Study of genetic diversity of *Aconitum heterophyllum* and its root microbiome

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BY

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UNDER THE GUIDANCE OF

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THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013
BENGALURU - 560064

DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "**Study of genetic diversity of *Aconitum heterophyllum* and its root microbiome**" submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide **Dr. Pavithra N**. I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged.

I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

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CERTIFICATE

This is to certify that the work incorporated in this thesis **"Study of genetic diversity of *Aconitum heterophyllum* and its root microbiome"** submitted by **Amandeep Kumar** was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged. I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

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LIST OF ACRONYMS

%P	: Percentage of polymorphic loci
AFLP	: Amplified Fragment Length Polymorphism
AMOVA	: Analysis of Molecular Variance
AMP-PCR	: Anchored Microsatellite-Primed Polymerase Chain Reaction
CAMP	: Conservation Assessment and Management Plans
F_{st}	: Fixation index
H_e	: Expected Heterozygosity
H_o	: Observed heterozygosity
I	: Shannon's information index
ISSR	: Inter Simple Sequence Repeat
ITS	: Internal Transcribed Spacer
IUCN	: International Union for the Conservation of Natural Resources
MCMC	: Markov Chain Monte Carlo
MPCDAs	: Medicinal Plants Conservation and Development Areas
N_a	: Number of alleles
N_e	: Number of effective alleles
NGS	: Next Generation Sequencing
N_m	: Gene flow
OUT	: Operational Taxonomic Unit
PBS	: Phosphate Buffered Saline
PCoA	: Principal Coordinates Analysis

PCR	: Polymerase Chain Reaction
PGPB	: Plant Growth Promoting Bacteria
PGPR	: Plant Growth Promoting Rhizobacteria
PGR	: Plant Growth Regulator
RAPD	: Randomly Amplified Polymorphic DNA
RFLP	: Restriction Fragment Length Polymorphism
rRNA	: ribosomal RNA
SCAR	: Sequence Characterized Amplified Region
SNP	: Single Nucleotide Polymorphism
SSR	: Simple Sequence Repeat
uH_e	: Unbiased Expected Heterozygosity
UPGMA	: Unweighted Pair Group Method with Arithmetic Mean
VNTR	: Variable Number of Tandem Repeat

ABSTRACT

In the present study, Genetic diversity and root microbiome of a medicinally important medicinal plant- *Aconitum heterophyllum* was studied. It belongs to the family Ranunculaceae and is commonly known as Atis or Ateesh. The roots of *A. heterophyllum* have been documented to possess various pharmacological properties. They are abundant in diterpene alkaloids and contain atisine, a low-toxicity alkaloid classified among the simpler aconite alkaloids. The species significant medicinal value fuelled extensive harvesting from the wild, ultimately leading to its critically endangered classification in natural habitats. The conservation and population status were evaluated by studying the genetic diversity along with the structure of population by utilizing the genetic markers (Microsatellites). This analysis encompassed 36 accessions from 8 distinct populations located in Himachal Pradesh and Uttarakhand, India. The characterization of the markers (Microsatellites) involved assessing their transferability and employing primers with a 5'-anchor designed with specific repeat motifs. Ten primers were identified through the transferability assessment from related Aconitum species, and another four primers were characterized using identified repeat motifs along with a 5’-anchor. Using these 14 primers across the studied populations, 55 alleles were observed in total with 40.18% average polymorphic loci. Molecular variance analysis demonstrated that most of the genetic variation (92%) occurred among populations, with only 8% within populations. Consequently, the species exhibited sparse genetic diversity, accompanied by significant differentiation at genetic level (Fst = 0.490) and restricted gene flow. Population structure and cluster analysis indicated two populations corresponding to their distant geographical locations. The findings suggest a potential decline in the species population stemming from this limited genetic diversity, restricted gene flow and high differentiation, likely driven by population fragmentation.

The root microbiome of the target species rhizosphere was examined using a 16S rRNA metagenomic approach. The rhizosphere is a dynamic zone of interaction between plants and microorganisms and plays a crucial role in plant health, growth, and nutrient cycling. By comparing the root microbiomes of wild and cultivated *A. heterophyllum*, unique microbes were identified. This study reports several beneficial rhizospheric bacteria, known as Plant Growth Promoting Rhizobacteria (PGPR), associated with the wild populations.

LIST OF PUBLICATIONS

1. Kumar, Amandeep & Narendran, Pavithra. (2022). **Ethnomedicinal properties and conservation issues of an endangered medicinal plant- *Aconitum heterophyllum* Wall. ex Royle.** *Medicinal Plants - International Journal of Phytomedicines and Related Industries*. 14. 374-381. 10.5958/0975-6892.2022.00042.9.

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CHAPTER 1

Introduction

Medicinal plants have played an important role in human medicine for centuries, providing the basis for conventional medicine and assisting in the formulation of contemporary medicines. Rich sources of bioactive compounds, they are the substances responsible for medicinal plants' efficacy. Used for alleviating chronic conditions and mundane ailments, medicinal plants provide natural and integrated avenues to good health. Most modern drugs owe their origin to or have been adapted from substances present in medicinal plants. For example, aspirin itself was first obtained from willow bark, and quinine was obtained from the bark of the cinchona tree. All these cases exemplify how substances present in plants have revolutionized medical treatment. The continued search for plant-derived substances continues to pose a strong possibility for finding new drugs.

The history of medicinal plants is deeply intertwined with various cultures and traditions. Ancient civilizations such as the Egyptians, Greeks, Chinese, and Indians carefully documented the use of plants for medicinal purposes. The traditional use of plants was inherited from generations to generations and currently serves as the foundation for modern medicine.

The global market for medicinal plants and their extracts is huge, and it gives livelihood to millions of individuals comprising of traditional healers, herbalists, farmers, collectors and scientists working in the production, harvesting and research on these plants. But despite of their tremendous importance, several medicinal plants face overexploitation leading to habitat loss. The destruction of these valuable plants not only threatens traditional practices and knowledge but also impedes the possible discovery of new therapeutic compounds. Hence, the conservation of medicinal plants is of utmost importance for a number of reasons which include sustainable healthcare, Preservation of Biodiversity and cultural heritage.

One of such important medicinal plant witnessing threats due to overharvesting and habitat destruction is *Aconitum heterophyllum* belonging to genus Aconitum. The genus *Aconitum*, commonly known as Aconite, with about 300 species worldwide, belongs to family Ranunculaceae and is a species-rich genera in India representing about 27 species and 5 varieties (Agnihotri et al., 2015). *Aconitum heterophyllum* Wall. ex Royle, (Illus. Bot. Himal. t. 13. 1833 & 86. 1834), is a perennial herbaceous medicinal herb of North-Western Himalayas (Dhar and Kachroo, 1983). *A. heterophyllum* is endemic to the Himalayan region at an altitude of 2400-4500 m (Bhatt et al. 2014; Jabeen et al. 2013). It commonly known as Atis in Hindi, Aruna of visa in Sanskrit and Atees in Urdu. *A. heterophyllum* is known for the medicinal value of its roots referred to as Ativisa. They have centuries-long history of use in traditional medicine, particularly in Ayurveda, Unani, and Tibetan medicinal systems. The roots of the plant are reported to exhibit a wide range of pharmacological activities but is mainly known for its Anti-pyretic (Verma et al., 2010) and Anti-diarrheal (Singh and Chaturvedi, 1982) potential. Studies indicate that the plant's root extracts can effectively lower body temperature in febrile conditions, aligning with its traditional use in treating fever. Additionally, roots of *A. heterophyllum* also exhibit antimicrobial activity against a range of pathogens, including bacteria and fungi. This property is the basis for its application in infection treatment and wound healing. Reports also indicate that the species possesses immunomodulatory effects to increase the immune system's response and provide other therapeutic benefits. Its root is also valued for its capability to promote appetite and maintain general digestion. In Unani medicine, *A. heterophyllum* finds application in febrifuge and analgesic uses. It is typically added to medicines intended for chronic fever and inflammatory disorders. The Tibetan medicinal system further identifies the plant as beneficial in addressing gastric troubles, infections, and inflammatory illnesses.

A. heterophyllum is a good source of diterpene alkaloids and is known to contain atisine (Brimton, 1877), an alkaloid belonging to the category of simpler aconite alkaloids of low toxicity, reason why the species is often regarded as non-poisonous (Khare, 2004), compared to poisonous Aconites containing complex toxic alkaloid- Aconitine (Jacobs and Craig, 1942). Another alkaloid- isoatisine is rather regarded as an artefact since it is isomeric form of atisine which isomerizes in alcohol even at room temperature (Pelletier et al., 1966). Phenols, tannins, flavonoids and saponins are also found in roots but are less in quantity (Prasad et al., 2012).

A. heterophyllum is categorized under the endangered category of International Union for the Conservation of Natural Resources (IUCN) of the Red Data Book (Ved et al., 2003). Its Rapid depletion can be attributed to its low population density in nature (Dhar and Kachroo, 1983) along with overexploitation due to unsustainable collection due to high market value (Nautiyal et al., 2002) of the tuberous roots. Sustainable use and conservation of its natural populations are urgently needed. Cultivation and propagation techniques, along with habitat protection measures, are essential strategies to conserve *Aconitum heterophyllum*.

Genetic diversity

Genetic diversity ensures species adaptation and evolution within their environments, enabling populations to change in response to environmental changes. Genetic diversity offers information on long-term survival and evolutionary potential of populations or species, which is of significant importance in conservation (Rivers et al., 2011). Genetic diversity comes about due to the heterogeneity of alleles included in a population for a given gene, often measured by frequency of heterozygosity or gene diversity (Rua, 2014).

To measure genetic diversity and structure, different traits or markers are employed, such as proteins, DNA (nuclear, mitochondrial, and chloroplast), quantitative characters, and chromosomes. These markers may have inheritance patterns similar to other traits, e.g., dominant/recessive or codominant. Codominant markers offer a significant advantage over dominant markers since they can differentiate between homozygous and heterozygous genetic patterns, giving more information than dominant markers. Methods that expose greater amounts of genetic diversity are most favoured in conservation biology for their higher precision (Frankham, 2010).

Cytological markers, which study features like chromosome size, secondary constriction, centromere position, arm ratio, heterochromatic patterns, and total genomic chromosome length, are employed to evaluate genetic diversity at both intraspecific and interspecific levels. However, their application is limited due to their low number and resolution (Bhandari et al., 2017). Biochemical markers, such as isozymes (allelic variants of enzymes distinguished by electrophoresis), are codominant and detect diversity at the functional gene level. Despite their simple inheritance, their use in assessment of diversity is limited because of restricted availability of markers, as only about 30% of DNA changes result in protein charge alterations (Frankham, 2010; Govindaraj et al., 2015; Kongkiatngam et al., 1995).

Molecular markers, including a wide range of DNA molecular markers, represent the most widely utilized approach for the evaluation of genetic diversity (Whitkus et al., 1994). These markers detect variations from chromosome deletions, duplications, inversions, and insertions. They are preferred for genetic diversity assessments due to their high variability, comprehensive genomic coverage, high reproducibility, and neutrality to environmental fluctuations (Bhandari et al., 2017; Nybom, 1994). Common molecular markers include Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs), Randomly Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplified Regions (SCAR), and Single Nucleotide Polymorphisms (SNPs).

Out of various DNA markers, the SSRs, also known as Microsatellites markers have emerged as a popular genetic marker for a wide range of applications in population genetics, conservation biology, and evolutionary biology. Their high mutation rates and simple Mendelian mode of inheritance make them particularly suitable for the studying population structure, mating systems and pedigrees. Microsatellites are simple sequence tandem repeats. The repeat units are generally di-, tri- tetra- or pent nucleotides. They are found in eukaryotes and prokaryotes. The basic unit lengths of microsatellites (or simple DNA sequence repeats) comprise up to six (or eight) nucleotides, and these motifs are perfectly repeated from 5 to more than 100 times. In contrast to single nucleotide polymorphism (SNP) and genotyping-by-sequencing methods, they are readily typed in samples of low DNA quality/concentration (e.g. museum/non-invasive samples), and enable identification of species, hybrids, clones and ploidy. Generally, microsatellite loci are abundant and distributed throughout the eukaryotic genome and each locus is characterized by known DNA sequence. These sequences consist of both unique DNA (which defines the locus) and repetitive DNA motifs (which may be shared among loci). The repetitive elements consist of tandem reiterations of simple sequence repeats (SSRs) and are typically composed of two to four nucleotides such as (AC)*n* or (GATA)*n* where *n* lies between 5 and 50. Within vertebrates, the dinucleotide repeats -GT and CA- are believed to be the most common microsatellites.

Microsatellites offer a significantly powerful and practical approach to evaluate genetic diversity in threatened species due to their high polymorphism, repeatability, and high PCR success rate (Frankham, 2010; Faria et al., 2016; Wang et al., 2017; Varshney et al., 2005; Agarwal et al., 2008). A critical aspect of microsatellite-based conservation genetics is developing species-specific or cross-species primer sets (Frankham, 2002; Castoe et al., 2012; Wang et al., 2017; Yang et al., 2017).

High throughput sequencing technology along with bioinformatics tools provide a method for developing SSR markers (Losada et al, 2023). Microsatellite primer sets are either identified by sequencing the complete genome or targeted partial genome. Earlier, identification of microsatellite regions was expensive and lengthy process. It necessitated construction of genomic libraries to target SSR motifs through restriction enzyme-based DNA fragmentation (Zane et al., 2002; Squirrell et al., 2003). Furthermore, the subsequent cloning of DNA fragments and sequencing of SSR-containing clones was also carried out (Santana et al., 2009), further lengthening the process. However, the advances in NGS (Next Generation Sequencing) technology and reduction in sequencing costs enabled easier, comparatively cheaper and rapid identification of microsatellite markers.

Several NGS platforms and different technologies are available, each with its strengths. The first commercial platform was 454 Roche, used for smaller genomes. Later, platforms like Illumina (for complex genomes) amongst others emerged, enabling a broad spectrum of applications (Techen et al., 2010). The development of DNA markers currently relies most heavily on sequencing by synthesis-based platforms, with Illumina being a notable example (Galperin, 2004; Fisher et al., 1996). The MinION (Oxford Nanopore Technologies) is a recently developed nanopore-based DNA sequencing platform. A nanopore is a nano-scale hole. In its devices, Oxford Nanopore passes an ionic current through nanopores and measures the changes in current as biological molecules pass through the nanopore or near it.

The high cost and labour involved in developing microsatellite repeats is a serious factor that restricts the wide-spread application of the SSRs based marker amongst different plants. Hence, identification and the use of SSRs based marker is often focused only to economically important crops. Therefore, microsatellite transferability is often explored for the low-cost generation of microsatellite markers consuming less time. Flanking sequences to microsatellites are reported to exhibit slower mutation rate than SSR region, permitting their sequence conservation across species or genera (Holmen et al., 2009). This homology allows the amplification of primers initially designed for a specific species to other members of a related species or genera (cross-species/cross-genera amplification or transferability).

Another method for developing microsatellite markers is by using Anchored Microsatellite-Primed PCR (AMP-PCR) (Zietkiewicz et al., 1994). In this method, 5′- or 3′-anchored repeats comprising of di or tri nucleotides serve as singular PCR primer. Anchored di or tri nucleotide repeats comprises of bases those are non-repetitive which ensures initiation of amplification in each cycle at the same position of nucleotide. Fingerprints obtained using this method resulted in inter- and intraspecific polymorphisms in a wide variety of eukaryotic taxa. This method presents significant benefits compared to non-anchored SSR-primed PCR. One key advantage is the structure of the primer, which restricts annealing to the microsatellite termini, avoiding internal priming and the resulting smear. Another benefit is the anchor's ability to selectively amplify a subset of inter-repeat regions, thereby mitigating the issue of numerous PCR products often seen with dinucleotide inter-repeat regions. Additionally, the functional 5′-anchors ensures the incorporation of the targeted microsatellite sequence within the amplified product (Fisher et al., 1996). Potential VNTR polymorphisms within microsatellites thus contribute to the inter-repeat variation. This considerably increases the chance of observing a polymorphism.

