Materials and Methods

3.1. Ethnobotanical data collection, area of study

Field study was done covering eight blocks of Dakshin Dinajpur district of West Bengal. The district lies between 25° 10´ N - 25° 40´ N latitude and 88° 10´ E - 89° 00´ E longitude and covering an area of 2162 sq. Km. The district is situated between Bangladesh on the east and south, Uttar Dinajpur district on the North and West and some southern part lies adjacent to Malda district (Figure 3.1). The main source of income is based on agriculture. The district witness annual temperature of 20-30 °C, relative humidity of 64-90% with average rainfall of 120 mm (data compiled from the Meteorological Department, Office of the ADA, NB Regional Office, Jalpaiguri, GoWB). The topography of the study site is mostly even plain land.

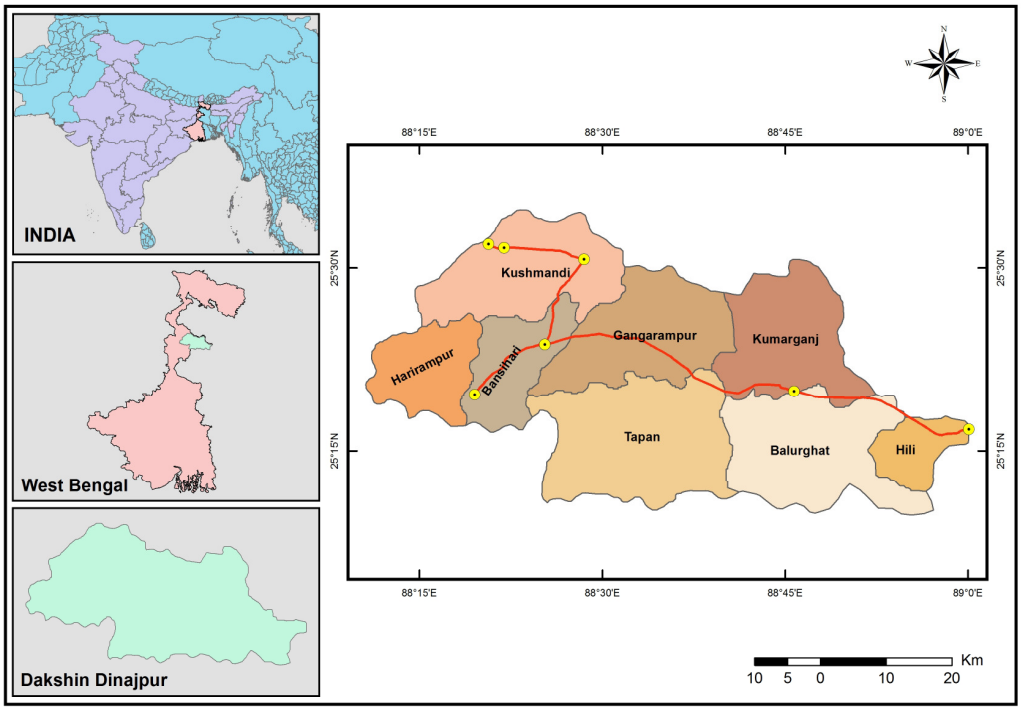

Figure 3.1: Map of the study area

Eight blocks of the study area are Hili, Balurghat, Kumarganj, Gangarampur, Tapan, Bangshihari, Harirampur and Kushmandi.

Various data collection techniques like personal interview, focused group discussion, semi-structured questionnaires following the method of participatory approaches were adopted in the present study (Jain, 1987; Jain and Mudgal, 1999). The structure of the questionnaire is given in Table 1 (Appendix A). Data were collected targeting mainly the knowledgeable tribal people locally known as Kabiraj/Baidya having traditional knowledge of using plants and their extracts for medicinal purposes. Total 160 persons were interviewed randomly taking 20 people from each block ensuring equal representation of the population in the sample targeted. The sample population targeted mostly belongs to the age group of 30 - 70 years.

