International Journal of Leading Research Publication
IJLRP

© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

# Ecology and population genetics studies of Alnus Nepalensis in sub-Himalayan West Bengal and Sikkim

*[1]Sanghati Bhattacharya, [2]Arnab Sen and [3]Malay Bhattacharya*

*[1]Molecular Genetics Laboratory, Department of Botany, University of North Bengal, Siliguri, 734013*

*[2]Bioinformatics facility, Department of Botany, University of North Bengal, Siliguri, 734013*

*[3]Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Siliguri, 734013*

**ABSTRACT**

***Alnus nepalensis, an actinorhizal plant, is an excellent example of successional plant found as early visitor of the landslide regions. Our present study includes a through survey of A. nepalensis in sub Himalayan West Bengal and Sikkim. The total study area was divided into three sections keeping in mind about the vehicle-route of Darjeeling and Gangtok. Soil from the bottom of collected A. nepalensis plants were taken during field visit to estimate the soil nutrients present within it. Analysis of soil nutrient revealed that extremely variable soil nutrient but with slightly high soil carbon was found to favour the growth of A. nepalensis in studied region. Germplasm collected from specified area were used for population genetics study. PCR followed by data analysis through coefficient matrix revealed that entire population of A. nepalensis has segregated depending on their geographical distribution and in this respect river Teesta act as geographical barrier for dispersal of germplasm.***

***Keyword:*** *Alnus nepalensis, Population genetics, Actinorhizal, Ecology*

## 1. INTRODUCTION

Actinorhizal plants are found in different geographical location of the earth, that covers arctic Tundra (Dryas spp.) and alpine forest (Alnus sp., Coriaria sp. etc) to costal and xeric regions (Casuarina sp.). In Nepal, Alnus nepalensis is found at a wide altitudinal range, descending as low as 500 m; but most common from 900m to 2700m. At lower altitude, A. nepalensis is mostly found in moist regions, such as riverside but also found abundant in rough rocky land exposed by landslides, and cultivable land (Sharma, 2012). A record regarding the presence of A. nepalensis in sub-himalayan West Bengal and Sikkim during the British period is available in the Bengal District Gazetteers by L S S O'MALLEY (1907). Alder being a major constituent of forest flora, particularly in the high altitude Himalayan region play a pivotal role in early succession of forest ecosystem and maintaining the fertility of forest soil (Benson and Silvester, 1993). Though reports of ecology and population genetics of Alnus nepalensis are available, but no work has been done on the ecological aspect, genetic diversity, population genetics studies of A. nepalensis in the region of Sub-Himalayan West Bengal and Sikkim.

The above information prompted us to an in-depth study of the ecological aspects, soil nutritional factors along with genetic diversity of A. nepalensis in Sub-Himalayan West Bengal and Sikkim.

## 2. MATERIAL AND METHODS

**The study area:**

Eastern Himalayas is famous for its rich biodiversity. The study area was selected in view of its rich biodiversity and comprises parts of eastern Himalayas like Darjeeling and Kalimpong districts of West Bengal and Gangtok with its adjoining areas in Sikkim. Darjeeling and Kalimpong districts lie in the northernmost part of West Bengal, a state in eastern India in the foothills of the Himalayas. The study area of Darjeeling and Kalimpong, lies in between 26°31′ to 27°13′N latitude and 87°59′ to 88°53′E longitude (Das and Ghose, 2011). It is situated on the flunks of the eastern Himalayas, and is bordered by the Tibetans plateau in the north, Nepal in west, Bhutan in the east and Jalpaiguri and Dinajpur district of West Bengal in south (Figure 1). Geographically, the districts are mostly mountainous with plains known as the Terai. Darjeeling district includes three subdivisions - Kurseong, Mirik and Siliguri. Kurseong and Mirik subdivisions are hill terrain while Siliguri lies in the plains. Gangtok, the capital of Sikkim, is the 22nd Himalayan state of India, located between 27°04′46″ to 28°07'48″N latitude and 88°00′58″ to 88°55′25″E longitude (Basistha et al., 2010). It is enclosed by the Tibetan plateau in the north, Nepal in west, Bhutan in the east and Darjeeling in the south. The state is predominantly mountainous; with no plain land spread over 7096 sq km (Basistha et al., 2010). However

© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

our study area covers Gangtok and adjoining regions, popularly known as East-Sikkim which lies between 27°17'25" N to 27°20'11" N latitude and 88°35'27" E to 88°36'55"E longitude (Table 1).

**Ecological studies:**

Literatures regarding sampling intensity of Alnus nepalensis of the study area are extremely lacking. A total of 18 different sites were selected throughout the study area to record different geophysico-chemical parameters. The morphological characters of plant were also recorded. The sites were named according to the information obtained from the local people. The study sites include gentle slopes, steep slopes, valley, landslides area and non-riverside areas. Different geo-physico-chemical parameters like altitude, latitude and longitude were recorded by Global Positioning System (GPS) (Gramin EXrex Vista H). Other data, such as aerial temperature and humidity were recorded using digital thermometer (Multi thermometer, CE make) and humidity meter (Model LR6 Mignon AA, England make). Site cover, vegetation type, management status and species association etc were also studied in each of the 18 sites.

**Germplasm collection:**

The germplasm (leaves), for carrying out experiments based on the objectives were collected from various location and altitude of geophysico-chemical study sites for DNA isolation. Collection was done in the middle of April. A. nepalensis leaves were collected from 17 plants of study area A, 14 plants of study area B and 12 plants of study area C. In the sampling area plants were located approximately 4-7 meters apart from each other (Table 2).

**Soil collection:**

Soils were collected from around the nodules of the germplasm collection sites of A. nepalensis in the month of April. For detail of the collection sites refer Table 3. Essential data like soil temperature, soil texture (field method) and colour were recorded during soil collection.

**Soil analysis:**

Soil analysis was conducted in laboratory. Soil pH, soil moisture, organic carbon, total organic matter, total Nitrogen, available Potassium, Phosphorus etc. were quantified during the soil analysis.

**Genomic DNA isolation from A. nepalensis:**

Previously stored A. nepalensis leaves were taken out from the cryogenic container (CRYOSEAL-IR-7, Model no. 023017) and used for isolation of genomic DNA by the following steps.

Five (5) grams of fresh leaves were taken in a motor and pestle, and ground it into a fine powder along with liquid N2. The pulverized material was taken in an Oakridge tube containing 15 ml of pre-warmed CTAB extraction buffer (65°C) and gently swirled. The tube was then incubated in a water bath (Rivotek) for 1 hour at 65°C with occasional mixing by gentle swirling. Following this, an equal volume of chloroform (Merck India, Cat# 822265): isoamyl alcohol (Merck India, Cat# 8.18969.1000) (24:4) was added and the mixture was mixed gently by inverting the tube upside down. The mixture was centrifuged (REMI make, Model No.C-24) at 6000 rpm for 15 minutes at 25°C and supernatant was transferred carefully to a fresh tube. Equal volume of ice cold Isopropanol (Merck India, Cat # 17813) was added to the final supernatant. Upon gentle swirling the DNA - CTAB complex precipitated as a whitish network and was spooled out of the solution using a bent Pasteur pipette. (For some strain, DNA was not observed as DNA- CTAB complex, but after adding ice cold isopropanol and incubation at 4°C for 30 minutes followed by centrifugation at 12000 rpm for 15 minutes at 4°C DNA precipitation was observed). DNA was then washed in 70% ethyl alcohol (BDH Cat#10107) and allowed to dry in air. It was finally dissolved in 500 µl of 1X TE buffer (pH 7.4).