A. heterophyllum has been indiscriminately exploited due to restricted distribution and destructive harvesting causing extensive depletion of their populations in the wild. Therefore, it is imperative to study its current diversity along with the population structure. The genetic diversity of *A. heterophyllum* in India has been studied sparsely. Consequently, the present investigation is designed to examine the same in *A. heterophyllum* obtained from diverse locations using Microsatellite markers. The information gained on the level of genetic variation in *Aconitum* would help in suggesting appropriate management strategies for the target taxa. An optimum diversity at genetical level in species would be helpful in *in-situ* conservation.

Microbiome

Microbiome refers to the collective genomes of microorganisms typically found living together, in and on a specific habitat that can be symbiotic or pathogenic. Plant microbiome, marine microbiome, human microbiome are a few to name. The plant microbiome has significant effects on seedling vigour, seed germination, plant health, diseases, growth and development, and food production (Compant et al., 2019). Plants live in association with varied microbial communities, known as the plant microbiota, living both inside as well as outside the plant tissues, known as endosphere and episphere, respectively (Turner et al., 2013). They contribute majorly to the physiology and ecology of the plant. The plant microbiome is composed mostly of bacteria, archaea, fungi, and oomycetes which can be pathogenic or beneficial for plant growth and health. A plant performs two functions simultaneously to grow healthy, one is- it needs to monitor the soil in proximity of the roots for the detection of harmful or pathogenic microbes, and second- it must make full use of the support of beneficial microbes in uptake of nutrients and growth promotion. To regulate their microbiome, plants use a range of mechanisms like the release of secondary metabolites (root exudates), structural modifications, and combined action of various defence responses.

Microbial communities are chiefly found predominant in three different regions of plant, influenced by the plant's habitat, namely phyllosphere, endosphere and the rhizosphere (Lata et al., 2022). The phyllosphere means the above-ground or aerial parts of the plant that are inhabited by microbial communities, endospheric microbiome is constituted by the microorganisms (called endophytes) that penetrate and then reside into the internal plant tissues, and rhizosphere can be defined as the narrow region of soil surrounding the plant roots containing a diverse and unique population of microbes.

Makeup of the root-associated microbiomes has been extensively studied in the last decade. The predominant methodology employed in these investigations involves NGS (Next-Generation Sequencing) of genes such as the 16S rRNA gene in bacteria and ITS region (Internal Transcribed Spacer) in fungi, which is a universal sequence for fungal barcoding. Few investigations made use of the Shotgun metagenomics sequencing in which not only the selected marker gene, rather all the genes present in the sample are sequenced. (Sessitsch et al., 2012; Ofek-Lalzar et al., 2014; Bai et al., 2015; Bulgarelli et al., 2015). This approach allows for both taxonomic profiling and the characterization of root-associated microorganisms.

Comparative root microbiome studies of wild and cultivated populations of *Aconitum heterophyllum* could help identify unique microbiome associated with the growth of the plant. Understanding the rhizosphere of medicinal, endemic plant species will be vital for bioprospection of ecto- and endophytic microbes and in selecting suitable sites for *ex-situ* conservation and cultivation. Effective agricultural practices could also be developed to improve root biomass of existing variety by studies on soil-microbe-plant interactions as specific microbe interactions are known to be associated to endemism of species.

OBJECTIVES

1. Characterization of microsatellite markers in *A. heterophyllum*.
    a. Utilization of PCR based molecular markers (SSRs derived from ISSR-SSR amplicons) for generating microsatellites and by exploring cross-species transferability- an approach for generation of the microsatellite markers for related species.

2. Assessment of genetic diversity in population of *A. heterophyllum*.
    a. Phenotypic assessment of accessions of *A. heterophyllum* from Uttarakhand and Himachal Pradesh states of India.
    b. Species authentication and the analysis of genetic diversity using characterized markers.

3. Study of the Root microbiome using metagenomic approach.
    a. Assessment of colonization pattern of the root microbiome in endemic *A. heterophyllum* and by targeted gene sequencing.
    b. Identification of unique microbes using targeted gene sequencing, by comparison of wild and cultivated root microbiome of *A. heterophyllum*.

CHAPTER 2
Review of Literature

Aconitum heterophyllum commonly known as Atees, is a medicinal plant from north-western Himalayan region used in Ayurvedic system of medicine. The medicinal properties of the herb are attributed to the alkaloids found in its tuberous roots. Use of roots results in its unsustainable collection from the wild and as a result the species is reported to be critically endangered. Medicinal properties of the tuberous roots of *A. heterophyllum* are well documented in ancient literature and are widely used in Indian traditional system of Medicine. The species is also majorly used in Japanese and Chinese traditional medicines.

Morphology

The morphological characteristics of *Aconitum heterophyllum* were documented in studies by Stapf (1905) and Chakravarty & Chakravarti (1954). The species produces biennial, tuberous roots, typically occurring in pairs. The daughter tubers, measuring up to 6.5 cm in length, depict a cylindrical to conical shape with thickening towards the upper end. Their fracture surface is white, with a scarcely farinaceous texture, and the cambium is discontinuous, forming a ring of strands. These strands are circular in cross-section for smaller strands and tangentially flattened for larger ones. Mother tubers share similar characteristics, featuring long filiform root fibres with a wrinkled and shrunken appearance. The tubers initially present a neutral taste and later a pronounced tingling sensation. The stem is erect, ranging from 15 to 90 cm in height, and can be simple or branched. It is terete, finely crispo-pubescent in the upper region, and glabrous below. Leaves are glabrous or sparsely pubescent on the nerves of the upper portion. The lower leaves typically senesce during flowering. The petiole is slender, measuring 5-7 cm, and dilated at the base. The leaf blade is reniform or ovate-reniform, with a wide sinus or nearly truncate base, and is 5-pedately partite almost to the base. The inflorescence is a straight and racemose is less dense. Sepals are predominantly blue, with the uppermost sepal helmet-shaped, obliquely depressed, slightly concave basally, and terminating in a short beak. Lateral sepals are oblique, suborbicular, and ciliate. Nectaries are hispidulous all over, with almost straight claw, a forward-leaning hood which is gibbous near the apex, and a short, broad, emarginate lip. Stamens are numerous, with hairy filaments in the upper part, winged beyond the midpoint. The species possesses three oblong, greyish pubescent and longcarpels. Follicles are contiguous, linear-oblong, straight, and elongated. Seeds, measuring 3-4 mm in length, are long, obconic and exhibit numerous small, short transverse lamellae.

The floral morphology of *A. heterophyllum* was re-assessed by Raina *et al.* (2011) as they noted that the floral description, particularly of perianth, as described earlier appeared to be incomplete. They reported flowers to be zygomorphic, bisexual, complete and bluish-purple in colour. The sepals are pedicellate, with elongated pedicels, and are subtended by bracts and bracteoles. The perianth-like bracts are ovate with acute apices, possessing a saccate base that sheaths the pedicel. Two opposite, elliptic-obovate bracteoles are also present. Perianth is described with five petaloid sepals and two petals. Laterally flattened sepals were reported to be blue or purple-green in colour, constituting the primary visual component of the flower and are arranged in three distinct whorls. The purple petals are positioned posteriorly and free, are modified into nectaries, mimicking stamens. These structures are characterized by a linear, cylindrical stalk culminating in a swollen apex, which features two openings for nectar secretion. A comprehensive description of the androecium, containing numerous stamens, and the gynoecium, with five carpels, was also provided.

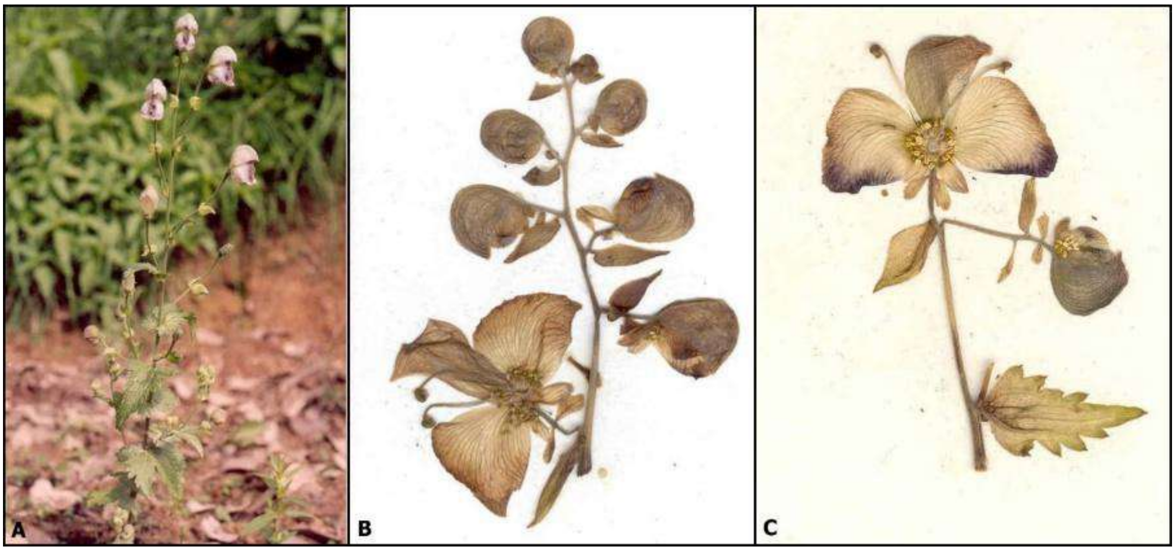

Figure 1: *Aconitum heterophyllum* A-plant, B- Inflorescence, C- Flowering twig (Raina *et al.*, 2011)

Geographical Distribution

Vegetation of *A. heterophyllum* occurs in the Himalayas stretching across Pakistan, India and Nepal. In India, distribution of *A. heterophyllum* is reported in the states of Jammu & Kashmir, Himachal Pradesh and Uttarakhand at altitudes ranging from 2400 to 4500 meters (Bhatt et al., 2014; Jabeen et al., 2013). Significantly, Stapf (1905), in his monograph- The Aconites of India, described its occurrence in the Himalayan alpine and subalpine regions, from the Indus to Kumaon, at an altitude of 1828 to 4572 meters. A chronological compilation of population trends, based on reported assessments, is given below to present the distribution and population status of the species in India, especially for Jammu & Kashmir, Himachal Pradesh and Uttarakhand.

a) Jammu & Kashmir

The plants of *A. heterophyllum* were reported by Stapf (1905) in Gulmarg at an altitude ranging from 2743-3048 m and in the surrounding areas covering, Liddar valley, Lidderwat and areas between Gulmarg and Kilan. Populations of the species were also reported, and specimens were collected by Beigh et al. (2006) from Gulmarg (Akad Patree Nallah, Kandwa Forest), Pir Panjal (Aharbal), Dachigam National Park and Dang Tangmarg. Jabeen et al. (2013) in their survey of the Aconitum genus reported that *A. heterophyllum* in Kashmir is represented by sparsely scattered, disjunct populations at 2125-3665 meters in elevation, limited to the subalpine and alpine habitats. Goraya et al. (2013) in their assessment reported the species at Saithal, Scoj, Klhoai (Anantnag) regions of Kashmir. In 2014, Baig et al. documented *A. heterophyllum* populations from Gurez, Harmukh, Khilanmarg, Thajwas, Munwarsar and Poshpather regions of Kashmir at an altitude of 2680 to 3500 m.

b) Himachal Pradesh

Stapf (1905) documented the presence of the species across a wide range of locations in the state of Himachal Pradesh, including the Kalatop forest (2438 meters) Chamba and Dalhousie (also at 2438 meters) and various points between Dalhousie and Chamba (starting at 1828 meters). Further, plantations were observed in Baili (2133 meters), near the Sauch Pass (3352 meters), in the upper Sanch valley of Pangi (between 3657 and 4572 meters), and within the meadows of the Lahaul-Bagha valley (2743 meters). The species was also noted in Sarahan within the Kangra-Kullu area (2438 meters), Sirmor-Muhassu (2133-3048 meters), and in the Shimla, Baghi forest, Jubal (3048 meters), Fagu, and Nagkanda regions. In 2007 a field survey on Medicinal plants of Himachal Pradesh was conducted by Samant et al. (2007) and presence of *A. heterophyllum* was reported in Subalpine (altitude 2801-3800 m) and Alpine (altitude >

3800 m) of the state. Populations of the species were also reported by Goraya et al. (2013) and Ved et al. (2003) in Chhota & Bara Bhangal, Great Himalayan National Park, Dainsar and Hamsar in upper Shimla, Panju Talram, Chhajpur and Khashadhar, Larot, Shagali Thatch, Kullu valley, Sheuthru and Bajouni, Thatch, Tod Valley, Chika, Mivar Valley, Miyar Nallah, Mani Mahesh, Patrair Valley (Lahaul and Spiti), Chamba and Pangi valley.

c) Uttarakhand

Presence of the species was reported by Stapf (1905) in several locations within the Garhwal and Kumaon regions. Specifically, it was observed at Dudu gach near Srikanta (ranging from 4267 to 4572 meters), in high-altitude meadows near Srikanta, Chinpul, and Bandrpunch (3657 to 3962 meters), at Derali in the Ganges valley (2743 to 3048 meters), and within open areas near the Milan Glacier and Surajkund in Kumaon (around 3962 meters). Later, Nautiyal et al. (2002) conducted a survey of the subalpine and alpine zones of the Garhwal Himalayas in Uttarakhand, during the plant's active growth period (May to November). Their population analysis revealed *A. heterophyllum* presence between 3400 and 4200 meters. They identified locations such as Hari Ki Dun (3450 meters) in Uttarkashi, Kedarnath (3450 and 3550 meters) in Rudraprayag, Tungnath (3500, 3600, and 3600 meters) also in Madhyamaheshwar (3400, 3500, and 4200 meters), and Panwali Kantha (3400 and 3550 meters) in Tehri. More recent surveys, including those by Goraya et al. (2013) and Ved et al. (2003), have confirmed its presence in locations like Dayara, Rudranath, Kuovari Pass, Kyarki, Bedani, Milam, Gidara, Kushkalyani (Uttarkashi), Bagi (Tehri), and the Malari and Milam regions within the Nanda Devi Biosphere Reserve.

Chemical Constituents

Roots of *A. heterophyllum* are notable for their high concentration of diterpene alkaloids, including atisine, a relatively low-toxicity compound within the simpler aconite alkaloid group. **Table-1** summarizes the compounds found in the roots of *A. heterophyllum*.

Table 1: Compounds identified in roots of *A. heterophyllum*.