Traverse walking was conducted during the initial phase interacting mainly with the old aged tribal people and Kabirajes/Baidyas. The main aim was to get an overall knowledge about the village and association of the tribal people in utilizing different locally available medicinal plants for curing various ailments. Several data were collected during the field study. For example, local name of the plant, plant part used for medicinal purpose, method of preparation, ingredients used and modes of administration etc. The duration of study covers two years from November 2013 to November 2015. The data collected were analysed employing appropriate statistical methods using Microsoft Excel version 2007 and represented as graphs, pie charts, tables etc.

3.2. Identification

The plants were collected at their reproductive stage, specimen herbarium prepared and preserved following the standard procedure of Jain and Rao (1977). The taxonomical study of the collected specimens and their characterization and identification was done following standard taxonomic literatures (Prain, 1903; Mukerjee, 1940; Hooker, 1973; Guha Bakshi, 1984; Mitra and Mukherjee, 2013). Nomenclature and correct author citation for all the species were thoroughly checked in the (The Plant List, 2013) database. Total 9 taxa of *Ocimum* were identified from the study area and included for the present study (Figure 3.2). Out of the 9 taxa, 2 morphotypes from *O. tenuiflorum* L. (Purple and Green type, commonly known as Krishna and Radha tulsi respectively), 2 morphotypes from *O. basilicum* L. (Babu and Marua tulsi), 2 morphotypes from *O. gratissimum* L. (Ram and Ajowan tulsi) and each from *O.* × *africanum* Lour. (Lebu

tulsi), *O. americanum* L. (Bon tulsi) and *O. kilimandscharicum* Guerke. (Karpur tulsi) species were considered for the present investigation (Table 3.1.). Their detailed characterization and classification were validated by the Botanical Survey of India (BSI), Howrah, West Bengal. The voucher specimens were deposited in the Department of Botany, University of North Bengal.

Table 3.1: *Ocimum* taxa collected from different places of the district Dakshin Dinajpur, West Bengal

Sl No.	Accession No.	*Ocimum* Taxa	Local name	Collection site	Longitude/ Latitude
1.	NBU-09801	*O. gratissimum* L.	Ram tulsi	Harirampur	88° 26´ 80´´ E 25° 37´ 73´´ N
2.	NBU-09802	*O. gratissimum* L.	Ajowan tulsi	Balurghat	88° 46´ 38´´ E 25° 13´ 16´´ N
3.	NBU-09795	*O. tenuiflorum* L.	Krishna tulsi	Gangarampur	88° 31´ 53´´ E 25° 23´ 58´´ N
4.	NBU-09796	*O. tenuiflorum* L.	Radha tulsi	Daulatpur	88° 19´ 36´´ E 25° 19´ 44´´ N
5.	NBU-09799	*O. basilicum* L.	Babu tulsi	Kushmandi	88° 22´ 06´´ E 25° 31´ 23´´ N
6.	NBU-09800	*O. basilicum* L.	Marua tulsi	Jordighi	88° 24´ 09´´ E 25° 26´ 28´´ N
7.	NBU-09797	*O. americanum* L.	Bon tulsi	Patiram	88° 76´ 14´´ E 25° 33´ 48´´ N
8.	NBU-09798	*O.* x *africanum* Lour.	Lebu tulsi	Bansihari	88° 24´ 04´´ E 25° 26´ 01´´ N
9.	NBU-09803	*O. kilimandscharicum* Guerke.	Karpur tulsi	Hili	89° 00´ 06´´ E 25° 16´ 39´´ N