**Purification of DNA:**

RNA, protein and polysaccharides are most important contaminants found in crude DNA preparation and it is importance to remove them as these will hamperd the further downstream processing. CTAB DNA extraction buffer helps in elimination of polysaccharides from DNA preparations to a large extent. The RNA was removed by treating the sample with RNase enzyme. Extraction with phenol : chloroform following RNase treatment was also employed for eliminating RNA and most of proteins by using the following protocol.

**Protein purification:**

For removal of protein from DNA samples, the dissolved DNA was extracted with an equal volume of equilibrated phenol (pH 8.0) (Sigma, Cat#P4557-400ML) and mixed properly for 10-15 minutes. It was then centrifuged at 12000 rpm for 15 minutes at 25°C. The upper aqueous layer was taken in a fresh tube and extracted with an equal volume of chloroform: Isoamyl alcohol (24:1) and then centrifuged at 10000 rpm for 10 minutes at 25°C. The upper aqueous layer was taken in a fresh tube. To it 0.1

© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

volume of 3M sodium acetate (pH 5.2) (Sigma, Cat#S-9513) and double volume of Ice cold absolute ethyl (BDH Cat#10107) was added and precipitated at 4°C for 30 minutes in a cooling centrifuge (REMI make, Model No.C-24) at 12,000 rpm. The pellet obtained was washed in 70% ethyl alcohol, dried and dissolved in 50 µl of 1X TE buffer (pH 7.4) (refer appendix C for composition).

**Purification of RNA:**

RNase-A (50 µg/ml) (Sigma, Cat#R-4875) was added to the genomic DNA dissolved in 500 µl of 1X TE buffer (pH 7.4) and it was incubated at 37°C for 1 hr in a dry water bath (GeNeiTM make, Cat#107173). An equal volume of chloroform : Isoamyl alcohol (24:1) was added and mixed properly. The content was centrifuged at 10000 rpm for 15 minutes at room temperature. The upper aqueous layer was transferred to a fresh microcentrifuge tube. To this aqueous phase 0.1 volume of 3M sodium acetate (pH 5.2) and double volume of ice cold ethanol (100%) was then added for DNA precipitation. It was then centrifuged at 13000 rpm for 30 minutes at 4°C. The DNA pellet obtained was washed in 80 % ethyl alcohol, air dried and finally dissolved in 50 µl of 1X TE (pH 7.4).

**Quantification of DNA using spectrophotometer:**

DNA quantification is important for several 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. Both the methods were employed in the present study. Spectrophotometer (Thermo UVI spectrophotometer, Thermo Electron Corporation, England UK) was calibrated at 260 nm and 280 nm by taking 600µl 1X TE buffer in a cuvette (*Photon cell, New Jersey, USA) DNA (6µl diluted in 594 µl of 1X TE) was taken in a cuvette and the optical density (OD) was recorded at both 260 nm and 280 nm.

**The DNA concentration (ng/µl) was measured by using the following formula:**

Amount of DNA (ng/µl) = (OD260 X 50 X DF) / 1000 (DF stands for "dilution factor"). The quality of DNA was judged from the OD values recorded at 260nm and 280 nm. The DNA showing A260/A280 ratio around 1.8 was chosen for further PCR-based methods.

**DNA analysis by gel electrophoresis:**

A pure molecular biology grade, DNase free Agarose (0.8%, gelling temperature 36°C) was used to cast the gel in 0.5 X TBE (Tris-Borate-EDTA) buffer (refer appendix C for composition) containing 7µl of Ethidium Bromide (10mg/ml) on gel platform (100x70mm) (Tarsons, Cat # 7024). Five (5µl) of DNA samples were mixed with 3µl of 6X gel loading dye (refer appendix C for composition) and loaded in the well carefully. Lambda DNA/ EcoRI/ HindIII double digest (2 µl) and 100 bp ladder were used as molecular markers to determine the size of genomic DNA in the adjacent well. The gel was run at 50 volt (V) and 100 milliampere for 1.5 hour in the midi submarine Electrophoresis Unit (Tarsons, Cat #7050) connected to the electrophoresis Power Supply Unit (Tarsons, Cat #7090). After the run time was over the gel was visualized on a UV Transilluminator (GeNeiTM, cat #SF805). The molecular size of the genomic DNA was detected in the form of bands. The size of the bands was estimated with Photo Capt Version 12.4, (Vi LHber lourmat, USA).

**Gel Photography:**

The gel was photographed by using an indigenously built Gel Documentation System fitted with Canon SLR camera (EOS 350D) bearing Marumi orange filter (58mm YA2, Marumi, Japan). The software in usage for the purpose was EOS utility software.

**RAPD-PCR analysis:**

RAPD - PCR were done to study the genetic diversity of A. nepalensis. A total of 40 oligonucleotide random primers (Chromus Biotech made) were screened for forty three (43) samples of A. nepalensis collected from different areas under study. Furthermore, successfully amplified primers were used for downstream amplifications (Table 4).

**PCR-RAPD amplification:**

In a sterile 0.2 ml thin walled PCR tube (Tarson, Cat #500050) following components were added sequentially for PCR reaction volume of 25 µl, in the order as given below.

Ready mix TM Taq PCR reaction mix with MgCl2 - 12.5µl
Primer – 1.25 µl (0.25 µM)
Template DNA – 2 µl (25 ng)
Pyrogen free water – to a final volume of 25 µl

One negative control tube was also prepared by PCR mix without DNA.

The ingredients were mixed evenly in a SpinWin PCR micro centrifuge (Tarson, Cat# 1000).

The PCR reactions were performed in Applied Biosystems, Thermal Cycler, 2720 PCR machine.

© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

The amplification cycle consisted of 35 cycles with following specifications:

Cycle 1: Denaturation at 94°C for 4 minutes, primer annealing at 58°C for 1 minute, primer extension at 72°C for 1 minute.

Cycle 2-34: Denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute, primer extension at 72°C for 1 minute.

Cycle 35: Denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute, primer extension at 72°C for 7 minutes.

**RAPD-PCR gel analysis:**

All the PCR product of RAPD, after their respective amplifications were separated on 1.5 % Agarose gel containing 7µl Ethidium Bromide solution run in 0.5 X TBE buffer (pH 8.0). PCR product (12µl) was mixed with 4µl of gel loading dye (refer appendix C for composition), mixed well and then loaded in the Agarose gel and run for 2.5 hours. Lambda DNA/ EcoRI/ HindIII double digest (2µl) and 100 bp (base pair) (2µl) ladder were used as molecular markers to determine the size of genomic DNA. The estimation of band sizes of the genomic DNA and photography were done as mentioned earlier. The PCR were carried out and only the clear and reproducible bands were compared with the adjacent marker DNA to estimate the sizes.