Compound	**Reported by**	**Category**
Atisine	Brimton, 1877	Diterpenoid alkaloids
Isoatisine		
Hetisine	Jacobs and Craig, 1942 Jacobs and Craig, 1943	Diterpenoid alkaloids
Heteratisine		
Benzoylheteratisine		
Atidine	Pelletier et al., 1968	Diterpenoid alkaloids
F-dihydroatisine		
Heterophyllisine		
Heterophylline		
Heterophyllidine		
Hetidine		
Hetisinone (dehydrohetisine)		
Atisenol	Pelletier et al., 1982	Diterpenoid alkaloids
6-dehydroacetylsepaconitine	Ahmad et al., 2008	Norditerpenoid alkaloids
13-hydroxylappaconitine		
Lycoctonine		
Delphatine		
Lappaconitine		
Heterophyllinine-A	Nisar et al., 2009	Diterpenoid alkaloids
Heterophyllinine-B		
Dihydroatisine		
2-O-cinnamoyl hetisine	Anmol et al., 2022	Diterpenoid alkaloids
4-oxabicyclo [3.2.2] nona-1(7),5,8-triene		
Atisinium cinnamate		
Atisinium formate		

Medicinal Uses and Pharmacological Activities

Traditionally, Indian medicine has utilized the powdered root of *A. heterophyllum*, Ativisa, to treat fevers and as a bitter tonic for convalescence after malaria and other febrile diseases (Chopra et al., 1958). Ancient Indian literature, like the *Charaka Samhita*, recommends its use in the treatment of conditions such as obesity, haemorrhoids, and gastrointestinal disorders. In addition, it is mentioned to be useful in the management of diarrhoea (*Sushruta Samhita*), dysentery, cough, chronic intestinal inflammation and indigestion (Pelletier et al., 1968). Ativisa is a main component of many Ayurvedic medicine preparations, such as *Chitrakadi vati, Rasnerandadi Kwatha, Sudarshana Churna, Balachaturbhadra Churna, Mahavisagarbha Taila, Panchatiktaka Guggulu Ghrta, Rodhrasava, Satyadi yoga* and *Kutajghav vati* (Lather et al., 2010; Ajanal et al., 2012; Joshi et al., 2014; Baishya et al., 2020). Many pharmacological studies have also unmasked a wide range of biological activities of the roots of *A. heterophyllum* (**Table 2**).

Table 2: Pharmacological activities shown by Ativisa

Activities	Reference
Anti-diarrheal	Singh and Chaturvedi (1982), Venkatasubramanian et al., 2010; Prasad et al., 2014
Anti-inflammatory	Verma et al., 2010
Anti-pyretic	Verma et al., 2010
Anti-helminthic	Pattewar et al., 2012
Anti-hyperlipidemic	Subash and Augustine, 2012
Anti-microbial	Ahmad et al., 2008; Munir et al., 2014
Anti-oxidant	Prasad et al., 2012; Munir et al., 2014; Konda et al., 2016
Immunomodulatory	Atal et al., 1986
Action on the nervous system	Hamet, 1938; Hamet, 1954; Nisar et al., 2009
Nephroprotective	Konda et al., 2016
Anti-obesity	Jain et al., 2019
Anti-plasmodial	Anmol et al., 2022

Chemical Constituents from Aerial Parts and their Pharmacological Activities

Although the roots of *A. heterophyllum* are well-documented for their pharmacological uses in traditional folk medicine, the aerial parts of the plant have been relatively underexplored. More recently, studies investigated the therapeutic benefits of the aerial parts. In a study, Srivastava et al. (2011) illustrated potent antimicrobial activity in methanolic extracts of the aerial parts, exhibiting both antibacterial and antifungal activity. Subsequent phytochemical investigation by Pande et al. (2014) resulted in the isolation of five alkaloids, four diterpenoids (two C19 and two C20) and atonce, all of which showed antidiarrheal activity comparable to that of the whole herb. Ahmad et al. (2017) also isolated eight diterpenoid alkaloids from the aerial parts, reporting their strong antioxidant and anticholinesterase activities. These compounds consisted of 6β-Methoxy, 1α,11,13β-trihydroxylhetisine; 9β-dihydroxylheteratisine; iso-atisine; 6,15β-dihydroxylhetisine; hetisinone; heteratisine; atidine and 19-epi-isoatisine.

In addition, Kumar and Chauhan (2016) identified steviol, a diterpene famous for its sweet-tasting steviol glycosides which are being employed as artificial sweeteners, in the leaves of *A. heterophyllum*. Interestingly, the intensity of steviol in this plant was observed to be equivalent to that of *Stevia rebaudiana*, presently the key commercial source of the compound.

Trade and Market Demands

The dried, tuberous roots of *A. heterophyllum*, which are sold commercially as Ativisa, fetch a market price of INR 3000 to 4000 per kilogram. A 2015 report, commissioned by the National Medicinal Plants Board (NMPB) and undertaken by Goraya and Ved (2017) under the Indian Council of Forestry Research and Education (ICFRE), found that the Indian herbal trade used an estimated 127.65 metric tons of the raw material in 2014-15, mainly obtained from wild Himalayan populations. Another 25.8 metric tons were used by rural homes. Although there are cultivation attempts, the research pointed out that wild harvesting is still the major source of Ativisa.

Figure 2: External morphology of material traded as Ativisa

The rhizomes of *Cyperus rotundus*, known as Musta and belonging to Cyperaceae, are sometimes used as a substitute for Ativisa. A 2010 study by Venkatasubramanian et al. suggested this practice is supported by comparable Ayurvedic pharmacological properties, phytochemical profiles, and anti-diarrheal effects between *A. heterophyllum* and *C. rotundus*. However, other adulterant plant materials are also sold as substitute for atees or ativisa in various Indian markets. For instance, *Cryptocoryne spiralis* corms are traded as Atividayam in Chennai and nattativitayam in Kerala (Anandakumar et al., 1982; Prasad et al., 2012). Similarly, *Chaerophyllum villosum* roots are reportedly sold as Atees, under the name Mithi-Patish, in Delhi (Goraya and Ved, 2017).

Conservation and Cultivation

Scattered populations of *A. heterophyllum* is found in the nature at restricted locations with low density (Dhar and Kachroo, 1983). This factor along with unsustainable collection (Nautiyal et al., 2002) of the tuberous roots due to its medicinal importance and high market value, has placed *A. heterophyllum* in endangered category of International Union for the Conservation of Natural Resources (IUCN) in the Red Data Book (Ved et al., 2003). During the Conservation Assessment and Management Plans workshop (CAMP) held in 2003 (Ved et al., 2003) at Shimla (Himachal Pradesh), it was observed that 70% of the wild population had declined over the previous ten years in India. Goraya and Ved (2017) assessed *A. heterophyllum* as Critically Endangered in Jammu & Kashmir, Himachal Pradesh, and Uttarakhand, and stressed upon sustainable use *A. heterophyllum* and conservation of its natural habitats.

Apprehension has been voiced concerning the swift decline of the species from its natural habitats, mainly contributed by overharvesting and uncontrolled gathering (Nautiyal et al., 2002). Observations point to the fact that populations are usually fragmented and scattered within their natural habitats (Nautiyal et al., 2002; Wani et al., 2022). For this reason, a critical imperative exists for the implementation of conservation measures and sustainable use techniques. Cultivation is seen as a sustainable method of both conserving wild populations and ensuring sustainable use of *A. heterophyllum* (Nautiyal and Nautiyal, 2004; Nautiyal et al., 2006; Goraya and Ved, 2017). Although seed-propagation is commonly regarded as the most effective means (Sharma et al., 2006), *A. heterophyllum* is challenging in this respect (Ved et al., 2003). These problems are caused by restricted seed supply, deficiency of quality germplasm (Nautiyal et al., 2009), and seed germination, dormancy, and viability-related problems (Nautiyal, 1986; Nautiyal and Dhyani, 1994) that also result in low rates of natural regeneration. Additionally, vegetative propagation through stem cuttings and tubers has given very poor success rates (Nautiyal, 1986; Nautiyal and Dhyani, 1994; Beigh et al., 2006).

Recognizing the limited seed availability of *A. heterophyllum*, scientists have studied the factors that impact its reproduction. Nautiyal et al. (2009) examined pollen germination, pollination, and fruit and seed development and concluded that low pollen viability can constrain seed production in alpine habitats (Cabin et al., 1991). They observed that optimal treatments with gibberellic acid (GA_3) improved pollen germination. In addition, they also revealed that the anther dehiscence is necessary at high temperatures and that *A. heterophyllum* is self-incompatible.

Improvement in seed germination has also been the focus of studies. Pandey et al. (2000) proved that germination could be promoted by high BAP concentrations. Beigh et al. (2006) investigated several treatments, such as scarification, coat puncturing, hot water treatment, washing with sulfuric acid, and chilling, to overcome seed dormancy. The seeds were found to show prolonged dormancy, which could be broken by chilling. Seeds collected in the wild were 60-70% viable, with 17% germination upon chilling (versus 2% for non-chilled seeds). Germination normally took place early in the spring (March-April) after winter dormancy. Seeds from ex situ cultivated plants showed very high germination (50-60%) upon chilling.

Beigh et al. (2006) also described the plant's cultivation cycle, noting that although it may be cultivated at lower elevations than where it occurs naturally, growth is slightly limited.

Pandey et al. (2005) were able to prove the successful cultivation and preservation of *A. heterophyllum* in ex-situ conditions, both open and under greenhouse, from its natural environment. They were able to do so by eliminating seed dormancy by hot water treatment. Significantly, plants grown in such ex-situ conditions showed a similar life cycle as plants in their natural habitat, showing successful acclimatization The researchers also observed that the plants growing in situ had smaller heights and wider leaves but had higher root biomass compared to their ex-situ counterparts.

Recognizing the significance of *A. heterophyllum* and other medicinal plants, India's National Medicinal Plants Board (NMPB) undertook in situ conservation activities during 2007-2008. This included the creation of Medicinal Plants Conservation and Development Areas (MPCDAs), with a particular 476-hectare area identified within the Dhauladhar and Zanskar ranges of Himachal Pradesh to conserve *A. heterophyllum* and 15 other endangered species (Biswas et al., 2017).

Molecular Markers and Importance of Studying Genetic Diversity of the species along with their Population Structure

Population studies by Nautiyal et al. (2002) found that *A. heterophyllum* to be severely population fragmented, with none of the subpopulations greater than 250 mature individuals. This trend of small, isolated populations is typical of those tendencies of endangered species, as defined by the IUCN. This type of fragmentation is also indicative of high extinction threats due to greater vulnerability to inbreeding and the loss of genetic diversity. Low genetic diversity can degrade both short-term reproductive performance and long-term evolutionary fitness to environmental change (Frankham, 2002; Ouborg et al., 2006). In addition, habitat fragmentation restricts gene flow which in turn increases genetic differentiation among populations and genetic structuring due to genetic drift (Wright, 1969; Ouborg et al., 2006). Smaller, endangered species, by contrast, tend to have lesser genetic diversity. For instance, Frankham (2010) demonstrated that endangered species from various plant and animal groups possess significantly lower heterozygosity at microsatellite loci than their non-endangered counterparts, averaging approximately 65% of the non-endangered species genetic diversity.

The ability of a population to adapt and change with environmental change is directly associated with its genetic diversity, or the amount of its gene pool. Therefore, measuring it within populations, and examining the population differentiation, has emerged as the key focus of conservation genetics (Sork et al., 2006). Such an understanding is the essential building block for creating successful conservation policies (Rivers et al., 2011).

Microsatellite markers (Simple Sequence Repeats- SSRs) are used widely in applications, including genetic diversity assessment, phylogenetic analysis, genotypic profiling, agronomic trait selection and varietal identification. Microsatellites have been used to evaluate genetic diversity along with the structure of population in a wide variety of endangered plant species (Kumar et al., 2014; Wu et al., 2016; Aboukhalid et al., 2017; Collin et al., 2017; Hatmaker et al., 2018; Litkowiec et al., 2018; Ni et al., 2018; Zhang et al., 2019). Studies are reported for the genetic assessments using SSR markers in various species of Genus Aconitum, however *A. heterophyllum* remains unstudied using this marker. **Table-3** summarizes the microsatellite maker characterized and genetic diversity studied in some species of Genus- *Aconitum*. Molecular markers other than microsatellites were also developed and used in some species of Aconitum genus e.g. Amplified-Fragment Length Polymorphism-AFLP markers for A. *heterophyllum*, *A. violaceum*, *A. balfourii*, and *A. ferox* (Dubey et.al., 2010); Inter Simple Sequence Repeats- ISSR markers for *A. leucostomum* (Gao et.al., 2014), *A. kongboense* (Meng et al., 2015) and *A. plicatum* (Mitka et.al., 2007); SCAR marker for validation of *A. heterophyllum* along with *C. rotundus* (Seethapathy et.al., 2014); Random Amplified Polymorphic DNA-RAPD markers in *A. vulparia*, *A. paniculatum*, *A. napellus* subsp. *tauricum* and *A. napellus* sub sp. *neomontanum* (Fico et.al., 2003).

Table 3: Microsatellite markers developed in different Aconitum species

Investigator	**Details**
Cadre et al., 2005	Characterized six polymorphic microsatellite markers study the polymorphism among six populations of *A. napellus* from France.
Xu et al., 2011	Identified sixteen polymorphic microsatellite loci in 66 individuals from six populations of *A. gymnandrum* endemic to Qinghai – Tibetan Plateau
Won et al., 2012	Reported Ten microsatellite markers and studied diversity in twelve populations of *A. coreanum* in Korea was assessed. Isolation of the species was reported due to heterozygosity deficit, low level of among-population differentiation and less gene flow.
He et al., 2015	Identified eighteen SSR markers in *A. vilmorinianum*, a Chinese traditional medicinal plant, using next-generation sequencing technology.
Li et al., 2015	Identified twelve microsatellite markers from two microsatellite-enriched libraries (AG, AC) and checked polymorphism of each locus 24 accessions of *A. brachypodum*, a critically endangered medicinal plant, endemic to China
Yun et al., 2015	Developed nine SSRs using NGS in *A. austrokoreense*, an endangered medicinal plant, endemic to Korea.
Ge et al., 2016	Developed thirteen microsatellite markers by genomic enrichment in three populations of *A. kusnezoffii*. They also observed that most of the primers designed for the identified loci could also amplify corresponding microsatellite loci in *A. barbatum* var. *puberulum* and *A. alboviolaceum*.
Park et al., 2017	Identified 36 species-specific microsatellite markers from the chloroplast genome in Korean species of Genus Aconitum- *A. pseudolaeve* and *A. longecassidatum*. Fifty-three indels and sixty two single-nucleotide polymorphisms (SNPs) were also observed between the two CP genomes.

Lee et al., 2018	Used microsatellites markers, five from nucleus and one from chloroplast for genetic assessment 0f seven populations throughout South Korea of *A. austrokoreense*, an endangered and endemic species in Korea. Low diversity was observed in the population. The pattern of isolation by distance was indicated by significant correlation between geographical and genetic distances. Results suggested recent population bottlenecks in populations of *A. austrokoreense*. Increased extinction risk was assessed due to limited dispersal ability and habitat fragmentation.
Zhou et al., 2018	Reported low interspecific transferability of the microsatellite markers developed by He et al., 2015 and Ge et al., 2016 in other Aconitum species using next generation sequencing and thus isolated and characterized 12 new microsatellites for A*. reclinatum* to investigate its genetic structure.

Root Rhizosphere and its importance in agriculture

The rhizosphere is a highly active zone of plant and microorganism's interaction, which has direct impact on the plant health, fitness, development and for nutrient cycling (Zhalnina et al., 2018). The soil nearest to the root system is the rhizosphere where the roots release vast variety of metabolites called root exudates that highly affect microbial activity. Studies have shown that plants via their roots, release these chemical compounds in the soil that selectively stimulate beneficial microbes for residing in the rhizosphere (Reinhold-Hurek et al., 2015: Sasse et al., 2018). The rhizospheric microbiome can have beneficial members (that protect from pathogens, help in providing nutrients from soil, and enhance tolerance to abiotic stress), and pathogenic (hazardous) organisms too. It can have a third group of microorganisms which are true and opportunistic human pathogens that can be found in or on plant tissue and may cause disease when ingested by humans (Mendes et al., 2013). In the rhizosphere, a wide variety of both macro and micro-organisms co-exist, like bacteria, virus, fungi, protozoa, algae, micro arthropods, and nematodes, and exhibit a range of interactions with the plant as well as among themselves. The rhizospheric bacteria having beneficial effects on the plant are called Plant Growth Promoting Rhizobacteria (PGPR). Studies have shown the benefits of rhizospheric interactions in providing pathogenic resistance to the plants and in increasing stress tolerance (Qu et al., 2020). The rhizospheric microbiome can also be used as an indicator of soil pollution and therefore soil health (Wang et al., 2019). Some studies have also shown the added advantage provided by plant-associated microorganisms as they tend to augment immune functions of their host plant (Vannier et al., 2019). A Classic example of rhizospheric plant-microbe interaction is Rhizobium symbiosis. Rhizobium-genus of Gram-negative soil bacteria known for fixation of nitrogen. Leguminous plants can form a symbiotic relationship with rhizobia. The rhizobium attaches to the roots of the plant and forms nodules. Rhizobium inside these nodules, converts atmospheric nitrogen to biologically useful form-ammonia. Nitrogen fixation cannot be done independently, which is why rhizobia require a plant host. Nitrogen fixation plays essential role for increasing soil fertility and productivity. Conversely, the plant provides photosynthetically fixed carbon, carbohydrates as energy source to the bacteria.