*Altitudinal range 24 to 39 m

Figure 3.2: Nine *Ocimum* taxa used in the present study

3.3. Ecology of *Ocimum*

3.3.1. Quadrat studies

Sites were selected based on natural occurrence of *Ocimum* species targeting all the eight blocks. Natural occurrence of *Ocimum* was found abundantly along the road side. Three sites (*viz*., Site-1, Site-2 and Site-3) were identified for the present study (Table 3.2). Twenty five quadrat plots were selected randomly from each site totaling 75 quadrat plots. Each quadrat plot is designed as per the method of Misra (1968). Each plot measures 1x1 m^2 in size and contains at least one *Ocimum* species. The study was conducted in the month of August-November. Each quadrat plot is thoroughly assessed and number of different plant species belong to each plot was recorded. Quadrat studies were applied to elucidate the ecosystem of *Ocimum*. Following parameters like Density (D), Relative Density (RD), Frequency (F), Relative Frequency (RF), Abundance (A) and Importance Value Index (IVI) were developed for phyto-association studies of *Ocimum* with other plant communities adopting the standard procedure of Cottom and Curtis (1956).

Density: Density represents the numerical strength of a species in the unit area or volume. The number of individuals of the species in any unit area is its density. Density gives an idea of degree of competition. It is calculated as:

$$\text{Density} = \frac{\text{Total number of individuals of a species in all quadrats}}{\text{Total number of quadrats studied}}$$

Relative density: Relative density is used to express the contribution of individuals of one species in relation to the total number of individuals of all species.

$$\text{Relative density} = \frac{\text{Density of individuals of a species}}{\text{Total density of all species}} \times 100$$

Frequency: It is the measure of commonness and distribution of a species within a study area. Frequency is the number of sampling units (as %) which a particular species occurs. Thus frequency of each species calculated as follows:

$$\text{Frequency (\%)} = \frac{\text{Total number of quadrats in which the species occur}}{\text{Total number of quadrats studied}} \times 100$$

Relative frequency: The dispersion of species in relation to that of all the species is termed as relative frequency of a species.

$$\text{Relative frequency} = \frac{\text{Frequency of individuals of a species}}{\text{Total frequency of all species}} \times 100$$

Abundance: This is the number of individuals of any species per sampling unit of occurrence. It is related to density but is a qualitative estimate. It is calculated as follows:

$$\text{Abundance} = \frac{\text{Total number of individuals of a species in all quadrats}}{\text{Total number of quadrats in which the species occurred}}$$

Importance value Index (IVI): Important Value Index (IVI) is the sum of quantities of relative frequency and relative Density.

Importance Value Index (IVI) = Relative frequency + Relative density

Table 3.2: Details of the study sites

Sites	Location	Latitude	Longitude	Altitude
		Range	Range	Range
S-1	Hili to Patiram (SH-10)	89° 10´ E to 88° 45´ E	25° 10´ N to 25° 19´N	25-30 m
S-2	Bangshihari to Ushaharan via Mahipal road	88° 25´ E to 88° 28´ E	25° 23´ N to 25° 32´N	29-34 m
S-3	Patirum to Daulatpur (NH-512)	88° 45´ E to 88° 46´ E	25° 19´ N to 25° 13´N	30-37 m

3.3.2. Soil sampling and analysis

Five hundred gram soil (from the upper soil layer not below 15 cm in depth) was collected from each quadrat plot. Sampling was done from the centre and four corners of each quadrat plot. Samples collected were air-dried, sieved in 2 mm mesh for analysis.

3.3.2.1. Soil pH

The pH of soil was determined using pH meter (Digital pH meter Model Systronics No. 802) adopting the method of Jackson (1967). 20 g soil sample was mixed with 40 mL

distilled water (1: 2 ratio). The suspension was stirred occasionally with glass rod for 30 minutes and settled down for one hour. The electrode was inserted in the clear supernatant and pH was measured.

3.3.2.2. Electrical conductivity

The electrical conductivity of a soil sample was determined utilizing digital electrical conductivity meter (303 Systronics) as depicted by Bower *et al*. (1952). 20 g soil sample was mixed with 40 mL of distilled water. The mixture was stirred with a glass rod for 30 min and kept it for settled down. Conductivity cell was embedded in the supernatant and EC was recorded.