**Data analysis:**

The RAPD-PCR fingerprints were scored in binary form i.e. the presence of the band as 1 and absence of band as 0 and assembled in data matrix. The data was primarily analyses by NTSYSpc2 and POPGENE programme package. Only distinct RAPD bands were recorded. Genetic variations in terms of genetic similarity as well as distance amongst individuals in each population and within population were also calculated by using binary matrix analysis.

## 3. RESULTS AND DISCUSSION

**Study area and ecology:**

Ecology and habitat related information of Alnus nepalensis in sub Himalayan West Bengal and Sikkim were depicted in Table 5. A. nepalensis plant was found to be growing naturally in area occupied by evergreen hilly forest, stony slopes, water channel areas and steep grassland. In the field study in Sub Himalayan West Bengal and Sikkim, A. nepalemsis was found to be naturally growing in the area with a geographical boundary of 26°87'78" to 27°34'71"N and 88°25'00" to 88°61'76"E and with relative humidity 19% to 35%. Sharma and Ambasht (1986) reported that this plant is commonly found at an altitude ranging from 5479ft to 9842.51ft in Nepal, with a temperate climate, but during field study, it was found that this plant grows mainly at an altitude ranging from 3616ft to 7598ft with soil temperature in between 3.4 to 10°C and air temperature 6 to 19°C. It was observed that young seedlings get defoliated by frost, and were very often killed. However, as it occurs naturally approximately up to 7600ft. in areas with low frost. These differences may be due to microclimate or germinating time. Particularly at lower elevations it is characteristic of moist sites such as riverside but also colonize in gravelly land exposed by landslides, and abandoned cultivable land (Sharma, 2012). Field work also confirms the altitudinal limit of 6000ft.-7000ft. as favorable height for growth of A. nepalensis in studies region. A. nepalensis was found to grow in varied soil types. The study area consisted of various combination of soil i.e. loamy, sandy, clayish etc, with colour variation from reddish brown to blackish brown in hilly vertical slopes. Slopes are important ecological component in identifying and evaluating potential environment impacts related to landform alteration along with vegetation successions. Soil pH of the study area was measured and it was found to be acidic with a range from 3.85 to 6.22. Similar soil pH range was observed in hilly regions of Darjeeling district of West Bengal by Mukherjee (2009). Soil moisture of the study area was extremely variable. Ranging from 10 to 60%. So, this wide range of variable soil moisture favors growth of the plant. It has also been observed that A. nepalensis grows mainly in the land-slide prone area, underlining its importance as successional plant. All matured A. nepalensis plants in the study area were found to bear root nodules due to symbiotic association with Frankia - an actinomycete to fix atmospheric nitrogen. Seeds produced in cones are abundant, winged and easily shed by slight shaking. This aid in easy dispersal and may be one of the important reason for its growth in landslide prone areas.

**Plant morphology:**

Morphological data of A. nepalensis were depicted in Table 5. Fully matured A. nepalensis trees from Sub-Himalayan region were found to around 25-33meters in length and 47±5cm in trunk circumference. Leaves are broad, dark green in colour, entire, linear, rough lower with slight shiny upper surface. Leaf size varied with different growing conditions and location. Leaf size of A. nepalensis was 6.54 to 12.86cm in length and 3.72 to

© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

6.34cm in width. Morphological study (Table 5) revealed that average inner branch distance of A. nepalensis trees were 23.63±10cm. It has been also noticed that inner branch distance of A. nepalensis trees were varied with altitude. In higher altitude inner branch distances found higher while in lower altitude, inner branch distance was comparatively shorter. Distances between root nodule clusters were also variable. Average inter nodular distance was found to be 3.5±1.5cm. A cluster of nodules borne in a matured plant measured 33.28 to 123.05gm (Figure 2). However, inter nodular distance found to be inversely correlated with altitude, but positively correlated with weight of nodules (Figure 3).

**Soil analysis:**

Soil samples were collected during the collection of nodules. Soil, around clumped and scatter root nodules of A. nepalensis were collected from different geographical location. Different soil parameters like pH, organic carbon, soil nitrogen, potash, phosphorus and sulphur content were estimated (Table 6). pH of the collected soil samples was found to be acidic (pH 3.85-6.22). Phosphate concentration varied considerably from 10 to 45ppm (parts per million). Amongst the study area lowest concentration of phosphate was estimated at Sonada (10ppm) and highest at Mongpoo (45ppm). Average sulphur concentration was estimated to be 30±15ppm. The soil sulphur content at Ranipool in Sikkim was found relatively high i.e. 45ppm. The test results were found extremely variable and were totally dependent on micro-ecological factors. Though, highly significant correlation between soil nitrogen with organic carbon has been found. A correlation study between the weight of nodules and amount of nitrogen estimated in particular nodulated soil was made. Result showed that, weight of nodules was found to be more in the places where amount of soil nitrogen were less (Figure 3). In Deorali bazaar soil nitrogen estimated were 0.057% (Table 6) and weight of nodule clusters were found to be 123.05gm (Table 5). Likewise in Lower Burtuk road weight of nodules were found to be 111.02gm and nitrogen content estimated to be 0.063%. However, the places like Upper Tadong, Ghoom, Sonada the amount of nodules were found to be very less i.e. 62.17gm, 68.29gm and 74.17gm and the soil nitrogen in those places were estimated to be 0.298%, 0.297%, and 0.245% respectively. So, from the result a strong negative correlation had been seen. This result may suggest that excellent quality nodulation in A. nepalensis takes place where the amount of nitrogen is less (Figure 4).

Population genetics and genetic diversity studies of A. nepalensis:

**DNA extraction:**

Genomic DNA form A. nepalensis leaves were isolated using the Doyle and Doyle, (1987) standard protocol with minor modifications. A whitish network of nucleic acid and starch, was precipitated as DNA-CTAB complex, and then subjected to purification process for complete elimination of polysaccharide. DNA obtained was used for further downstream processing. The presence of DNA bands were observed by using agarose gel electrophoresis.

**DNA quantification and quality check:**

The DNA quantification was done by UV spectrophotometer using 260nm and 280nm filters. The ration of A260/A280 was calculated for each sample. All the experiments were performed in three or more replicates and the samples showing the ratio of around 1.8 were chosen for further studies. The concentration of the isolated DNA samples was varied from 215-2465ng/µl. This showed the isolated DNA samples were reasonably pure. The intactness of the DNA was determine with the help of 0.8% Agarose gel electrophoresis using λ DNA-EcoRI+Hind III double digest and 100bp ladder. The size of the bands was found to be approximately around 20-21kb (Figure 5).

