probable areas for reintroduction of rare and threatened species of the genus, the GPS coordinates were recorded during the species collected from the studied areas. The GPS coordinates were recorded with the help of Global Positioning System machine (Grahmin) with an accuracy of 10–30 m. Then the coordinates were converted into decimal degree to be used in habitat distribution modelling software. The environmental datasets used in distributional modelling of species were taken from public domain website (Global Land Cover Facility GLCF; University of Maryland; CGIAR-CSI (http://srtm.csi.cgiar.org, Jarvis *et al*. 2008). To get a potential habitat and to improve the conservation status of rare and threatened species of *Ophiorrhiza*, Maximum Entropy (MaXent) distribution modelling algorithm was used (Adhikari & Barik 2012; Sharma & Tanti 2022).