3.3.2.3. Organic carbon (%)

The amount of organic carbon of the soil was measured by technique for Walkley and Black (1934) portrayed by Jackson (1967). 1 g soil sample was taken into 500 mL conical flask, to which 10 mL of 1 N potassium dichromate and 20 mL concentrated H_2SO_4 was included. The mixture was shaken for a minute and permitted to set for 30 minutes. 200 mL distilled water, 10 mL concentrated orthophosphoric acid and 1 mL diphenylamine indicator was included. The solution was titrated against standard ferrous ammonium sulfate (FAS) till colour appears from blue violet to brilliant green. The clear titration (without soil sample) was done initially.

$$\% \text{ organic carbon} = (B - S) \times N \times 0.003 \times \frac{100}{\text{wt. of dry soil}}$$

Where,

B = mL of std. 0.5 N ferrous ammonium sulphate required for blank.

S = mL of std. 0.5 N ferrous ammonium sulphate required for soil sample.

N = Normality of std. ferrous ammonium sulphate (0.5N).

3.3.2.4. Available nitrogen (N)

Available N of soil sample was determined adopting the method of Subbiah and Asija (1956). 20 g soil sample and 20 mL distilled water were added in 1000 mL Kjeldahl flask. Then 100 mL potassium permanganate (0.32 %) and 100 mL NaOH (2.5%) solution were mixed thoroughly and attached it to Kjeldahl flask for boiling. The solutions were distilled in a kjeldahl and collected in the Erlenmeyer flask that contains boric acid with methyl red and bromocresol green indicator. After 30 minutes the

solution was titrated with 0.02 N H_2SO_4 until the colour appeared from green to pink. Blank (without soil) sample was run simultaneously. Available nitrogen was calculated from the following equation,

$$\% \text{ available N} = (S - T) \times (\text{N, of Acid}) \times 0.014 \times \frac{100}{W}$$

$$\text{Available N kg/ha} = \% \text{ of N} \times \frac{2240000}{100}$$

Where,

W = Weight of dry soil

S = Blank titration, mL standard NaOH required for 25 mL H_2SO_4, blank used for received the distillation of blank

T = Titration of sample, ml standard NaOH required for 25 mL H_2SO_4 receiving the sample

N = Normality of sulphuric acid

3.3.2.5. Available phosphorus (P)

Available phosphorus was measured by Olsen's technique modified by Watanbe and Olsen (1965). 2.5 g soil, 50 mL of Olsen's reagent (0.5M $NaHCO_3$ Solution, pH 8.5) and one teaspoonful of activated charcoal were mixed in a conical flask. The flask was shaken for 30 minutes and the solution was filtered through Whatman filter paper (No. 40). The filtrate (5 mL) was taken in a volumetric flask (50 mL) and neutralized with H_2SO_4. The volume was made up by adding distilled water. Blue colour developed when few drops of ascorbic acid-mixed solution was added to the solution. After 10 minutes optical density was read by spectrophotometer using 730-840 nm wave length. Blank (without soil) sample was run simultaneously. The measure of phosphorus was estimated by utilizing equation,

$$\text{P (ppm in soil)} = \text{ppm P in aliquot} \times \frac{\text{Total volume of extract}}{\text{Aliquot taken (ml)}} \times \frac{1}{\text{Wt.of soil (g)}} (R \times F)$$

$$\text{P (Kg/ha)} = \text{ppm P in soil} \times 2.24$$

$$P_2O_5 \text{ (Kg/ha)} = \text{P (kg/ha)} \times 2.24 \times 2.29$$

3.3.2.6. Available potassium (K)

The flame photometric method was utilized to estimate the available K present in a sample (Jackson, 1958). 5 g soil sample and 25 mL of 1 N ammonium acetate was added in 150 mL Erlenmeyer flask. The solutions were shaken for 30 minutes and

filtered through Whatman filter paper (No. 40). 5 mL of filtrate was diluted with 25 mL distilled water. Atomized the solution to flame photometer (Digital flame Photometer-130, Systronics) and recorded the reading. The amount of potassium was estimated by the following formula:

Available K (Kg/ha) = $\frac{x \times v \times 2.24}{w}$

Where,

x = ppm K_2O obtained from standard curve

v = volume of extractant [i.e. 1 N CH_3COONH_4 (pH 7.0) taken for extraction of K_2O from soil (mL)]

W = weight of the soil sample taken (g)

3.4. Morphological studies

Morphological study was carried out during the flowering period (Septembers to January) of different *Ocimum* collections. Total 35 morphological characters including both the qualitative and quantitative ones were recorded (Table 3.3) based on the standard descriptor developed by NBPGR (Singh *et al*., 2003) (Table 1 in Appendix B).

Table 3.3: List of qualitative/quantitative data recorded for morphological evaluation

Sl. No.	Qualitative character	Quantitative character
1	Habit	Plant height (cm)
2	Growth habit	Canopy (cm)
3	Mode of reproduction	Leaf length (cm)
4	Stem colour	Leaf width (cm)
5	Stem shape	Leaf area (cm^2)
6	Stem pubescence	Petiole length (cm)
7	Leaf colour	Inflorescence length (cm)
8	Leaf surface	No. of whorls/inflorescence
9	Leaf margin	Bract length (cm)
10	Leaf tip	Bract width (cm)
11	Leaf shape	Peduncle length (cm)
12	Inflorescence type	Sepals length (cm)
13	Inflorescence colour	Sepals width (cm)
14	Flower colour	Petal length (cm)
15	Anther colour	Petal width (cm)
16	Seed mucilage	Stamen length (cm)
17	Seed colour	Style length (cm)
18	Seed shape	- - - -

All the 18 qualitative characters were converted into quantitative ones adopting the numerical data matrix developed by Singh *et al*. (2003) for the purpose of statistical analysis (Table 2 in Appendix B). Principal Component Analysis (PCA) was carried out to measure the significant variation of morphological diversity among the *Ocimum* taxa. The identical ordinal variables detected and eliminated particularly for the qualitative characters (e.g. mode of reproduction and plant growth habit). Further, Agglomerative Hierarchical Cluster (AHC) was constructed using dissimilarities with Euclidean Distance by Ward's method. Data were analyzed using of XLSTAT software (2015).

3.5. Chemical components analysis through GC-MS

3.5.1. Preparation of ethanolic extracts

Leaves were collected from all the 9 taxa of *Ocimum* during inflorescence period. Laves collected were shade dried and ground to powder. 100 g from each sample was dissolved in 500 mL of ethanol and kept for 7 days at room temperature (Emamuzo *et al*., 2010). The extract was then filtered using Whatman filter paper (No. 41). The solvent was recovered using a rotary evaporator (Buchi Rotavapor R-3; Buchi Labortechnik AG, Flawil, Switzerland) at 40 °C. Finally, the yellow-greenish ethanolic extracts were lyophilized and kept in a sealed labeled vial at 4 °C in dark condition until tested and analyzed.

3.5.2. Gas chromatography/mass spectrometry (GC-MS)

The GC–MS was done at Indian Institute of Science (IISc.), Bangalore. A composition of the ethanolic extracts of each *Ocimum* taxa was assessed by GC–MS. 10 µL sample was diluted in 1 mL of ethanol (1:100 dilutions). From this 100 µL of the ample was completely dried using nitrogen. Sample was derivatised using 30 µL pyridine and 50 µL of BSTFA: TMCS (99:1) and incubated at 60 °C for 60 min. Derivatised samples were subjected to GC-MS. The GC analysis was done using an Agilent Technology 7890A equipped with a DB 5 MS capillary column (30 m L x 0.25 mm ID x 0.25 µm film thicknesses dimension). The carrier gas was helium with the flow rate of 1.0 mL/min. Initial column temperature was maintained at 70 °C with 2 min hold time. Then ramp the temperature to 150 °C at the rate of 5 °C/min and again to 280 °C at the rate of 3 °C/min with 2 min hold time and finally to 20 °C temperature at the rate of 10