**RAPD analysis:**

RAPD fingerprinting was used to study the genetic diversity among indigenous A. nepalensis species from sub-Himalayan study area of my interest. All the collected DNA samples were tried to amplify with 40 different 10-mer primers (refer materials and methods for primer sequences). Of the 40 primers screened, 34 resulted in producing distinct and scorable bands. Representatives of RAPD profile photograph of the A. nepalensis were given in Figure 6. Total number of amplified bands, number of monomorphic, polymorphic bands, and size of the amplified bands and percentage of polymorphism generated by the RAPD primers is tabulated in Table 7. In study area A, 32 primers resulted in producing of 367 bands ranging in between 220bp to 2100bp of which 16 were monomorphic while rests were polymorphic (Table 7). The percentage of polymorphism was found to be 95.64%. The number of polymorphic bands generated by each decamer primers ranged in between 8 and 17. Likewise, 30 primers resulted in production of 356 bands in study area B. The fragments found, ranging in between 200 bp to 1950 bp of which 17 were monomorphic and

© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

rest 339 were polymorphic (Table 7). The percentage of polymorphism was found to be 95.22%. The number of polymorphic bands generated by each decamer primers ranged in between 7 and 16. However, 28 monomorphic bands and 324 polymorphic bands were recorded from study area C by successful amplification of 31 primers. The amplified products ranged in size from 140 bp to 2000 bp. The amplification pattern showed that the percentage of polymorphism was 92.04% and the number of polymorphic bands generated by each decamer primer ranged in between 6 and 15 (Table 7). Total numbers of 1075 major scorable bands were obtained, among which 61 were monomorphic and 1014 polymorphic bands were recorded from the whole study and the amplification product ranged from 140 bp to 2100 bp (Table 7). A. nepalensis collected from population I showed highest polymorphism while population III showed lowest polymorphism amongst all three populations.

**Similarity matrix analysis:**

The similarity matrix was obtained using Dice coefficient of similarity (Nei and Li 1979). The lowest similarity (43.5%) was observed between samples collected from Tibetian monastery (C10-6700ft) and Lower Sichey (C32-4857ft), while the highest similarity (95%) found between samples collected from Upper Sonada (C12-6915ft) and Ghoom (C13-7091ft). The resulting dendrogram, generated by using 34 oligonucleotide primers has been shown in Figure 7. Dendrogram showed three distinct clusters and represented by 15, 15 and 13 samples respectively. It is to note that while collecting samples, the collection sites were divided into two parts. In study area I, samples collection were conducted from Darjeeling hills which was further divided into route from Darjeeling to Kurseong (collection route A) and the other was from Ghoom to Mirik via Pashupathi (collection route B). The study area II collections were conducted from Kalimpong to Gangtok via Rangpo (collection route C) which is the left side of Tista river. It was observe that in RAPD analysis the samples clustered almost exactly on the basis of their geographical locations. The samples C1 to C17 (other than C14 and C15) grouped together as cluster I were from collection route A. C18 to C31 and C14 grouped as cluster II (collection route B) and C32 to C43 along with C15 as cluster III (collection route C). Henceforth, they will be called as POP A, B and C respectively. However, sample C14 (Dooteria forest at 7033ft.) and sample C15 (Senchal at 7362ft.) though belonged to cluster II and cluster III respectively, but both the samples were collected from collection route A (POP A).

The above result clearly showed that the genetic distance and similarity are clearly dependent on the geographical location of the populations. The route, which is via Pashupathi, is relatively less popular and runs through the western part of Darjeeling hills. Whereas, the POP A is the more popular route runs through the eastern part. It has been shown the population of A. nepalensis, the eastern part of Darjeeling hills is different than that of the western part. Similarly POP C which is on the left side of river Teesta (incidentally POP A and B are situated at the right side of Teesta river) clustered in a completely different manner (cluster C).

**Genetic similarity and distance between populations:**

Closeness between the three populations in terms of Genetic similarity & distance were also measured. Of the 34 primers producing distinct scorable bands in all the three populations, 26 primers were found to be common, producing scorable bands in all three populations individually (Table 8). Therefore it was considered that, bands produced by 26 primers were used to measure the genetic variation between populations. Table 8 shows that population of POP A and B acquired 6 common bands whereas POP A and POP C shared 5 bands. POP B and C on the other hand shared 4 common bands with each other. Table 8 also revealed that primer sequences of OPA01, OPA04, OPA13, OPB04, OPB06, OPB12, and OPB13 were found to be present in the A. nepalensis samples present in POP A and B. Likewise A. nepalensis of POPA and C shared primer sequence of OPA10, OPA13, OPA15, OPB141 and OPB12. Similarly, primer sequence of OPB05, OPB07, OPB12, and OPB14 were found in A. nepalensis samples present in POP B and C. A binary matrix has been prepared by counting the presence and absence of common monomorphic bands in all three populations and subsequently the similarity between populations was calculated (Table 9). The similarity coefficient amongst three populations has been found to be 81.14-88.57%. The similarity between population A and B was found to be highest and was 88.57%, while the similarity between population B and C was found to be lowest at 81.14%. The similarity between population A and C was found to be 83.42%. The resulting dendrogram (Figure 8) showed POP A and B are relatively closer than POP C. In this case Tista River may be considered as geographical barrier for dispersal of germplasm on its two sides.

**7. CONCLUSION**

The distribution of A. nepalensis is restricted within altitudinal boundary ranging from 3616ft to 7598ft in sub

Himalayan West Bengal and Sikkim. micro-ecological factors were also found to be equally important for proper growth of this plant in the fragile landslide prone areas. Specifically 19% to 31% relative humidity and acidic soil conditions favoured its growth. Study of Alnus population showed that they can exist under extremely variable soil nutrient conditions but favors moderate to high soil carbon.

Population genetics study revealed that entire population of A. nepalensis in sub-Himalayan West Bengal and Sikkim clusters depending on their geographical distribution. The individuals collected from specific area were found to be genetically close to each other with minor exceptions. A. nepalensis collected from study area A showed high and C showed low percentage of DNA polymorphism. It has been also found that A. nepalensis collected from eastern part (study area A) of Darjeeling hills were genetically more similar to western part (study area B) of Darjeeling hills, while genetically distant from Kalimpong and Gangtok hills (study area C), which situated other side of river Teesta. River Teesta bisects collection sites, keeping population I and II on its right and population III on its left bank. So, river Teesta may act as a geographical barrier for dispersal of A. nepalensis germplasm.