Application of Nitrogen-fixing bacteria

Nitrogen is an essential element for living beings. It is needed to make important structural components of cells including amino acids, nucleic acids, hormones, chlorophyll, and several vitamins. With increasing population, the demand for non-leguminous plants like rice, wheat, maize, sugarcane, etc. is exponentially growing. The nitrogen-fixing microbes, collectively called as Diazotrophs, can be symbiotic or free-living fixers. The ability of NF (nitrogen-fixing) bacteria to interact with leguminous plants roots, develop a symbiotic relationship and assist in nitrogen fixation has been exploited greatly in agricultural fields. For instance, *Azospirillum* spp. are PGPB, nitrogen-fixers, associate with several cereals and crops like rice, wheat, maize, sugarcane, etc. One of the most exploited PGPB is *Azospirillum brasilense,* being used in Brazil, India, Argentina, Europe, and Mexico.

CHAPTER 3

Material and Methods

Identification of habitats and collection of samples

Habitat identification of the targeted species- *Aconitum heterophyllum* was done by exploring literature and local leads from Uttarakhand and Himachal Pradesh states of India. Botanical Identification was done and documented through Herbarium preparation. The voucher specimens of each population were deposited in the herbarium of Taxonomy department of the University of Trans Disciplinary Health Sciences and Technology, Bangalore. The Geographical coordinates were mapped using the geographical coordinates in geographic information system program QGIS. Random sampling was done from each population based on availability of individuals, as population were observed to carry very low population density of the species.

Collection of samples for Genetic diversity studies

Leaf samples were collected and washed with water to remove dirt and other foreign material. They were further wiped with 70% ethanol, surface dried and stored in packets containing silica gel for 24 hours to remove moisture from the collected leaves. The leaf samples were stored in silica gel under room temperature until further processing.

Collection of plants for root microbiome studies

Samples of *A. heterophyllum* root tip and base parts were collected form the identified populations representing the wild and cultivated population. Excess soil was removed from the roots by shaking, leaving roots and firmly adhering soil, the rhizosphere soil. Samples were stored at 4°C for no more than 24 hours before processing.

Characterization of microsatellite markers and assessment of genetic diversity

Microsatellite (SSR) markers were characterized following the DNA isolation. The characterized markers were used for the assessment of diversity in the collected samples. The outline of the study for characterization of microsatellite markers and assessment of genetic diversity is depicted in **figure 3** and detailed in the sections below.

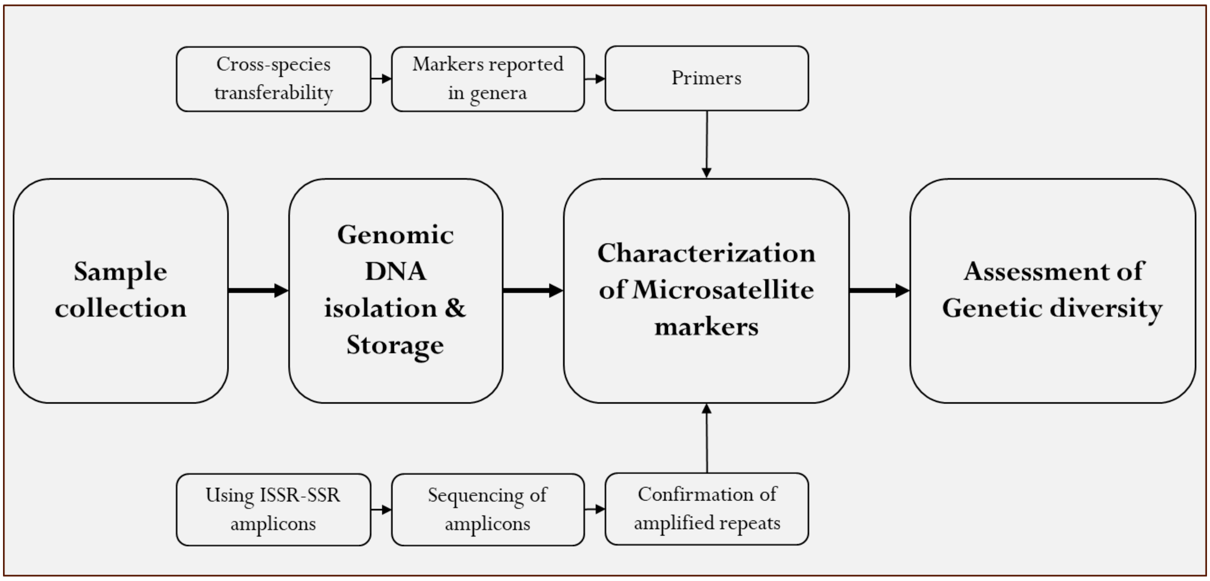

Figure 3: Outline of study for characterization of microsatellite markers and targeted assessment

DNA extraction and Selection of primers for assessment of genetic diversity

DNA was isolated from the collected accessions using standard procedure (**Annexure 1)** of Qiagen DNeasy plant DNA isolation mini kit (Qiagen, USA) and stored at -20°C. To detect polymorphism among the collected accessions, microsatellite loci was identified by utilizing the approaches listed below:

Transferability of SSR markers- Microsatellite flanking regions, which have lower mutation rates than SSR regions, tend to be sequence-conserved among related species or genera (Holmen et al., 2009). This conservation allows for the application of primers from one species to amplify homologous regions in other species, a process referred to as cross-species or cross-genera transferability. To detect microsatellites in *A. heterophyllum*, 44 primer pairs (summarized in **Table 4**) were chosen, based on earlier reported numbers of alleles in different Aconitum species. The suitability of these primers for *A. heterophyllum* was subsequently tested. Out of the selected primers, four were characterized by Cadre *et al.*, (2005) in *A. napellus* (Lu12, Lu13, Lu15 and Lu28), seven by Xu *et al.*, (2011) in *A. gymnandrum* (Ag01, Ag03, Ag05, Ag07, Ag12, Ag14 and Ag15), three by Won *et al.*, (2012) in *A. coreanum* (AK_AT13, AK_CT07 and AK_CA03), seven by He *et al.*, (2015) in *A. vilmorinianum* (Av_7306, Av_11592, Av_19762, Av_32854, Av_38749, Av_40439 and Av_84257), five by Li *et al.*, (2015) in *A. brachypodum* (AcoB01, AcoB03, AcoB05, AcoB11 and AcoB12), four by Yun *et al.*, (2015) in *A. austrokoreense* (Aau_CA04, Aau_ATA02, Aau_AT37 and Aau_ACA03), seven by Ge *et al.*, (2016) in *A. kusnezoffii* (Ak1, Ak2, Ak6, Ak7, Ak13, Ak17 and Ak19) and seven by Zhou *et al.*, (2018) in *A. reclinatum* (AR01, AR03, AR04, AR06, AR09, AR10 and AR11).

Table 4: Details of 44 sets of primers used for accessing transferability

Primer No.	Code	Sequence (5'-3')	T_a (° C)
1	Lu12f	CAACCGACCTATCCTTCGAC	55
	Lu12r	AAGGCTACACCATGGACTGTG	
2	Lu13f	CGTTTTGTTGGGTTTTCACG	55
	Lu13r	TGGTTGTCCTGGGCATAAAC	
3	Lu15f	AGGAAACACTGTCACGGTACG	55
	Lu15r	GGGTGGAGACACCAATTCAG	
4	Lu28f	GATGGAGAGTGTGCTTTCTCG	55
	Lu28r	CCGTCGATGGACTCTAGTTAGG	
5	Ag01f	ACATCATTACTCGCACAACG	60
	Ag01r	CTAGACATGCCCAGACTTAA	
6	Ag03f	GTGAGCGAGTCGAGTTGTG	53
	Ag03r	CTCTTCCCTTTCTCGTGTT	
7	Ag05f	TCACGAGCATCCGAAGACAA	50
	Ag05r	TCATGTGGGCAATATCAAAG	
8	Ag07f	GTGAGCCGCAGGGACTGTTCT	60
	Ag07r	TGGCATCTACAAACGCCGACA	
9	Ag12f	TCTCCTCTTCCTCTTGGTC	50
	Ag12r	ATGAATCTAGTGATGCTTT	
10	Ag14f	TTGTGGTGTCCTCCATCCTT	56
	Ag14r	GGACCCTCAGAGCCCAAGAG	
11	Ag15f	AACAGCCACCACCGGCAGAC	60
	Ag15r	ACAATAGCATTGGAGGGACAT	
12	AK_AT13f	GCAGAAACCAATACTGACCACT	56
	AK_AT13r	GGAATTACCCAAGAGATGTCAA	
13	AK_CT07f	TTCATATTGATCAAGTCCACCA	56
	AK_CT07r	TCAGCAGGTTCTGTCCTTATTT	
14	AK_CA03f	AATAAGCAATGTTGGTTGGAAG	56
	AK_CA03r	TTGAGTACCACTTGGTCGAAAT	
15	Av_7306f	AGTTTCCATATTTTCGTG	45
	Av_7306r	GGAGTTCTACAAGGTTTT	
16	Av_11592f	CTAAACGACCGACCTTGT	51
	Av_11592r	GTTGTTCTGCTGCCTCTT	
17	Av_19762f	TAGTCCGTGCTTGTATGA	47
	Av_19762r	CGGTGTTTAGAAGGTTAG	
18	Av_32854f	GGTGCCTTATCTGCGTGT	47
	Av_32854r	TCTTCTCGGGTCTTCTCC	
19	Av_38749f	AAAGGAGGATGGGTCAAA	52

	Av_38749r	CAAGTTGTTCGCAGTGTTC	
20	Av_40439f	AATCTCCCACCTTGTTCT	48
	Av_40439r	AAGCATCGGTAAGTCGTT	
21	Av_84257f	CCAGGGTCAGCATCTTCTT	52
	Av_84257r	CACTCAGTAATCCGAAACG	
22	AcoB01f	AATAACGCCGTCCAATCTCA	55
	AcoB01r	TTGTGTGCTTTCTCTCTCTTCA	
23	AcoB03f	TTTGAGGTTTCGCATTGAGG	50
	AcoB03r	CGTCGTTTATGATTTTCTC	
24	AcoB05f	TACAAGAAATGAGAACCAGTT	51
	AcoB05r	GTAAGCCAAAAGATGAAGAC	
25	AcoB11f	ATGCGATTAGAGATGGATGC	55
	AcoB11r	CCAAGCGAACACCGAATGAT	
26	AcoB12f	GGAATCACTGAGAAACCTAT	50
	AcoB12r	GCTTTCCATTATTTCCCCTA	
27	Aau_CA04f	F: AAGATTCCATCCCGATCTTTAT	56
	Aau_CA04r	R: ATATCAACGGATCAAACCAAAT	
28	Aau_ATA02f	F: GCTTGAGGGGATTAGAGAGATT	56
	Aau_ATA02r	R: TCTTTTCCAGTGTTGGAAACTT	
29	Aau_AT37f	F: CCATTGGATGATGGTGTTTT	56
	Aau_AT37r	R: CGCAGCAATCACACAAGTAA	
30	Aau_ACA03f	F: GCTCGACGATTCCACAGTA	56
	Aau_ACA03r	R: TAACTTCTCCGTTCCGCTCT	
31	Ak1f	GGACAAGTAATCGCCGTGGA	60
	Ak1r	CTAGTGTGATGTGGAGCTTA	
32	Ak2f	TGTGTTGGTCCTTACATGTG	57
	Ak2r	GTTGCACTCATGCACAGACA	
33	Ak6f	CTTGTTATTATGATTGGTGATGGGAT	49
	Ak6r	CTATCATCACTTGCCGTACTTTTCAG	
34	Ak7f	AATCAAAGTCTACAGCGG	60
	Ak7r	GAATAGGATGCGTCAGTT	
35	Ak13f	AATACGTCGTCGTCACGCAAAC	49
	Ak13r	TCTGTTCCTGGGCTTCTCATCTC	
36	Ak17f	GTTGTAGACTCGGTGGGAGCATTG	50
	Ak17r	GGGTGGATTCGGTTATAGAGGGTG	
37	Ak19f	CCTACCCGGCCTCCTTTCTTC	50
	Ak19r	CCATCGGTTTCTCACCTGAATCTTG	
38	AR01f	TTAGACTTACACGGCCCAGG	60
	AR01r	GTTCCGGGCTTCTCATAACA	
39	AR03f	ATGAATGCAAAGTCCCTTGG	60
	AR03r	GAAGGAGTGCGGTTGATGAT	
40	AR04f	TGCTGCTTTCAGGAACAATG	60
	AR04r	AGGAGGACATTGGTGAATCG	
41	AR06f	CGATCTGACTAGGCCCACAT	60
	AR06r	GGAGAGGGTGGGAATTAGGA	
42	AR09f	CGAGCCATTTCACTTGTGTG	60

	AR09r	AGGAGCGAATGTGAGTTGCT	
43	AR10f	GAAGGGTATTTTCTCCCCCA	60
	AR10r	ATCCACAGGGACAAACTTGC	
44	AR11f	ACCAACTCAGGCATTTGGTC	60
	AR11r	CTCCTCCAATCCCATCAGAA	

Anchored microsatellite-primed PCR (AMP-PCR)- Single primers in PCR, using di- or trinucleotide repeats with 5' or 3' anchors, are able to amplify microsatellite areas that have those particular repeats (Zietkiewicz et al., 1994). This research used 5'-anchored primers, adhering to the design guidelines of Fisher et al. (1996), where the 5'-anchor allows for the target microsatellite to be part of the amplified product. In particular, the degenerate primer PCT4, with the sequence KKVRVRV$(CT)_6$ (K = G/T, V = G/C/A, R = G/A), was tried in *A. heterophyllum*. The $(CT)_6$ section of this primer is intended to bind to (GA)n repeats. To investigate the possibility of different microsatellite repeat types, as suggested by Fisher et al. (1996) for genomes of different kinds, some di- and trinucleotide repeat motifs were also tried. Six degenerate 5'-anchored primers, which are listed in **Table 5**, were employed, covering varied repeat motifs (Caldeira et al., 2002; Kale et al., 2012): [KKVRVRV$(CT)_6$, KKVRVRV$(CT)_{10}$, KKVRVRV$(GA)_{10}$, KKVRVRV$(CA)_6$, KKVRVRV$(AGA)_6$ and KKVRVRV$(GT)_6$]. Integrated DNA Technologies (Iowa, USA) had these primers synthesized. The primers were then used in PCR amplification of *A. heterophyllum* as described in the protocol given by Fisher et al. (1996).