°C with 3 min hold time. 1.0 µL of sample was subjected to GC-MS using the split mode (split ratio 10:1). The GC-MS analysis was done on the Agilent Technologies 5975CMSD (Mass selective detector). Ionization for MS was Electron Impact Ionization and mass analyzer was single quadrupole. Mass spectra scan range was from 30 m/z to 600 m/z with +ve polarity.

3.5.3. Identification and quantification of components

The compounds were identified based on the comparison of mass spectra (MS) obtained with those of the mass spectra from the library. The relative percentage of each component was calculated by the relative percentage of the total peak area in the chromatogram. AMDIS was used as a deconvolution tool and National Institute Standard and Technology (NIST 2011) was used to identify the compounds.

3.6. Genetic variation based on RAPD fingerprinting

3.6.1. DNA extraction and purification

DNA was isolated from tender fresh leaves of all the nine taxa of *Ocimum* adopting the Cetyl trimethyl ammonium bromide method as per Murray and Thompson (1980). Please see Table 1 in Appendix C for CTAB buffer preparation. Leaves were surface sterilized with teepol (Extran) for 5 minutes and rinsed with sterile double distilled water and wiped off with clean tissue paper to remove surface water completely. Isolation of DNA from the leaves was carried out with following procedures-

a. Crushed 1 g of fresh young surface sterilized leaf tissue in liquid nitrogen with the help of pre-chilled mortar and pestle.

b. Transferred immediately the paste leaf tissue to clean autoclaved 10 mL polypropylene tube containing 5 mL of extraction buffer and mixed well to form slurry (Table 2 in Appendix C for preparation of extraction buffer).

c. Incubate homogenate at 65 °C for 1 hour in a water bath with intermittent shaking.

d. The homogenate mixture was cooled at room temperature and added an equal volume of chloroform : isoamyl alcohol (24:1) and mix by inversion for about 10 minutes.
e. The tube was centrifuged at 6500 rpm for 10 minutes at 25 ℃. The upper aqueous layer was transferred to a fresh polypropylene tube and added 2/3 volume of ice cold Isopropanol by quick gentle inversion for about 2 minutes.
f. The tube was incubated overnight at -20 ℃.
g. After overnight incubation, the tube was centrifuged at 6500 rpm for 10 minutes at 4 ℃.
h. The supernatant was discarded gently and the pellet was washed with 70% chilled ethanol.
i. Again centrifuged at 10000 rpm for 5 minutes at 4 ℃. The step repeated about 3-4 times.
j. Air dried the pellet for about 30 minutes at room temperature followed by dissolving in 1 mL of TE buffer (pH-8.0).

Purification of extracted DNA

a. DNase free RNaseA (10 µL of 10 mg/mL) was added to the genomic DNA dissolved in 1 mL TE buffer (pH 8.0) and incubated at 37 ℃ for 1 hour in a dry water bath.
b. An equal volume of Phenol: Chloroform (1:1) was then mixed to each sample by gentle inversion for 2 min and centrifuged at 10000 rpm for 10 min at 4 ℃.
c. The aqueous phase was then transferred to a sterile micro centrifuge tube and repeats the extraction twice with Chloroform: Isoamyl alcohol (24:1) and centrifuged at 6000 rpm for 5 minutes at 4 ℃.
d. Each sample was mixed with 0.1 volume of 3M sodium acetate (pH 5.2) and double volume of isopropanol and stored overnight for DNA precipitation. It

was then centrifuged at 10000 rpm for 5 minutes at 4ºC. The supernatant was discarded and the pellet was washed with 1 mL of 70% chilled ethanol thrice and dried at room temperature.

e. Finally, the DNA pellet was dissolved in 100 µL TE buffer (pH 8.0) and stored at -20 °C for quantification.