## REFERENCES

1. Basistha, B. C., Sharma, N. P., Lepcha, L., Arrawatia, M. L., & Sen, A. (2010). Ecology of Hippophae salicifolia D. Don of temperate and sub-alpine forests of North Sikkim Himalayas—a case study. Symbiosis, 50(1-2), 87-95.
2. Benson, D. R., & Silvester, W. B. (1993). Biology of Frankia strains, actinomycete symbionts of actinorhizal plants. Microbiological reviews, 57(2), 293-319.
3. Das, A. P., & Ghosh, C. (2011). Plant wealth of Darjiling and Sikkim Himalayas vis-avis conservation. NBU J. Pl. Sci, 5(1), 2533.
4. Doyle, J. J., & Doyle, J. L. (1987). CTAB DNA extraction in plants. Phytochemical Bulletin, 19, 11-15.
5. Mukherjee, D. (2009). Medicinal plant in Darjeeling hills. Krishi Sandesh - Miazik International Volunteer Center, Japan, 118121.
6. Nei, M., & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences, 76(10), 5269-5273.
7. O'malley, L. (1907). Bengal District Gazetteers -Darjeeling.
8. Sharma, E., & Ambasht, R. S. (1986). Root nodule age-class transition, production and decomposition in an age sequence of Alnus nepalensis plantation stands in the Eastern Himalayas. Journal of Applied Ecology, 689-701.
9. Sharma, R. P. (2012). Modelling dry matter allocation within Alnus nepalensis D. Don trees in Nepal. International Journal of Biodiversity and Conservation, 4(2), 4753.

**Figure legend:**

Figure 1: The area covered under study.

Figure 2: Clusters of nodules collected from study area.

Figure 3: Plant morphology.

Figure 4: Correlation of weight of nodules with soil nitrogen.

Figure 5: The size of the bands.

Figure 6: Representatives of RAPD profile photograph of the A. nepalensis.

Figure 7: The resulting dendrogram, generated by using 34 oligonucleotide primers.

Figure 8: The resulting dendrogram showed POP A and B are relatively closer than POP C

Table 1: Detail information of study area

| Study area | Altitude (ft.) | Rainfall (mm) | Soil type | Ecological region |
|---|---|---|---|---|
| Darjeeling | 5473-7431 | 82 - 3092 | Loamy | Temperate |
| Kurseong | 4888-5473 | 36 - 3736 | Sandy | Worm & temperate |
| Mirik | 5267 | 24 - 2876 | Clayish | & temperate |
| Kalimpong | 3811-7598 | 34 - 2395 | Clayish | Mild, worm & temperate |
| Gangtok | 3926-4857 | 56 - 3626 | Rocky | Mild - temperate |
| Namchi | 4154 | 50 - 2699 | Loamy | Temperate |

Table2: Detail description of collection site (Sl no=Serial number)

| Sl no. | Study area | Region | Site name | Altitude(ft) | Latitude (°N) | Longitude (°E) |
|---|---|---|---|---|---|---|
| A-1 | Study area - A | Kurseong | Baghgora | 4959 | 26.879509 | 88.278594 |
| A -2 | | | Hill cart road | 4905 | 26.880552 | 88.277335 |
| A -3 | | | Montiviot | 4888 | 26.886389 | 88.277721 |
| A -4 | | | St. Marys post office | 5096 | 26.894273 | 88.279787 |
| A -5 | | | Chaita Pani Tea garden | 5383 | 26.903776 | 88.292014 |
| A -6 | | | Edenvale Tea garden | 5473 | 26.916533 | 88.289731 |
| A -7 | | Sonada | Lower Sonada | 5842 | 26.936511 | 88.290210 |
| A -8 | | | Sonada khasmahal | 6238 | 26.957725 | 88.269532 |
| A -9 | | | Sonada post office | 6389 | 26.960081 | 88.271320 |
| A -10 | | | Tibetian monastery | 6700 | 26.966750 | 88.274087 |
| A -11 | | | Sonada forest | 7174 | 26.971903 | 88.280224 |
| A -12 | | | Upper Sonada | 6915 | 26.984010 | 88.278330 |
| A -13 | | Ghoom | Ghoom | 7091 | 26.989249 | 88.270367 |
| A -14 | | | Dooteria forest | 7033 | 26.995121 | 88.259360 |
| A -15 | | | Senchal | 7362 | 27.003892 | 88.260622 |
| A -16 | | | Bhalikhop | 7347 | 27.008180 | 88.259458 |
| A -17 | | | Ghoom monastery | 7293 | 27.011612 | 88.250448 |
| B-1 | Study area - B | Darjeeling | Saint Josephs College | 6492 | 27.059512 | 88.250667 |
| B -2 | | | Richmond hill | 6451 | 27.057831 | 88.256503 |
| B -3 | | | Chauk bazar | 6904 | 27.047997 | 88.265206 |
| B -4 | | | Pandam Limbu | 6577 | 27.042177 | 88.269932 |
| B -5 | | | Limbugaon | 7052 | 27.038853 | 88.266641 |
| B -6 | | | Jalapahar | 7431 | 27.03207 | 88.264865 |
| B -7 | | | West point | 6738 | 27.026802 | 88.254201 |
| B -8 | | | Batasia loop | 6968 | 27.018308 | 88.248157 |
| B -9 | | Ghoom | Lepcha jagat | 6952 | 27.010235 | 88.196913 |
| B -10 | | | Majdhura | 7077 | 27.004332 | 88.171849 |
| B -11 | | Sukhia pokhri | Sukhia pokhri | 7102 | 26.997813 | 88.167230 |
| B -12 | | Pashupati | Mim Nagri | 6756 | 26.973733 | 88.130303 |
| B -13 | | | Pashupatinagar | 6669 | 26.946805 | 88.125566 |
| B -14 | | Mirik | Mirik | 5267 | 26.886290 | 88.187986 |
| C -1 | Study area - C | Gangtok | Lower sichey | 4857 | 27.336767 | 88.606937 |
| C -2 | | | Tadong | 4131 | 27.311819 | 88.597223 |
| C -3 | | | Namchi | 4154 | 27.160503 | 88.369701 |
| C -4 | | | Ranipool | 3926 | 27.296625 | 88.595803 |
| C -5 | | Rishop | Rishop | 7598 | 27.183197 | 88.521206 |
| C -6 | | | Icha forest | 6663 | 27.126697 | 88.580521 |
| C -7 | | Kalimpong | Delo | 5088 | 27.086460 | 88.498359 |
| C -8 | | | Baghdhara | 3811 | 27.067951 | 88.471776 |
| C -9 | | | Sunwar | 3880 | 27.037848 | 88.447891 |
| C -10 | | Mongpoo | Upper Mongpoo | 6144 | 26.972187 | 88.339870 |
| C -11 | | | Chinchona plantation | 3643 | 26.973322 | 88.370000 |
| C -12 | | | Mongpoo bazar | 3616 | 26.978898 | 88.360149 |

© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

Table3: Geographical location from where soil samples were collected (Sl no=Serial number)

| Sl no. | Zone | Site name | Altitude (ft) | Latitude (°N) | Longitude (°E) |
|---|---|---|---|---|---|
| 1 | East Sikkim (Gangtok and adjoining region) | Enchey monostry | 5909 | 27.3357 | 88.6176 |
| 2 | | Upper Sichey | 5428 | 27.3349 | 88.6144 |
| 3 | | Lower Burtuk | 5183 | 27.3471 | 88.6126 |
| 4 | | Deorali | 4574 | 27.3219 | 88.6063 |
| 5 | | Gairi Gaon | 4418 | 27.3144 | 88.6012 |
| 6 | | Tadong | 3720 | 27.3203 | 88.5967 |
| 7 | | Upper Tadong | 4131 | 27.3118 | 88.5972 |
| 8 | | Samdur | 3569 | 27.2975 | 88.5923 |
| 9 | | Rani pool | 3926 | 27.2966 | 88.5958 |
| 10 | Kalimpong | Kalimpong | 5088 | 27.0864 | 88.4983 |
| 11 | Darjeeling | Mongpoo | 3616 | 26.9788 | 88.3601 |
| 12 | | Kurseong | 4888 | 26.8863 | 88.2777 |
| 13 | | Sonada | 6238 | 26.9577 | 88.2695 |
| 14 | | Ghoom | 7347 | 27.0081 | 88.2594 |