Table 5: Details of 6 sets of primers for accessing 5’- anchored primers

Primer No.	Code	Sequence (5'-3')	Annealing Temperature
1	5ADPIR-01	KKVRVRV$(CT)_6$	60
2	5ADPIR-03	KKVRVRV$(CT)_{10}$	60
3	5ADPIR-04	KKVRVRV$(GA)_{10}$	60
4	5ADPIR-05	KKVRVRV$(CA)_6$	60
5	5ADPIR-12	KKVRVRV$(AGA)_6$	60
6	5ADPIR-18	KKVRVRV$(GT)_6$	60

To confirm microsatellite region amplification, DNA sequencing analysis was conducted using the Oxford Nanopore MinION sequencer (ONT, Oxford Nanopore Technologies, UK). Quantified of the amplified DNA was done by using Qubit HS Kit (Thermo-Fisher Scientific, Waltham, US), and 1 µg amplicon was utilized for library preparation. The ligation sequencing kit (SQK-LSK109) was used for library preparation, following the manufacturer's standard protocol (see **Annexure 2**). Multiplexing of samples was done with the Native Barcoding Expansion 1-12 kit (ONT, EXP-NBD104). Sequencing was done using a R9.4 flow cell. Guppy basecaller (v3.6.0, ONT) was utilized for Basecalling, barcoding, and Q7 filtering. Quality control along with diagnostic analysis of the data resulting from sequencing was done using the MinIONQC program (Lanfear et al., 2019). Targeted SSR repeats were verified using Perl scripts with default parameters from the MIcroSAtellite (MISA) identification tool (Beier et al., 2017).

PCR, Electrophoresis and Data documentation

DNA-fragment amplification was performed by polymerase chain reaction (PCR) using the selected primers in a 50 µl reaction volume with Taq PCR Master-Mix kit (Qiagen, USA). The PCR cycling protocol involved an initial denaturation step for 5 minutes at 94°C, then 35-cycles for denaturation step for 30 seconds at 94°C, primer annealing for 30 seconds (temperature conditions are provided in **tables 4** and **table 5**), and the extension for 45 seconds at 72°C. The last step of final extension was done for 10 minutes at 72°C. The PCR products thus produced were separated by electrophoresis on 2% (w/v) agarose. GeneRuler 100 bp plus DNA Ladder (Thermo Fisher Scientific, Waltham, US) served as a molecular weight marker. UV illumination with an AlphamagerEC imaging system (Protein Simple, CA, USA) was employed for visualization of separated DNA fragments.

Data analysis

Fragment lengths of DNA bands, which were visualized and documented with 100bp ladders, were calculated using Alphaview software version 3.4 (Protein Simple, CA, USA). Population genetic parameters for Na-number of alleles, Ne-effective number of alleles, Shannon's information index (I), observed & expected heterozygosity (Ho and He), unbiased expected heterozygosity (uHe), and the percentage of polymorphic loci (%P) were calculated with GenAIEx 6.5 (Peakall and Smouse, 2012). Genetic relations within and between populations were investigated via analysis of molecular variance (AMOVA) with GenAIEx 6.50 (Peakall and Smouse, 2012). Population structure was studied with STRUCTURE 2.3.40 (Pritchard et al., 2000), with a Bayesian clustering method and an admixture model. Microsatellite marker information was analysed by fixing the assumed population (K) between 1 and 16, with five repetitions for each K, with 10,000 burn-in steps and 10,000 Markov Chain Monte Carlo (MCMC) iterations. The optimum population (K) was identified by computing Delta K values according to Evanno et al. (2005), employing the STRUCTURE HARVESTER program (Earl and Von Holdt, 2012). Accessions were grouped into subpopulations on the basis of their probability of association ≥60% to the group assigned by the program and individuals with a lower value were placed in admixtures. Cluster analysis was done using the NTSYSpc 2.2 (Rohlf, 1990) programme. Genetic similarity was estimated using the Dice coefficient (Nei and Li, 1979), and UPGMA was used to construct a dendrogram illustrating phylogenetic relationships. The similarity coefficients were tested with NTSYSpc 2.2.

Root microbiome studies

An outline of the root microbiome study is outlined in **figure 4** and detailed further in the sections below.

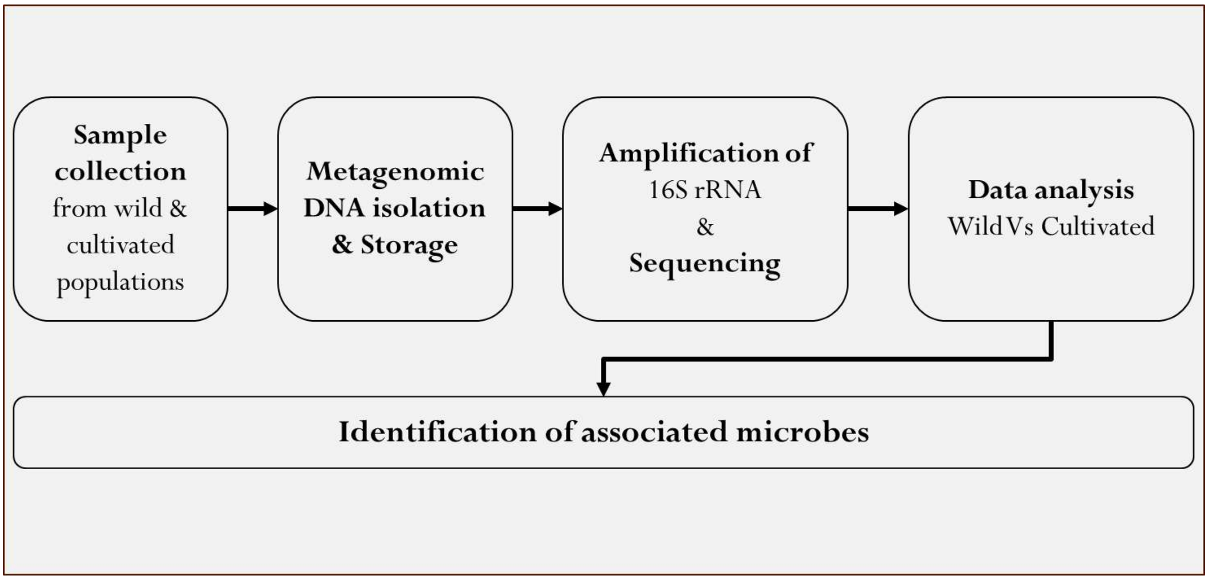

Figure 4: Outline of root microbiome study

DNA isolation of microbes associated with the roots

The rhizosphere fraction of microbes associated with the roots was separated using the protocol outlined by Edwards et al. (2015), which is based on Lundberg et al.'s (2012) method for isolation of endospheric bacteria. Upon harvesting the plants, vigorous shaking was performed on the roots to dislodge loose soil, with only the tightly bound soil remaining. Using scissors (sterilized), approximately 5cm of root following the root-shoot joint was cut and used further for isolation of DNA following separation of rhizosphere compartment in sterilized PBS (Phosphate Buffered Saline) solution. The roots were vortexed for 15-20 secs to mix the isolated fraction with PBS solution. DNA was isolated from the resulting soil suspension using standard procedure of Qiagen DNeasy PowerSoil kit (Qiagen, USA). 500ul of soil suspension generated earlier was used as starting material to be loaded to the PowerBead Pro Tubes.

The Identification of Root-Associated Microbiomes

In the present study the marker genes, 16S rRNA was used to study the root-associated bacterial microbiome in wild and cultivated populations of *A. heterophyllum*. The 16S ribosomal RNA (rRNA) gene is a component of the small subunit of the bacterial ribosome. Though it is highly conserved among bacteria and archaea, it also contains variable regions that can be used to distinguish different species and strains of microorganisms. By sequencing and analysing the 16S rRNA, bacteria and archaea can be identified and classified in the sample. The 16S rRNA gene comprises nine variable regions denoted as V1 to V9, which are separated by highly conserved sequences that remain consistent across various taxa. In the process of identification, the 16S rRNA gene is initially amplified through polymerase chain reaction (PCR) using primers designed to bind to these conserved regions, followed by subsequent sequencing.

Amplification of 16S rRNA gene

The forward and reverse set of primers used for amplification of the complete 16 rRNA gene are represented in **table 6**.

Table 6: Details of primers used for amplification of complete 16 rRNA gene

Primer No.	Code	Sequence (5'-3')
1	16S_27F	5'- AGAGTTTGATCATGGCTCAG -3'
2	16S_1492R	5'- CGGTTACCTTGTTACGACTT -3'

Primers were synthesized by Integrated DNA Technologies (Iowa, USA) and PCR was performed using the selected primers in a reaction volume of 50 µl using Taq PCR Master-Mix kit (Qiagen, USA). The PCR program had an initial cycle of five minutes at 94°C and then 35 cycles at 94°C for 45 Seconds, 45 Seconds at annealing temperature and 45 Seconds at 72°C. The final extension was performed at 72°C for 10 minutes. Resulting Amplicons were then separated on 2% (w/v) Agarose gel. GeneRuler 100 bp plus DNA Ladder (Thermo Fisher Scientific, Waltham, US) was used as a size fragment standard. Gels were visualized under UV with AlphamagerEC imaging system (Protein Simple, CA, USA) and checked for amplification of targeted 16 rRNA gene.

PCR amplicon purification:

Purification of 16 rRNA gene amplicons was done using AMPure beads (Beckman Coulter, USA). 50µl from PCR reaction mixture was taken in a 1.5ml centrifuge tube and 1X AMPure XP beads were added, tubes were continuously tapped gently for 5 minutes and spun briefly in a centrifuge. The tubes were then transferred to the magnetic rack to separate and withdraw and discard the clear transparent solution. The pellet attached to the magnetic rack was washed twice using 80% ethanol. The pellet was then dissolved in 50µl molecular grade water. The water containing purified amplicons were withdrawn to a fresh centrifuge using the magnetic rack.

Sequencing of amplicons:

Amplified fragments were sequenced using Oxford Nanopore MinION sequencer (ONT, Oxford Nanopore Technologies, UK), Amplicons were quantified using Qubit HS Kit (Thermo Fisher, Waltham, US), and 1 µg amplicon was used for library preparation. For library preparation, ligation sequencing kit (SQK-LSK109) was used according to the standard protocol provided by the manufacturer (**Annexure-II**). Samples were multiplexed using native Barcoding Expansion

1-12 kit (ONT, EXP-NBD104). The prepared library was sequenced on R9.4 flow cell. Basecalling, Barcoding and Q7 filtering was done using Guppy basecaller (v3.6.0, ONT). Quality check and diagnostic analyses was done utilizing the program- MinIONQC (Lanfear *et al.*, 2019).

Taxonomic analysis:

The raw reads (fastq) were processed to remove adapter sequences and low-quality bases using Porechop- a tool used to identify and eliminate adapters from the reads. The trimmed reads were processed to Kraken2 tool (Wood et al., 2019), which classifies the reads using k-mer method against GREENGENE and SILVA databases. Kraken2 results were visualized using Pavian package in R (Breitwieser and Salzberg, 2020). Pavian gives the taxonomic classification in the form of sankey plots. Pavian was used to generate abundance tables and processed for further downstream analysis.

Alpha diversity

It summarizes the measurement of an ecological community with respect to its richness (number of taxonomic groups), evenness (distribution of abundances of the groups) within the sample. Alpha diversity was derived using indices of which Chao1 and ACE represent the sample richness and Shannon, Simpson, InvSimpson and Fisher represent richness as well as relative abundance.

Beta diversity

Beta diversity is ratio in between the regional and local species diversity. To identify difference among OTUs abundance between the samples, STAMP was used to perform Fisher's exact test. It is a graphical software package that provides statistical hypothesis tests and exploratory plots (Parks *et al.,* 2014).

CHAPTER 4

Result and Discussion

Identification of habitats and collection of samples

High altitude regions (2500-4800 m) of Uttarakhand and Himachal Pradesh were identified as habitats of *A. heterophyllum* as reported in the literature. Sample collection from the identified habitats was done between August 2020 and October 2021, from eight populations detailed in **table 7**. The population sites mapped using the geographical coordinates in geographic information system program QGIS and are represented in **figure 5**. Random sampling was performed from each population based on availability of individuals, as population was found to be highly fragmented and small, witnessing extremely low population density of the species. Voucher specimens were deposited in the herbarium of Taxonomy department of the University of Trans Disciplinary Health Sciences and Technology, Bangalore. An herbarium from one population (Pangi valley, Chamba, Himachal Pradesh) along with field photographs is shown in **figure 6** while the herbarium for each population is attached as **Annexure 3**. The voucher numbers of the herbariums are shown in **table 7**.

Collection of samples for characterization of microsatellite markers and assessment of genetic diversity

Thirty-six leaf samples were collected and stored as per the protocol. The number of samples collected from each population is depicted in **table 7**.

Collection of plants for rhizospheric studies

Samples of *A. heterophyllum* root tip and base parts were collected form the identified populations. The rhizosphere soil from a total of 10 plant sample was harvested. Out of this, 5 sample represented the wild population and the remaining 5 represented the cultivated population. To ensure sufficient material for the downstream applications, pooled samples, each containing the rhizosphere soil of two plants, were prepared, overall representing the 10 populations. Number of samples collected from each population is depicted in **table 7**.

Table 7: Details of samples collected

Population code	**Location**	**Latitude/ longitude**	**Elevation (m)**	**Herbarium voucher no.**	**Number of samples (for genetic diversity)**	**Number of samples (for rhizospheric studies)**
AHD	Dayara-Uttarkashi, Uttarakhand	30°50'17.88''N, 78°33'18''E	3200	126423	4	2
AHBC	Bedni-Chamoli, Uttarakhand	30°12'22.32''N, 79°39'48.24''E	3300	126424	5	1
AHGC	Ghes-Chamoli, Uttarakhand	30°6'59.76''N, 79°42'2.52''E	2300	126425	6	1
AHKM	Khaliya-Munsyari, Uttarakhand	30°3'36.36''N, 80°11'18.96''E	3400	126426	5	2
AHCT	Chandrashila, Rudraprayag, Uttarakhand	30°29'42.72''N, 79°12'59.04''E	3600	126427	4	2
AHS	Sunderdhunga, Bageshwar, Uttarakhand	30°12'50.4''N, 79°54' 45''E	4000	126428	5	0
AHLS	Tindi-Lahaul spiti, Himachal Pradesh	32°43'48.36''N, 76°28'9.12''E	3200	126429	4	1
AHPC	Pangi Valley (Killar), Chamba, Himachal Pradesh	33°0'1.44''N, 76°31'6.24''E	3400	126430	3	1

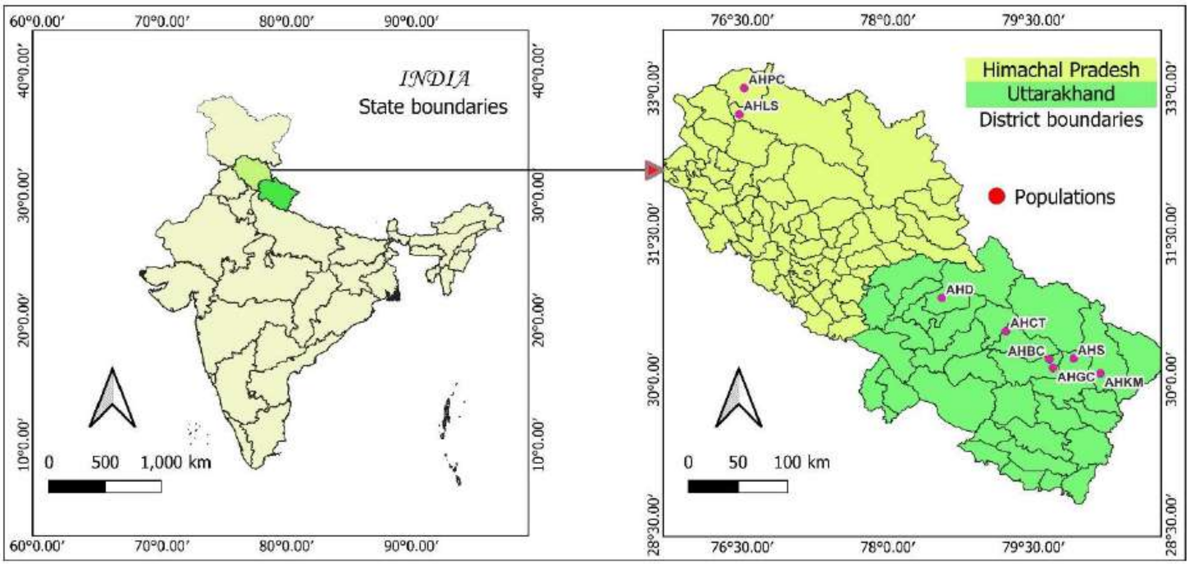

Figure 5: Map representing study area (8 populations of *A. heterophyllum*)

Figure 6: Herbarium from Pangi valley, Chamba (A) Field photographs (B to D)

Characterization of microsatellite markers

Through transferability of SSR markers- Based on amplification and polymorphism observed in the targeted species, 10 primers were selected. **Table 8** enlists the selected primers along with number of alleles observed in *A. heterophyllum* and the species in which the primer was originally reported. Transferability offers potential for the low-cost generation of microsatellite markers for related or distant species. Although low rate of cross-genera SSR transferability is reported (Aiello et al. 2020), cross-species transferability is reported to be successful, and a good rate is observed in various species (Kuleung et al. 2004; Gurcan et al. 2010; Lebedev et al. 2019). The present study reports successful cross-species transferability of microsatellite markers from different species of the genus *Aconitum* to *A. heterophyllum* species.