3.6.2. Quantification of DNA

Reliable measurement of DNA concentration is important for applications in molecular biology including amplification of target DNA by polymerase chain reaction and complete digestion of DNA by restriction enzymes. DNA quantification is generally carried out by spectrophotometric measurements or by agarose gel analysis for DNA qualification. Both the methods were used in the present study.

i. Spectrophotometric measurement

The isolated DNA was measured using UV-Spectrophotometer (CECIL, CE 7200, Germany). 998 µL of TE buffer was taken in a quartz cuvette and 2 µL of extracted genomic DNA was added to it. The optical density (absorbance A) was taken at 260 nm (A_{260}) and 280 nm (A_{280}). For an ideal DNA preparation the A_{260}/A_{280} ratio should be ≥ 1.8 (Sambrook and Russed, 2001). DNA concentration was estimated by employing the following formula:

$$\text{Amount of DNA (ng/µL)} = \frac{\text{OD260} \times 50 \times \text{dilution factor}}{1000}$$

For double stranded genomic DNA, 1 O.D. corresponds to 50 µg/mL of DNA (Sambrook and Russed, 2001).

$$\text{Dilution factor} = \frac{998\ \mu\text{L TE buffer } 2\ \mu\text{L DNA}}{2\mu\text{L}} = 500$$

ii. Quality check through agarose gel

Qualification of isolated genomic DNA of nine genotypes was performed by 1 % agarose gel electrophoresis. 1 % agrose mixed with 1X TAE buffer for gel casting. 5 µL Ethidium bromide (Genei) was added and pour to the gel casting tray. 2 µL DNA of each genotype was mixed with 8 µL 1x TAE and 2 µL gel loading dye on a 0.2 mL tube and loaded onto each well for electrophoresis. The gel was allowed to run at constant voltage of 60v for about 1.5 hours till the tracking dye reaches end of the gel. Then the

gel was analysed under UV-translluminator to check the quality. Photographs were taken using Gel Doc system (Bio-Rad, Gel Doc-2000).

3.6.3. RAPD-PCR analysis

RAPD amplification was performed with extracted and purified genomic DNA from nine taxa of *Ocimum* using 10 RAPD primers (Genei) (Table 3.4). Primers were selected on the basis of previous works on *Ocimum* species (Saha et al., 2012; Singh, 2004). Each RAPD PCR mixture (25 µL) contained 25 ng genomic DNA as template, 2.5 µL PCR Assay buffer (1X), 2.5 µL of each dNTPs (final concentration of 200 µM each dNTP), 1µL $MgCl_2$ (final concentration of 1.0 mM $MgCl_2$), 0.5 µL Taq DNA polymerase (3U/µL) and 2.5 µL of each primers (final concentration of 1µM) (Table 3 in Appendix C for composition). The final volume of 25 µL was made up with PCR grade water (Genei, Pvt. Ltd., Bangalore, India).

The PCR were performed in a thermo-cycler (Perkin Elmer Gene Amp 2400 PCR system) programmed as follows.