Table 4: Primer sequences producing successful amplification

| Primer ID | Sequence (5'-3') | Primer ID | Sequence (5'-3') |
|---|---|---|---|
| OPA 1 | CAGGCCCTTC | OPB 1 | GTTTCGCTCC |
| OPA 2 | TGCCGAGCTG | OPB 2 | TGATCCCTGG |
| OPA 3 | AGTCAGCCAC | OPB 3 | CATCCCCCTG |
| OPA 4 | AATCGGGCTG | OPB 4 | GGACTGGAGT |
| OPA 5 | ATTTTGCTTG | OPB 5 | TGCGCCCTTC |
| OPA 7 | GAAACGGGTG | OPB 6 | TGCTCTGCCCC |
| OPA 9 | GGGTAACGCC | OPB 7 | GGTGACGCAG |
| OPA 10 | GTGATCGCAG | OPB 8 | GTCCACACGG |
| OPA 11 | CAATCGCCGT | OPB 10 | CTGCTGGGAC |
| OPA 12 | TCGGCGATAG | OPB 11 | GTAGACCCGT |
| OPA 13 | CAGCACCCAC | OPB 12 | CCTTGACGCA |
| OPA 15 | TTCCGAACCC | OPB 13 | TTCCCCCGCT |
| OPA 16 | AGCCAGCGAA | OPB 14 | TCCGCTCTGG |
| OPA 17 | GACCGCTTGT | OPB 15 | GGAGGGTGTT |
| OPA 18 | AGGTGACCGT | OPB 17 | AGGGAACGAG |
| OPA 19 | CAAACGTCGG | OPB 18 | CCACAGCAGT |
| OPA 20 | GTTGCGATCC | OPB 20 | GGACCCTTAC |

Table 5: Morphologycal data of *A. nepalensis* recorded during field study

| Place | Altitude (ft.) | Average leaf length (cm) | Average width (cm) | Average inter branch distance (inch) | Average inter nodular distance (cm) | Mass of a cluster of nodules (gm) | Average tree height (m) | Average tree trunk circumference (cm) |
|---|---|---|---|---|---|---|---|---|
| Rani pool | 3926 | 8.45 | 5.75 | 12.72 | 4.8 | 92.15 | 25 | 45.23 |
| Samdur | 3569 | 7.30 | 4.02 | 14.45 | 5.1 | 95.08 | 27 | 47.64 |
| Upper Tadong | 4131 | 9.11 | 5.45 | 14.87 | 4.7 | 62.17 | 31 | 50.85 |
| Tadong | 3720 | 10.25 | 6.12 | 13.82 | 4.5 | 85.93 | 30 | 49.44 |
| Gairi Gaon | 4418 | 9.76 | 4.03 | 22.43 | 3.9 | 85.07 | 29 | 42.87 |
| Deorali bazaar | 4574 | 11.88 | 5.02 | 21.19 | 4.2 | 123.05 | 31 | 53.03 |
| Upper Sichey | 5428 | 10.41 | 5.89 | 25.09 | 3.5 | 89.49 | 33 | 50.17 |
| Enchey monostry | 5909 | 12.75 | 6.34 | 27.93 | 3.1 | 94.75 | 31 | 44.23 |
| Lower Burtuk | 5183 | 12.23 | 5.22 | 23.67 | 2.8 | 111.02 | 30 | 52.01 |
| Kalimpong | 5088 | 10.54 | 5.38 | 21.05 | 4.1 | 96.72 | 27 | 50.91 |
| Mongpoo | 3616 | 8.97 | 4.86 | 26.74 | 3.8 | 106.09 | 25 | 49.99 |
| Kurseong | 4888 | 6.54 | 3.72 | 24.65 | 3.3 | 92.12 | 33 | 45.16 |
| Sonada | 6238 | 7.95 | 4.14 | 29.28 | 3.1 | 74.17 | 26 | 41.91 |
| Ghoom | 7347 | 12.86 | 5.78 | 33.09 | 2.3 | 68.29 | 32 | 52.06 |
| Darjeeling | 6968 | 10.02 | 4.32 | 31.23 | 2.6 | 57.09 | 31 | 39.98 |
| Sukhia pokhri | 7102 | 11.98 | 5.74 | 31.97 | 2.1 | 55.26 | 29 | 47.62 |
| Pashupati | 6669 | 9.48 | 5.02 | 30.45 | 2.9 | 48.05 | 30 | 38.97 |
| Mirik | 5267 | 7.15 | 4.18 | 24.23 | 3.4 | 33.28 | 28 | 51.06 |

Table 6: Estimation and analysis of nutrients present in soil collected from the base of *A. nepalensis* in studied area (Alt. =Altitude)

| Place | Alt. (ft.) | pH | Carbon (%) | Potash (ppm) | Phosphate (ppm) | Sulphur (ppm) | Nitrogen (%) |
|---|---|---|---|---|---|---|---|
| Enchey monostry | 5909 | 3.85 | 1.52 | 70 | 25 | 30 | 0.130 |
| Lower Burtuk | 5183 | 4.58 | 0.74 | 200 | 35 | 35 | 0.063 |
| Deorali bazaar | 4574 | 5.13 | 0.66 | 340 | 15 | 20 | 0.057 |
| Upper Sichey | 5428 | 4.74 | 1.63 | 385 | 30 | 30 | 0.140 |
| Gairi Gaon | 4418 | 5.70 | 2.30 | 395 | 25 | 25 | 0.197 |
| Tadong | 4131 | 6.20 | 1.98 | 240 | 15 | 30 | 0.170 |
| Upper Tadong | 3720 | 4.75 | 3.47 | 235 | 25 | 35 | 0.298 |
| Samdur | 3569 | 6.22 | 1.39 | 75 | 40 | 35 | 0.119 |
| Rani pool | 3926 | 4.25 | 1.56 | 53 | 35 | 45 | 0.134 |
| Kalimpong | 5088 | 4.03 | 1.03 | 230 | 15 | 25 | 0.089 |
| Mongpoo | 3616 | 5.34 | 1.19 | 155 | 45 | 35 | 0.103 |
| Kurseong | 4888 | 6.18 | 1.59 | 87 | 35 | 30 | 0.136 |
| Sonada | 6238 | 5.72 | 2.85 | 72 | 10 | 25 | 0.245 |
| Ghoom | 7347 | 5.53 | 2.88 | 67 | 15 | 30 | 0.247 |