Table 8: Selected microsatellites from cross-species Transferability

Locus Name	**Primer sequence (5′–3′)**	**Repeat Motif**	**Alleles observed**	**Species**	**Reference**
Lu12	**F:** CAACCGACCTATCCTTCGAC **R:** AAGGCTACACCATGGACTGTG	$(GT)_3GGG(TG)_4$	3	*A. napellus*	Cadre et al. 2005
Ag07	**F:** GTGAGCCGCAGGGACTGTTCT **R:** TGGCATCTACAAACGCCGACA	$(AG)_8$	6	*A. gymnandrum*	Xu et al. 2011
Av_38749	**F:** AAAGGAGGATGGGTCAAA **R:** CAAGTTGTTCGCAGTGTTC	$(CT)_5$	3	*A. vilmorinianum*	He et al. 2015
Av_40439	**F:** AATCTCCCACCTTGTTCT **R:** AAGCATCGGTAAGTCGTT	$(GA)_{11}$	2	*A. vilmorinianum*	He et al. 2015
Av_84257	**F:** CCAGGGTCAGCATCTTCTT **R:** CACTCAGTAATCCGAAACG	$(TC)_5$	2	*A. vilmorinianum*	He et al. 2015
AcoB11	**F:** ATGCGATTAGAGATGGATGC **R:** CCAAGCGAACACCGAATGAT	$(TC)_{12}$	4	*A. brachypodum*	Li et al. 2015
Ak1	**F:** GGACAAGTAATCGCCGTGGA **R:** CTAGTGTGATGTGGAGCTTA	$(TC)_{12}$	3	*A. kusnezoffii*	Ge et al. 2016
Ak6	**F:** CTTGTTATTATGATTGGTGATGGGAT **R:** CTATCATCACTTGCCGTACTTTTCAG	$(TA)_7(TG)_{13}$	2	*A. kusnezoffii*	Ge et al. 2016
Ak19	**F:** CCTACCCGGCCTCCTTTCTTC **R:** CCATCGGTTTCTCACCTGAATCTTG	$(CA)_5$	7	*A. kusnezoffii*	Ge et al. 2016
AR09	**F:** CGAGCCATTTCACTTGTGTG **R:** AGGAGCGAATGTGAGTTGCT	$(CA)_{13}$	4	*A. reclinatum*	Zhou et al. 2018

Through Anchored microsatellite-primed PCR (AMP-PCR)- 5′-anchored primers with the targeted repeat motifs [KKVRVRV$(CT)_6$, KKVRVRV$(CT)_{10}$, KKVRVRV$(GA)_{10}$, KKVRVRV$(CA)_6$, KKVRVRV$(AGA)_6$ and KKVRVRV$(GT)_6$] successfully amplified the microsatellite region in *A. heterophyllum*. This was confirmed by the analysis of the sequence of amplified fragments which showed the presence of SSR sequences. Out of six 5′-anchored primers tested, four were selected (**Table 9**) based on the polymorphism observed in *A. heterophyllum*. DNA sequencing of the amplified fragments generated 3.6 M reads with 2.98 Gb estimated bases. Q7 filtering using MinIONQC recovered 2.71 Gb bases with mean quality (Q) score of 9.6. Identification of microsatellites using MISA identification tool confirmed the presence of targeted SSR repeats in the amplicons (**Table 10**). Predominant dinucleotide and trinucleotide repeat with targeted repeat motifs CT, CA, AGA, and CT were observed on examining the total identified SSRs.

Table 9: Selected microsatellites using AMP-PCR

Locus Name	**Primer sequence (5′–3′)**	**Repeat Motif**	**No. of Alleles observed**
5ADPIR-03	KKVRVRV$(CT)_{10}$	$(CT)_{10}$	8
5ADPIR-05	KKVRVRV$(CA)_6$	$(CA)_6$	2
5ADPIR-12	KKVRVRV$(AGA)_6$	$(AGA)_6$	6
5ADPIR-18	KKVRVRV$(GT)_6$	$(GT)_6$	3

The AMP-PCR method is reported to detect polymorphism in wide variety of taxa (Kostia et al. 1995; Sharma et al. 1995; Wang et al. 1998; Zietkiewicz et al. 1994). The method successfully detected polymorphism in the case of the targeted species, *A. heterophyllum* in the present study.

Table 10: Confirmation of targeted repeat motifs CT, CA, AGA, and CT using MISA

ID	SSR	size	Start	end	FORWARD PRIMER1 (5'-3')	Tm(°C)	REVERSE PRIMER1 (5'-3')	Tm(°C)	PRODUCT size (bp)	Alignment1
42	(CT)62	124	8	131	GGGGCGGCTCTCTCTCTC	61	GTACCCCCATCCTTGGTTGG	60	206	Select seq NC_053920.1 Aconitum kusnezoffii
89	(CT)17	34	8	41	GGAAAGGCTCTCTCTCTCT	58	ACTGGGGTAGTGTCGAGTTT	58	174	Select seq NC_053368.1 Anemone maxima
368	(CT)7	14	8	21	TGAAAGGCTCTCTCTCTCT	57	TCCCAGCATGATTAGTATGGGC	60	152	Select seq XR_003364516.1: Papaver somniferum
439	(CT)6	12	8	19	GGGGCAGCTCTCTCTCT	60	GAGAGGGGACGAGGAGGATT	60	111	Select seq AC253884.1 Aquilegia coerulea
560	(CT)7	14	8	21	TGCGCGGCTCTCTCTC	62	GGAGAGGGCGTCTACAGAGA	60	110	Select seq NC_053920.1 Aconitum kusnezoffii
613	(CT)16	32	8	39	GGGAGGGCTCTCTCTCT	59	CCAACCCTTGGTCGTGATGA	60	168	Select seq NC_053920.1 Aconitum kusnezoffii
825	(CT)7	14	8	21	GTAGCACCTCTCTCTCTCT	57	GGTTTTGTTGGTGCCACCTC	60	152	Select seq XM_026590174.1: Papaver somniferum
899	(CT)51	102	8	109	GGAAGGCCTCTCTCTCTCT	60	ACCTAGCGGCTTTTCAACCA	60	253	Select seq MH837999.1 Papaver somniferum
6565	(CA)7	14	8	21	GGAAAGGCACACACACAC	60	CACACACCAAGTAAACGAGCA	59	208	Select seq MH837999.1 Papaver somniferum
4291	(CA)6	12	9	20	GTAACGGCACACACACAC	60	CCACCATTGCTCCTGTTCCT	60	241	Select seq AY631962.1 Aconitum napellus
948	(CA)6	12	8	19	GTGAAGGCACACACACAC	60	GCTTTAGTTGGTGGGGTGGT	60	197	Aquilegia coerulea
83	(CA)6	12	8	19	TGAAAGCCACACACACACA	58	ACCACCTCACCAAACAAGCA	60	262	Papaver somniferum
16	(CA)7	14	8	21	TGCACAGCACACACACAC	59	AGGCCTCGGAAAACACTCAA	60	133	Select seq KT384162.1 Tinospora cordifolia
48662	(AGA)7	21	8	28	GGAAAGGAGAAGAAGAAGAAGA	58	GGTAGCTGGTAGGTGAGGGA	60	223	Select seq MH837997.1 Papaver somniferum
1	(AGA)6	18	8	25	GGAAGGCAGAAGAAGAAGAAGA	58	AGGTCAGTTTCAGCACCTGC	61	242	Select seq XR_003336381.1 Papaver somniferum
72725	(AGA)5	15	8	22	GGAGAGCAGAAGAAGAAGAAGA	57	TCGATGATGCACACGCCATA	60	206	Select seq NC_053368.1 Anemone maxima
48	(AGA)11	33	8	40	GTGAGGCAGAAGAAGAAGAAGA	58	CGGCGATGAGTGCTATGTGA	60	161	Select seq AC253965.1 Aquilegia coerulea
2165	(AGA)6	18	8	25	TGGGAGCAGAAGAAGAAGAAG	57	TCGCGCACTGTGTATATGCT	60	243	Select seq NC_053368.1 Anemone maxima
595	(GT)11	22	8	29	GGACCTCGTGTGTGTGTG	58	TGCTTTCACAAACTTGCCGG	60	104	Aconitum kusnezoffii
8898	(GT)9	18	8	25	GGAGATAGTGTGTGTGTGTGT	58	TCGTTGGGGGTATTGTGAGC	60	117	Select seq MH837997.1 Papaver somniferum
8216	(GT)6	12	8	19	GGCTCGAGTGTGTGTGTG	58	AACGCGTTTTGCACTGTAGC	60	160	Select seq KU700181.1 Hypecoum procumbens
5	(GT)6	12	8	19	GGTCATCGTGTGTGTGTGT	58	GCGTTTTGCACTGTAGCGAA	60	156	Select seq MH837999.1 Papaver somniferum
713	(GT)9	18	8	25	TGTTCCCGTGTGTGTGTGT	60	CAATGGGCATACACGCATGG	60	223	Select seq KJ188172.1 Aconitum austrokoreense

Genetic diversity

Four AMP-PCR primers targeting di- and trinucleotide repeats along with ten microsatellite markers identified by accessing transferability, were used to study the genetic diversity and population structure in *A. heterophyllum*. Fingerprints were obtained by amplification of the collected 36 accessions of the targeted species using the 14 characterized set of primers. The accession codes used during analysis of 36 accessions of *A. heterophyllum* are listed in **table 11**. A Fingerprint profile of the 36 accessions using one set of primers (5ADPIR-12) is shown in **figure 7**, while the fingerprints obtained from each set of primers along with the band analysis using the size fragment standard are attached as **Annexure 4**.

Table 11: Accession codes for Genetic Diversity studies

Population code	**Location**	**Accession code**
AHD	Dayara-Uttarkashi, Uttarakhand	D 1 to 4
AHBC	Bedni-Chamoli, Uttarakhand	BC 1 to 5
AHGC	Ghes-Chamoli, Uttarakhand	GC 1 to 6
AHKM	Khaliya-Munsyari, Uttarakhand	KM 1 to 5
AHCT	Chandrashila, Rudraprayag, Uttarakhand	CT 1 to 4
AHS	Sunderdhunga, Bageshwar, Uttarakhand	S 1 to 5
AHLS	Tindi-Lahaul spiti, Himachal Pradesh	LS 1 to 4
AHPC	Pangi Valley (Killar), Chamba, Himachal Pradesh	PC 1 to 3

Using the data obtained from fingerprints from these microsatellite markers, different statistical parameters to study genetic diversity were calculated (**Table 12**). Highest genetic diversity was observed in AHBC (Bedni-Chamoli, Uttarakhand) population with Shannon's information index (I) of 0.290 and unbiased expected heterozygosity (uH_e) of 0.221. The lowest genetic diversity was observed in AHS population with I=0.207 and uH_e=0.159. Thus, at species level, I was 0.246 and uH_e was observed to be 0.194. The Number of alleles (Na) and Number of effective alleles (Ne) varied from 1.357 (AHS) to 1.5 (AHBC) and 1.264 (AHS) to 1.371 (AHCT), respectively. The average percentage of polymorphic loci (%P) was observed to be 40.18%. The results from AMOVA (**Table 13**) indicated higher variation among the populations (92%) and very low variation of 8% within the population with total estimated variance (EV) of 1.886. Further, observed genetic differentiation coefficient (F_{st}) and gene flow (N_m) at species level was 0.490 and 0.932, respectively.

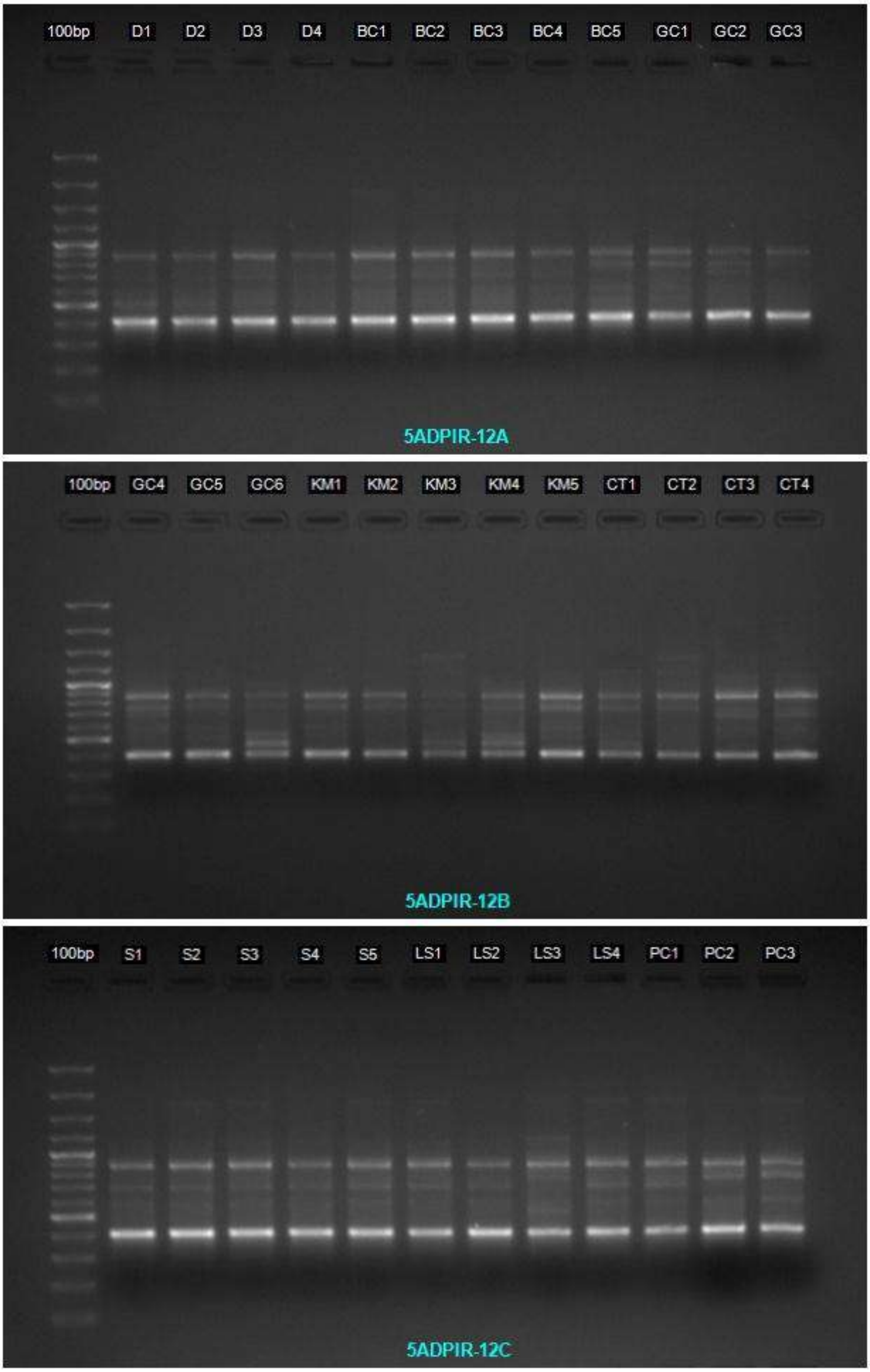

Figure 7: Fingerprints of 36 accessions of *A. heterophyllum* using microsatellite primer 5ADPIR-12