Step 1: Initial denaturation at 94 °C for 4 min

Step 2: Denaturation at 94 °C for 40 sec
Annealing at 36 °C for 40 sec
Extension at 72 °C for 60 sec
} 35 cycles

Step 3: Final extension at 72 °C for 7 min

Step 4: 4 °C forever

Table 3.4: List of RAPD primers with their sequence

Sl. No	Primer code	Sequence (5´-3´)
1	BGM-1	TGCCGAGCTG
2	BGM-3	GTGACGTAGG
3	BGM-4	AGGTCTTGGG
4	BGM-5	GGTGCTGCGC
5	BGM-7	CTGGGCAACT
6	BGM-9	GAAACGGGTG
7	BGM-12	GGAACGGGTG

8	BGM-13	CATCCCGACA
9	BGM-15	GCACGCCGGA
10	BGM-17	CTATCGCCGC

Note: All the primers (oligos) RAPD primers were supplied as lyophilized powder form from Genei, Pvt. Ltd., Bangalore, India. The hydration was performed by adding 100 µL sterile PCR grade water (Genei) and incubation the vial at 65 °C for 10 minutes. After tapping and vortexing for a few seconds, the vials were stored at -20 °C.

3.6.4. Agarose gel electrophoresis of RAPD-PCR band profiling

The PCR amplified products were separated on horizontal gel electrophoresis using 1 % agarose gel containing 0.5 µg/mL ethidium bromide (EtBr). 7µL EtBr was mixed with warmed agarose before pouring in casting tray. Solidified gel was placed within the submerge gel and immersed with sufficient 1X TAE buffer (Table 4 in Appendix C for composition). 10 µL PCR products of each sample mixed with 2 µL gel loading dye and then loaded in the wells of the agarose gel. A low range DNA ladder (100-3000bp) (Genei) was used as known molecular weight marker and loaded separately. Gel electrophoresis was done at a constant voltage of 70V for 1.5 hours until the tracking dye reaches at the end of the gel. The gel was observed in a UV-transilluminator and photographed by Gel Doc System (Bio-Rad, Gel Doc-2000).

The clear and visible amplified bands from the photographic gels were considered for the analysis. The amplified bands were scored as 1 or 0 on the basis of present and absent of bands to generate a binary data matrix. Only reproducible bands were considered for the analysis. The binary data matrix was used to calculate Jaccard's similarity coefficient among 9 *Ocimum* taxa using the Simqual module of NTsys-PC (Numerical Taxonomy System version 2.1) (Rohlf, 2000). These distance coefficients were used to construct dendrogram using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) employing the Sequential Agglomerative Hierarchical and Nested (SAHN) algorithm for determining the genetic diversity and relationships among the genotypes. Principle coordinate analysis (PCA) was performed using the EIGEN and PROJ modules of NTsys-PC.

The polymorphic information content (PIC) is generally used in genetics as a measure of polymorphism for a marker locus utilizing linkage analysis. The PIC value was

computed using the formula- PIC= $1-\Sigma p_i^2$, where, p_i is the frequency of the i^{th} allele of the locus in the set of nine *Ocimum* taxa (Anderson *et al*., 1993).

3.7. Propagation and conservation of *Ocimum*

3.7.1. Germination of seeds

Seeds from all the 9 taxa of *Ocimum* were collected from the AASM garden of Raiganj University. The collected seeds were allowed to germinate on moistened cotton bed in petri dishes for 7 days. The bed was kept moistened with periodic addition of distilled water as required. Seed germination was carried out during summer and winter seasons at room temperature. Three replicates were maintained keeping 50 seeds in each replicate. Seed germination percentage was calculated using the following formula-

$$\text{Germination percentage } (\%) = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds tested}} \times 100$$

3.7.2. Multiplication through stem cutting

The healthy apical portion of each taxa of *Ocimum* was selected for preparing the cutting. Length of the cutting was kept 8-10 cm in height keeping at least 4-5 leaves at the top. The bottom portion of the cuttings were submersed in water and monitored regularly to record the root growth. Care was taken to maintain proper hygienic condition. The samples were kept at room temperature and 8 hours in day light. Three replicates were maintained for each taxa keeping 50 cuttings in each replicate. The rooting percentage was calculated using the following formula-

$$\text{Rooting percentage } (\%) = \frac{\text{Total number of cutting rooted}}{\text{Total number cutting planted}} \times 100$$