Table 7: Total number and size of amplified bands, number of monomorphic and polymorphic bands and percentage of polymorphism generated by the RAPD primers (MB=Monomorphic bands, PB= polymorphic bands)

| Primers | Sequence | Amplified RAPD loci | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Study area I | | | | Study area II | | | | Study area III | | | |
| | | Band No | MB | PB | Band size (bp) | Band No | MB | PB | Band size (bp) | Band No | MB | PB | Band size (bp) |
| OPA 01 | CAGGCCCTTC | 13 | 2 | 11 | 250-1900 | 9 | 2 | 7 | 210-1300 | 13 | 2 | 11 | 210-1300 |
| OPA 02 | TGCCGAGCTG | 11 | 0 | 11 | 230-1600 | 10 | 0 | 10 | 280-1400 | 10 | 0 | 10 | 220-1580 |
| OPA 03 | AGTCAGCCAC | - | - | - | - | 11 | 0 | 11 | 230-1600 | 15 | 2 | 13 | 200-1320 |
| OPA 04 | AATCGGGCTG | 10 | 1 | 9 | 250-1580 | 17 | 1 | 16 | 220-1670 | 9 | 1 | 8 | 230-1860 |
| OPA 05 | ATTTTGCTTG | 12 | 0 | 12 | 240-1700 | 9 | 0 | 9 | 400-1880 | - | - | - | - |
| OPA 07 | GAAACGGGTG | 9 | 0 | 9 | 230-1400 | 12 | 0 | 12 | 250-1540 | 8 | 0 | 8 | 400-1800 |
| OPA 09 | GGGTAACGCC | - | - | - | - | 10 | 0 | 10 | 390-1320 | 10 | 0 | 10 | 190-1370 |
| OPA 10 | GTGATCGCAG | 10 | 2 | 8 | 280-1300 | 15 | 0 | 15 | 220-1300 | 14 | 1 | 13 | 220-1900 |
| OPA 11 | CAATCGCCGT | 9 | 0 | 9 | 270-1920 | 13 | 1 | 13 | 210-1700 | 13 | 0 | 13 | 250-1690 |
| OPA 12 | TCGGCGATAG | 9 | 0 | 9 | 310-1850 | 9 | 0 | 9 | 210-1920 | 11 | 0 | 11 | 260-1800 |
| OPA 13 | CAGCACCCAC | 11 | 2 | 9 | 320-1900 | 10 | 1 | 9 | 250-1700 | 14 | 2 | 12 | 230-1320 |
| OPA 15 | TTCCGAACCC | 9 | 1 | 8 | 300-1640 | 14 | 1 | 13 | 230-1680 | 10 | 1 | 9 | 240-1700 |
| OPA 16 | AGCCAGCGAA | 14 | 0 | 14 | 250-1430 | 8 | 0 | 8 | 380-1880 | 9 | 0 | 9 | 220-1860 |
| OPA 17 | GACCGCTTGT | 10 | 0 | 10 | 260-1680 | 11 | 2 | 9 | 220-1650 | 11 | 0 | 11 | 190-1900 |
| OPA 18 | AGGTGACCGT | 12 | 0 | 12 | 240-1600 | - | - | - | - | - | - | - | - |
| OPA 19 | CAAACGTCGG | 9 | 0 | 9 | 240-1900 | 12 | 0 | 12 | 250-1320 | 8 | 0 | 8 | 210-1700 |
| OPA 20 | GGACCCTTAC | 10 | 0 | 10 | 300-1850 | - | - | - | - | 8 | 1 | 7 | 310-1886 |
| OPB 01 | GTTTCGCTCC | 9 | 0 | 9 | 230-1900 | 10 | 0 | 10 | 220-1320 | - | - | - | - |
| OPB 02 | TGATCCCTGG | 13 | 0 | 13 | 240-1950 | 9 | 0 | 9 | 310-1950 | 10 | 0 | 10 | 200-1820 |
| OPB 03 | AGTCAGCCAC | 10 | 0 | 10 | 260-1700 | 9 | 1 | 8 | 230-1800 | 13 | 1 | 12 | 300-1800 |
| OPB 04 | GGACTGGAGT | 10 | 2 | 8 | 270-1500 | 9 | 2 | 7 | 310-1860 | 15 | 1 | 14 | 200-1450 |
| OPB 05 | TGCGCCCTTC | 9 | 0 | 9 | 320-1800 | 14 | 1 | 13 | 320-1600 | 10 | 2 | 8 | 210-1490 |
| OPB 06 | TGCTCTGCCCC | 15 | 1 | 14 | 310-1400 | 16 | 0 | 16 | 310-1790 | 9 | 1 | 8 | 420-1900 |
| OPB 07 | GGTGACGCAG | 9 | 0 | 9 | 220-2100 | 12 | 2 | 10 | 210-1580 | 10 | 2 | 8 | 310-1800 |
| OPB 08 | GTCCACACGG | 13 | 0 | 13 | 220-1680 | 9 | 0 | 9 | 200-1600 | 14 | 2 | 12 | 280-1380 |
| OPB 10 | CTGCTGGGAC | 11 | 0 | 11 | 240-1960 | 10 | 0 | 10 | 210-1390 | 8 | 2 | 6 | 140-1600 |
| OPB 11 | GTAGACCCGT | 18 | 1 | 17 | 250-1650 | 14 | 0 | 13 | 240-1760 | 12 | 1 | 11 | 230-1930 |
| OPB 12 | CCTTGACGCA | 12 | 1 | 11 | 310-1800 | 19 | 1 | 18 | 200-1900 | 11 | 1 | 10 | 220-1600 |
| OPB 13 | TTCCCCCGCT | 16 | 1 | 15 | 220-1450 | 12 | 1 | 11 | 240-1530 | 9 | 1 | 8 | 290-1840 |
| OPB 14 | TCCGCTCTGG | 15 | 0 | 15 | 250-2000 | 16 | 1 | 15 | 220-1730 | 11 | 2 | 9 | 210-1740 |
| OPB 15 | GGAGGGTGTT | 13 | 0 | 13 | 250-1770 | 12 | 0 | 12 | 200-1940 | 13 | 0 | 13 | 210-1930 |
| OPB 17 | AGGGAACGAG | 10 | 0 | 10 | 220-1500 | - | - | - | - | 17 | 1 | 16 | 300-2000 |
| OPB 18 | CCACAGCAGT | 16 | 1 | 15 | 300-1400 | - | - | - | - | 15 | 0 | 15 | 200-1680 |
| OPB 20 | GGACCCTTAC | 10 | 1 | 9 | 220-1900 | 15 | 0 | 15 | 240-1750 | 12 | 1 | 11 | 220-1640 |
| Total | | 367 | 16 | 351 | | 356 | 17 | 339 | | 352 | 28 | 324 | |
| %of Polymorphism | | 95.64 | | | | 95.22 | | | | 92.04 | | | |

Table 8: Primers found in all three populations producing distinct scorable bands