Table 12: Genetic diversity statistics in 8 populations of *A. heterophyllum* (N-Number, Na-Number of alleles, Ne-Number of effective alleles, I-Shannon's information index, H_o-Observed heterozygosity, H_e-Expected heterozygosity, uH_e-unbiased expected heterozygosity and %P-Percentage of polymorphic loci)

Populations	**N**	**Na**	**Ne**	**I**	**H_o**	**H_e**	**uH_e**	**%P**
AHD	4	1.357	1.306	0.225	0.304	0.158	0.181	35.71
AHBC	5	1.500	1.358	0.290	0.271	0.199	0.221	50.00
AHGC	6	1.429	1.282	0.241	0.238	0.163	0.177	42.86
AHKM	5	1.357	1.301	0.221	0.271	0.156	0.173	35.71
AHCT	4	1.429	1.371	0.278	0.286	0.196	0.224	42.86
AHS	5	1.357	1.264	0.207	0.257	0.143	0.159	35.71
AHLS	4	1.429	1.334	0.267	0.196	0.185	0.212	42.86
AHPC	3	1.357	1.329	0.239	0.214	0.171	0.205	35.71
Total/Average	36	1.402	1.318	0.246	0.255	0.171	0.194	40.18

Table 13: AMOVA in 36 accessions of *A. heterophyllum* (DF-Degrees of freedom, SS-Sum of squares, MS-Mean of squares, EV-Estimated variation)

Source	**DF**	**SS**	**MS**	**EV**	**Variation**
Among Pops	7	55.233	7.890	1.727	92%
Within Pops	28	4.433	0.158	0.158	8%
Total	**35**	**59.667**		**1.886**	**100%**

Structure of the Polulation and Genetic relationships

The assessment of the structure of population of 36 accessions of *A. heterophyllum* from eight distant geographical locations estimated optimum subpopulation of K=2 (**Figure 8**) using Evanno method in STRUCTURE HARVESTER Program.

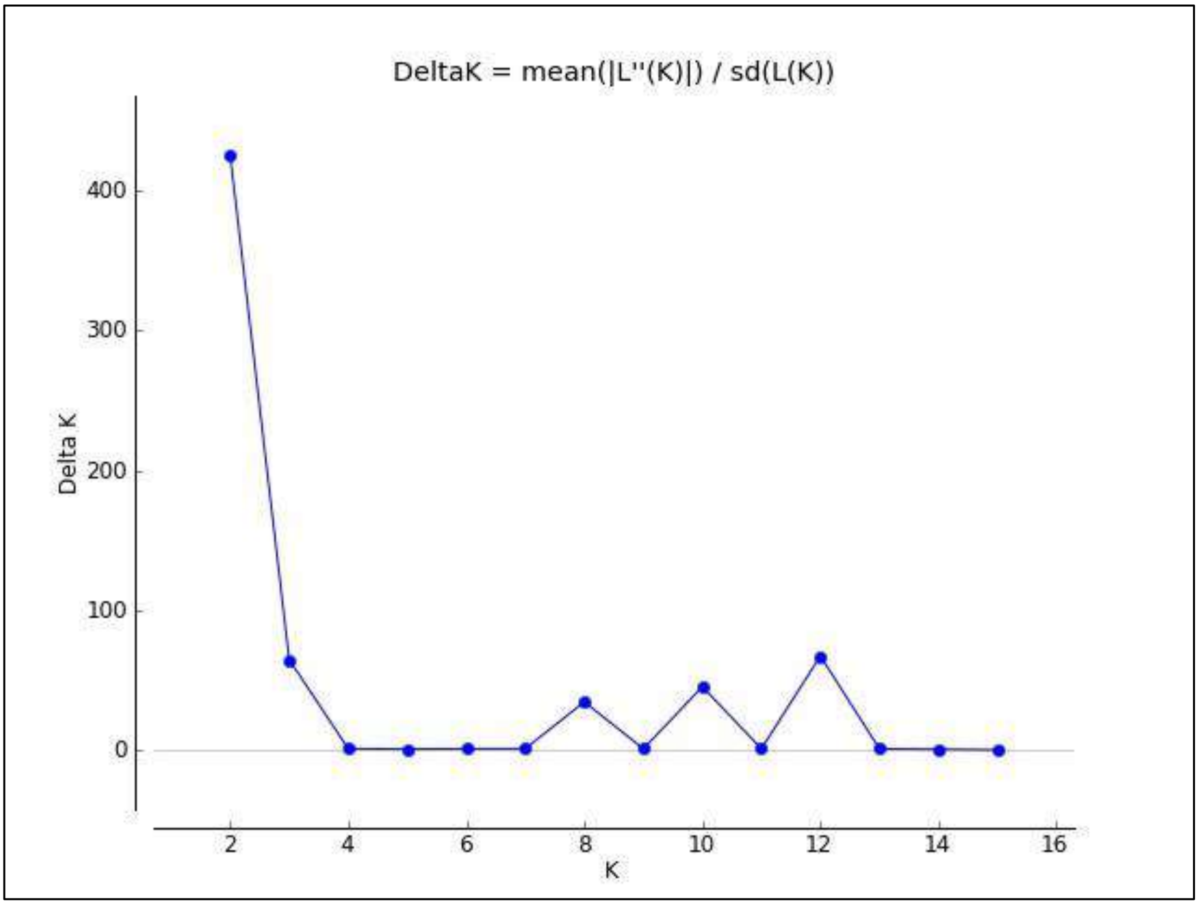

Figure 8: Estimated delta K values and the optimum population (K=2) using STRUCTURE HARVESTER

The accessions were grouped in two subpopulations and no admixtures were reported (**Figure 9**). Subpopulation one consisted of 24 accessions from various locations of Uttarakhand. While subpopulation one covered maximum accessions from Uttarakhand, five accessions belonging to Khaliya-Munsyari region of Uttarakhand (AHKM, accessions 16 to 20) were grouped along with seven accessions of Himachal Pradesh, representing subpopulation two. Reported allele frequency divergence among the subpopulations was 0.2039 with higher average distances (expected heterozygosity) between accessions of subpopulation two (0.2328) compared to expected heterozygosity of subpopulation one (0.0972). Fixation index (F_{st}) of each subpopulations suggested significant degree of differentiation within both subpopulations. However, fixation index was higher in subpopulation one (0.6628) indicating higher differentiation compared to fixation index in subpopulation two (0.3075).

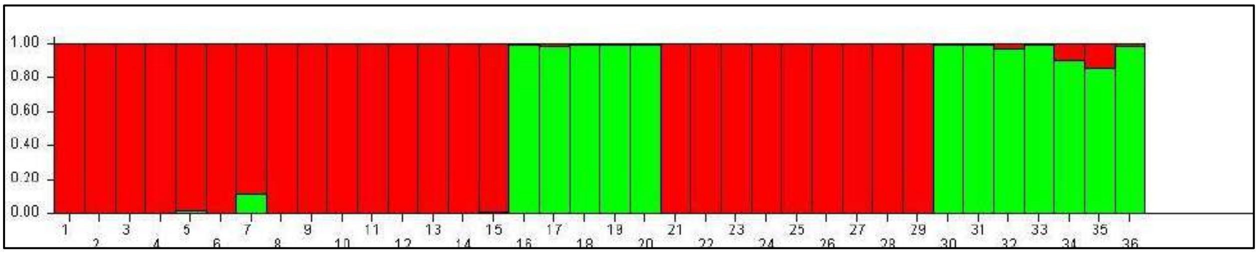

Figure 9: Population structure of 36 accessions of *A. heterophyllum*

Cluster analysis using UPGMA by estimating Dice coefficient of similarity is represented in **figure 10**. The dendrogram reported two major clusters with cluster-1 representing 24 accessions (all from Uttarakhand) with similarity coefficient ranging from 0.90 to 1.00. Cluster-2 included 12 accessions, out of which, seven were from Himachal Pradesh (all) and five were from Uttarakhand (AHKM, Khaliya-Munsyari region) with similarity coefficient ranging from 0.70 to 1.00. Cluster two was further grouped in two sub-clusters, with sub-cluster one representing accessions from Uttarakhand (AHKM, Khaliya-Munsyari region) and sub-cluster two representing all seven accessions from Himachal Pradesh. Overall, the clusters identified were consistent to the results of structure analysis. The clusters identified through Principal Coordinates Analysis (PCoA) (**Figure 11**) were identical to those identified by UPGMA cluster analysis and also from STRUCTURE analysis.

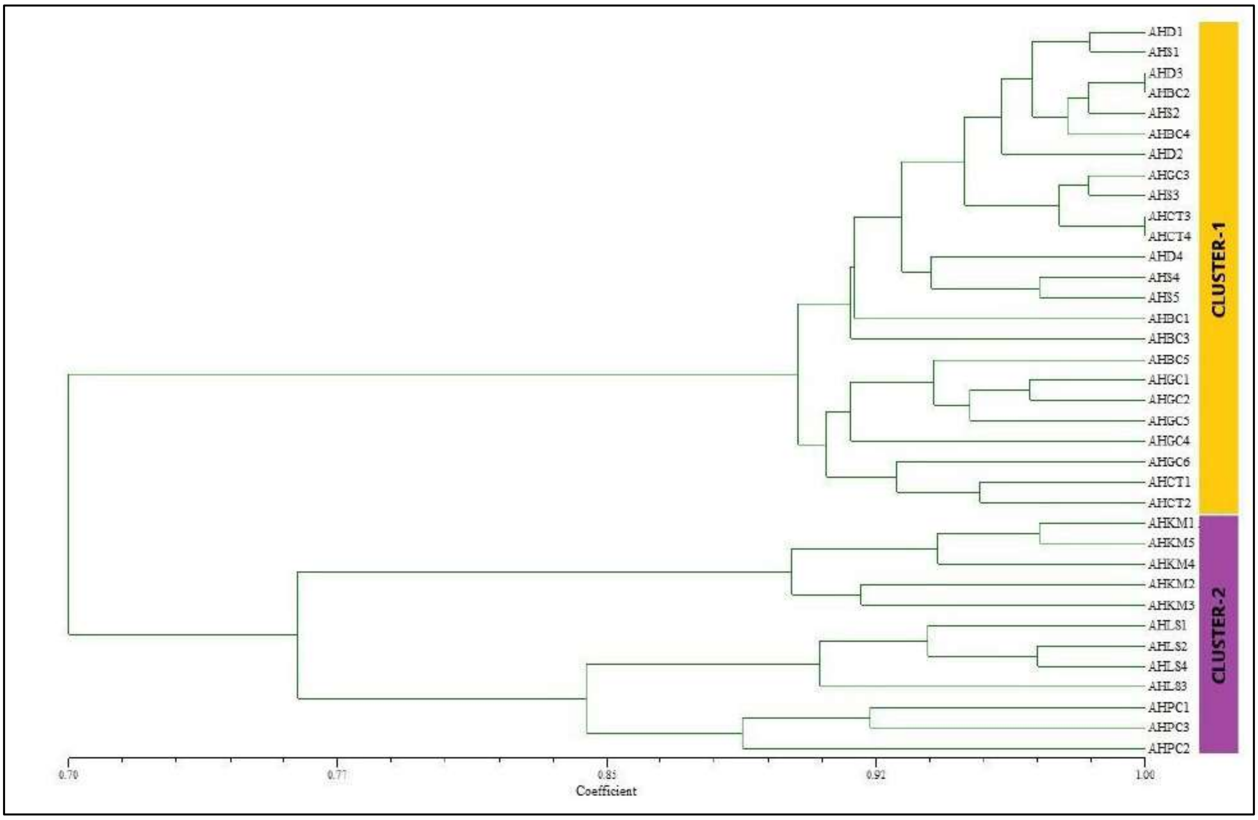

Figure 10: Dendrogram (UPGMA) showing the genetic relationship between the 36 accessions of *A. heterophyllum*

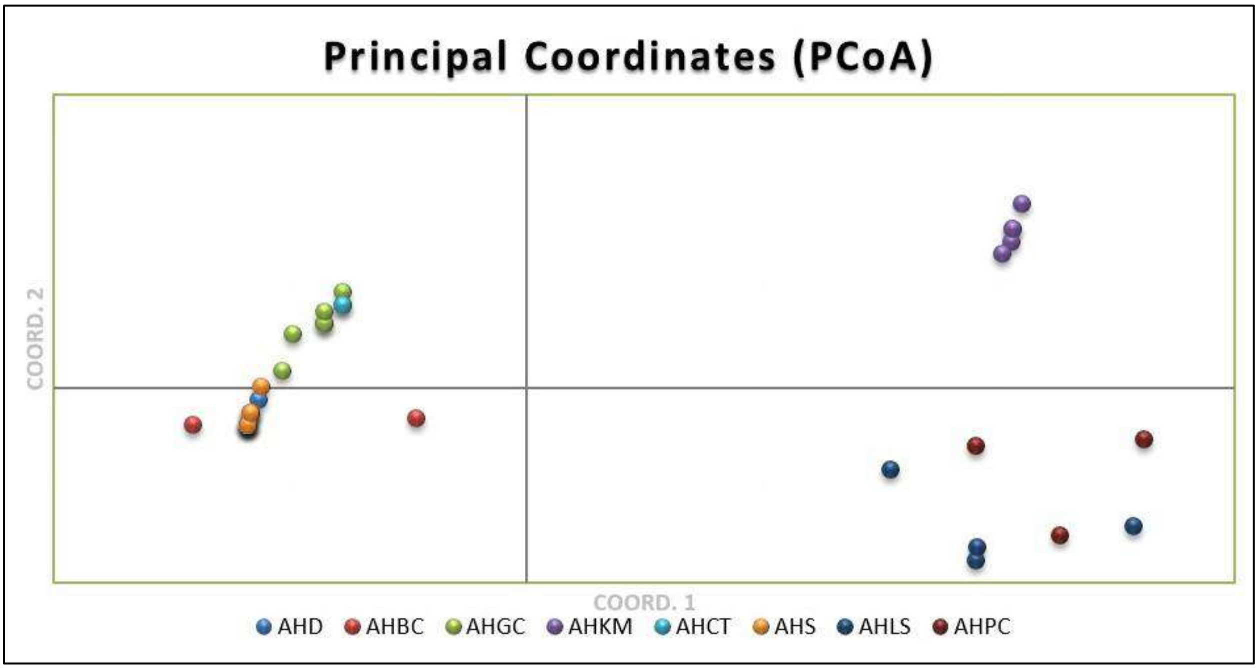

Figure 11: Principal Coordinates Analysis (PCoA) of 36 accessions of *A. heterophyllum* from 8 populations based on two-dimensional coordinates

Overall, the results indicated low genetic diversity, both at population (uH_e=0.159- 0.224) and species level (uH_e=0.194) as expected in an endangered species. The results were consistent to the reports in some critically endangered plant species in which low genetic diversity was reported e.g. *Monoon tirunelveliense* (uH_e=0.1428) (Viswanathan et al. 2015), *Commiphora wightii* (uH_e=0.294) (Harish et al 2014), *Salix taishanensis* (uH_e=0.2227) (Liu et al. 2020) and *Decalepis salicifolia* (uH_e=0.255) (Gokul et al. 2020). This indicates that the reduction in number of populations of *A. heterophyllum* has resulted in the decrease of genetic diversity. It was also observed that the variation within the populations is very low (8%) compared to among the population's variation of 92%, indicating inbreeding within population. Genetic differentiation with F_{st} value of 0.490 indicated highly differentiated populations as F_{st} value of more than 0.25 indicates high genetic differentiation (Wright, 1978). Further, higher differentiation was observed Uttarakhand subpopulation (F_{st}=0.6628) compared to Himachal Pradesh subpopulation (F_{st}=0.3075). High differentiation was also witnessed by grouping of populations in two clusters, specific to distant geographic locations (Uttarakhand and Himachal Pradesh), although with exception of one population of Uttarakhand (Khaliya-Munsyari) grouped along with other accessions of Himachal Pradesh. Also, the gene flow (N_m) was relatively lower (Wright 1978) with value of 0.932. Limited flow of gene between populations is responsible for genetic differentiation and same may in inferred in case of *A. heterophyllum* as the populations are separated by geographic and ecological factors (Large spatial distance between all 8 populations) that may strongly restrict dispersal, thus resulting in decline in populations. Populations of *A. heterophyllum* are declining rapidly (Nautiyal et al. 2002) and thus the species is at elevated extinction risks as it is suffering from inbreeding which may lead to inbreeding depression and further to erosion of the genetic diversity coupled with human activities.