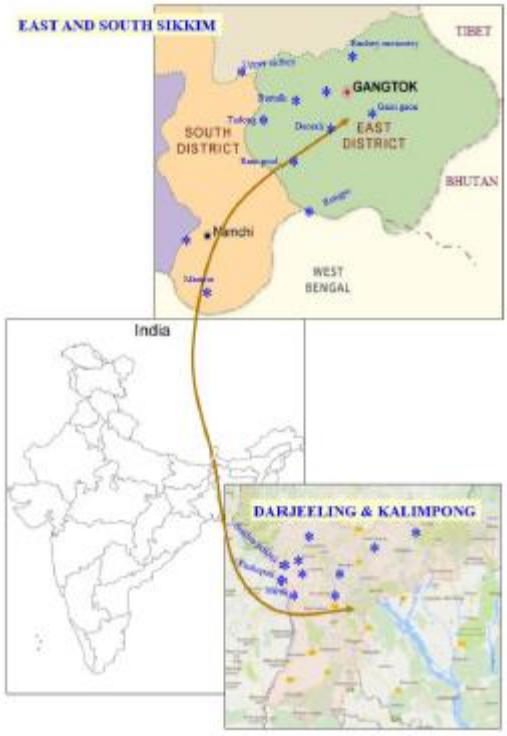
| Primers | Common bands in population I & II | Common bands in population I & III | Common bands in population II & III |
|---|---|---|---|
| OPA 01 | 1 | 0 | 0 |
| OPA 02 | 0 | 0 | 0 |
| OPA 04 | 0 | 0 | 0 |
| OPA 07 | 0 | 0 | 0 |
| OPA 10 | 0 | 1 | 0 |
| OPA 11 | 0 | 0 | 0 |
| OPA 12 | 0 | 0 | 0 |
| OPA 13 | 1 | 1 | 0 |
| OPA 15 | 0 | 1 | 0 |
| OPA 16 | 0 | 0 | 0 |
| OPA 17 | 0 | 0 | 0 |
| OPA 19 | 0 | 0 | 0 |
| OPB 02 | 0 | 0 | 0 |
| OPB 03 | 0 | 0 | 0 |
| OPB 04 | 2 | 0 | 0 |
| OPB 05 | 0 | 0 | 1 |
| OPB 06 | 0 | 0 | 0 |
| OPB 07 | 0 | 0 | 1 |
| OPB 08 | 0 | 0 | 0 |
| OPB 10 | 0 | 0 | 0 |
| OPB 11 | 0 | 1 | 0 |
| OPB 12 | 1 | 1 | 1 |
| OPB 13 | 1 | 0 | 0 |
| OPB 14 | 0 | 0 | 1 |
| OPB 15 | 0 | 0 | 0 |
| OPB 20 | 0 | 0 | 0 |
| Total | 6 | 5 | 4 |

Table 9: Similarity coefficient amongst studied populations of *A. nepalensis*

| | Population I | Population II | Population III |
|---|---|---|---|
| Population I | 1.00 | | |
| Population II | 0.88 | 1.00 | |
| Population III | 0.83 | 0.81 | 1.00 |

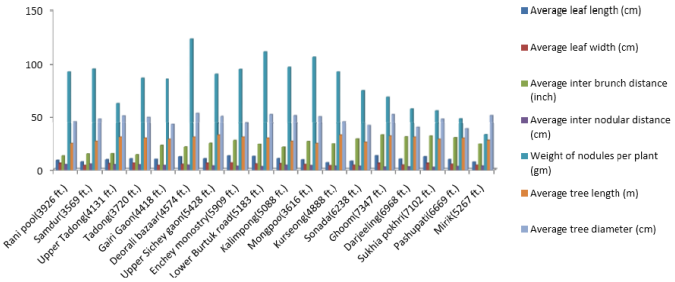
© IJLRP | ISSN (O) - 2582-8010
September 2020 | Vol. 1 Issue. 3
www.ijlrp.com

**Fugure1:**

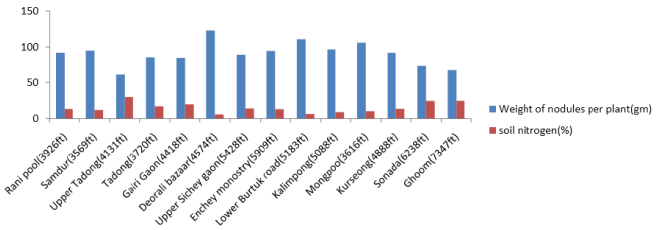



**Fugure2:**

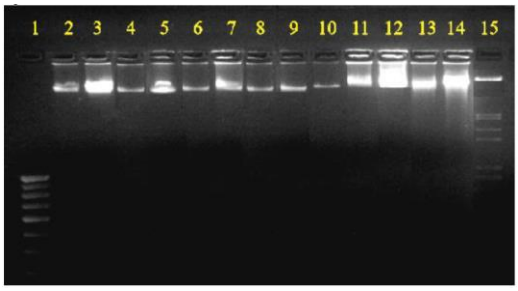
**Fugure3:**



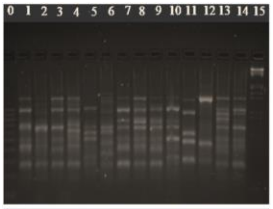

**Fugure4:**



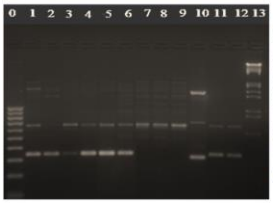

**Fugure5:**




**Fugure6:**

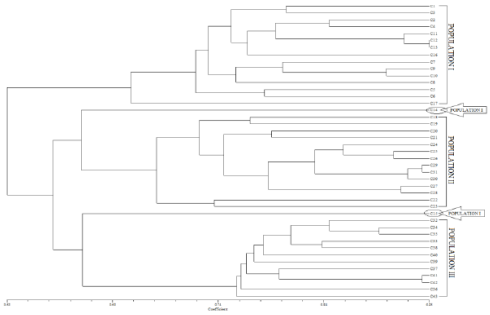



Study area-II (OPA02) Lane 0:100 bp DNA ladder; 1:Saint Josephs College; 2:Richmond hill; 3:Chauk bazaar; 4:Pandam Limbu; 5:Limbugaon; 6:Jalapahar; 7:West point; 8:Batasia loop; 9:Lepcha jagat; 10:Majdhura; 11:Sukhia pokhri; 12:Mim Nagri; 13:Pashupatinagar; 14:Mirik; 15:λ DNA-EcoRI+*Hind* III double digest DNA ladder




Stusy area-III (OPA02) Lane 0:100 bp DNA ladder;1:Lower sichey; 2:Tadong; 3:Namchi; 4:Ranipool; 5:Rishop; 6:Icha forest; 7:Delo; 8:Baghdhara; 9:Sunwar; 10:Upper Mongpoo; 11:Chinchona plantation; 12:Mongpoo bazaar; 13:λ DNA-EcoRI+*Hind* III double digest DNA ladder

**Fugure7:**




**Fugure8:**