Root microbiome studies

16S rRNA gene amplification from the genomic DNA of samples was conducted using a pair of primer that targets full length of 16S gene. Amplification was checked by Agarose gel electrophoresis and amplicons of approx. 1500 bp observed (**Figure 12**).

Figure 12: Amplification of 16S rRNA gene

Sequencing of the amplified fragments generated 5.72 M reads with 5.12 Gb estimated bases. Q7 filtering using MinIONQC recovered 4.18 Gb bases with mean quality (Q) score of 10.1 (**Figure 13**). The summary of sample codes, raw sequence data and quality is laid out in **table 14**.

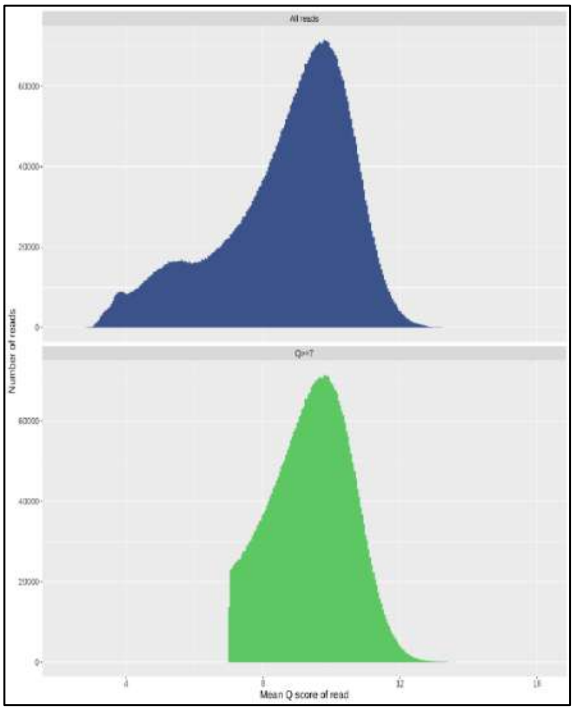

Figure 13: Q7 filtering by MinIONQC

Table 14: Accession codes for Root microbiome studied and Summary of Raw sequence data and quality

Area	**Collection site and demarcation**	**Analysis code**	**No. of reads**	**GC%**
Chamoli, Uttarakhand	Wild (Bedni)	1A-BC-W	52000	54
	Cultivated (Ghes)	1B-GC-C	36000	54
Dayara-Uttarkashi, Uttarakhand	Wild	2A-DU-W	132990	55
	Cultivated	2B-DU-C	128145	56
Khaliya-Munsyari, Uttarakhand	Wild (Khaliya)	3A-KM-W	123968	55
	Cultivated (Musyari)	3B-KM-C	114651	55
Chandrashila, Uttarakhand	Wild	4A-TCU-W	63166	56
	Cultivated	4B-TCU-C	154429	55
Tindi-Lahaul spiti	Wild (Pangi)	5A-LHP-W	56000	54
	Cultivated	5B-LHP-C	52000	53

Classification summary

Table 15 summarises the classification of reads of each sample. An average of 99.76% reads were classified out of which 100% were bacterial reads. While no viral, fungal, or protozoan reads were reported, 0.24% reads were unclassified.

Table 15: Summary of classification

Analysis Code	**Raw reads**	**Classified reads**	**Unclassified reads**	**Microbial reads**	**Bacterial reads**	**Viral reads**	**Fungal reads**	**Protozoan reads**
1A-BC-W	52,645	99.83%	0.17%	99.83%	99.83%	0%	0%	0%
1B-GC-C	36,474	99.83%	0.17%	99.83%	99.83%	0%	0%	0%
2A-DU-W	1,36,661	99.73%	0.27%	99.73%	99.73%	0%	0%	0%
2B-DU-C	1,31,628	99.73%	0.27%	99.73%	99.73%	0%	0%	0%
3A-KM-W	1,26,795	99.76%	0.24%	99.76%	99.76%	0%	0%	0%
3B-KM-C	1,17,848	99.71%	0.29%	99.71%	99.71%	0%	0%	0%
4A-TCU-W	64,716	99.66%	0.34%	99.66%	99.66%	0%	0%	0%
4B-TCU-C	1,58,887	99.72%	0.28%	99.72%	99.72%	0%	0%	0%
5A-LHP-W	56,652	99.84%	0.16%	99.84%	99.84%	0%	0%	0%
5B-LHP-C	52,759	99.81%	0.19%	99.81%	99.81%	0%	0%	0%

Detection of Operational Taxonomic Units (OTUs)

The OTUs were detected in the samples using Greengenes and Silva reference databases. The Greengenes detected the OTUs up to the species level, while Silva reported classification up to genus level.

Phylum abundance and distribution

Phylum abundance using Greengenes and Silva reference databases reported similar phylum in the samples. Major phylum reported by the reference databases are Proteobacteria, Cyanobacteria, Acidobacteria, Planctomycetes, Bacteroidetes, Actinobacteria, etc. The top 10 phylum abundance and distribution across different samples are shown in **figure 14a** (Results using Greengenes as reference database) and **figure 14b** (Results using Silva as reference database). Further, all the samples were observed to contain similar phylum.

Genus abundance and distribution

Genus abundance using Greengenes and Silva reference databases reported similar genus in the samples. Similar genus was observed in all wild and cultivated accessions. The top genus observed across the studies samples are shown in **figure 15a** (Results using Greengenes as reference database) and **figure 15b** (Results using Silva as reference database). Some of the major genus observed using the reference databases in the samples were Agrobacterium (Rhizobium in Silva), Bacillus, Bradyrhizobium, Flavisolibacter, Gemmata and Massilia (Unclassified under family Oxalobacteraceae in Greengenes).

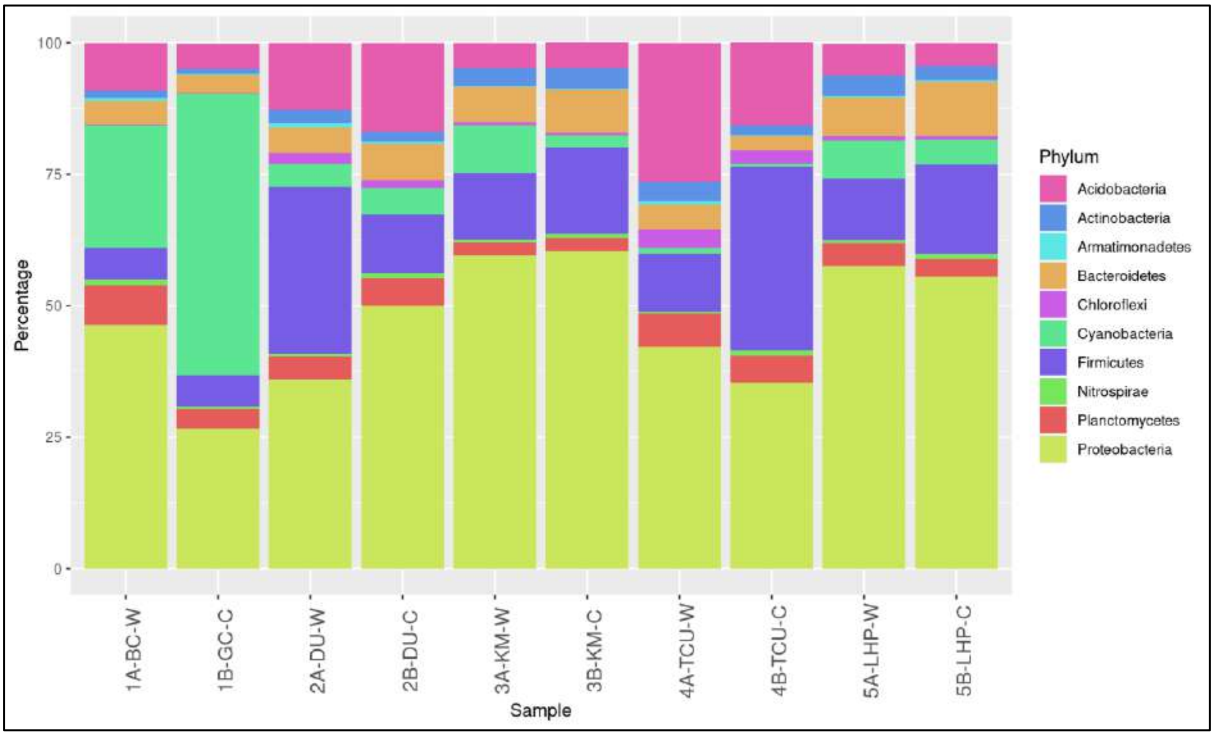

Figure 14a: Top 10 phylum abundance distribution (Greengenes)

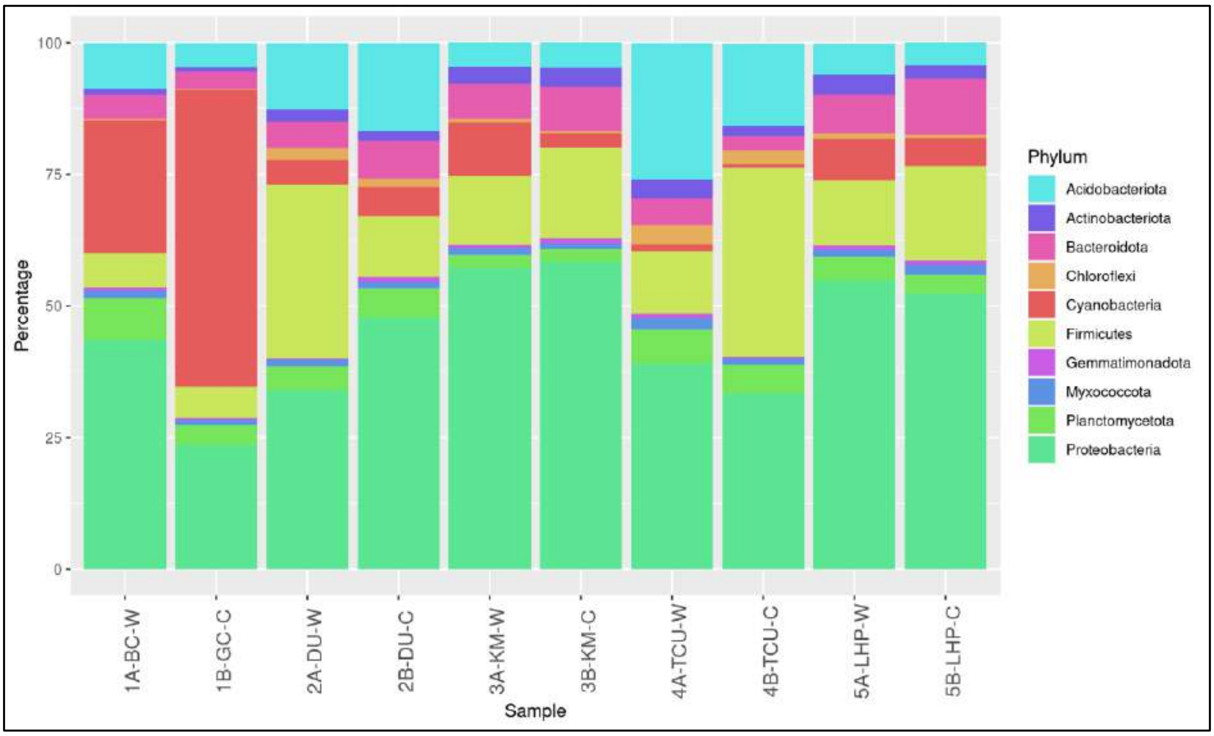

Figure 14b: Top 10 phylum abundance distribution (SILVA)

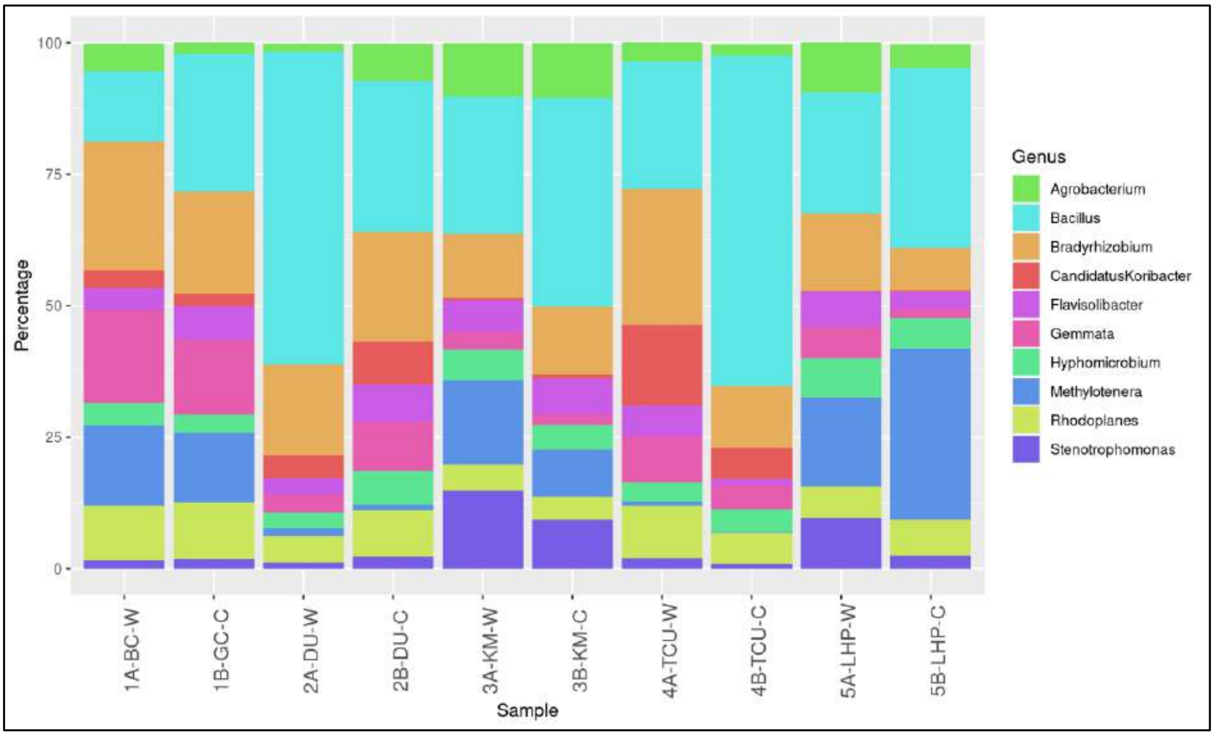

Figure 15a: Top 10 genus abundance distribution (Greengenes)

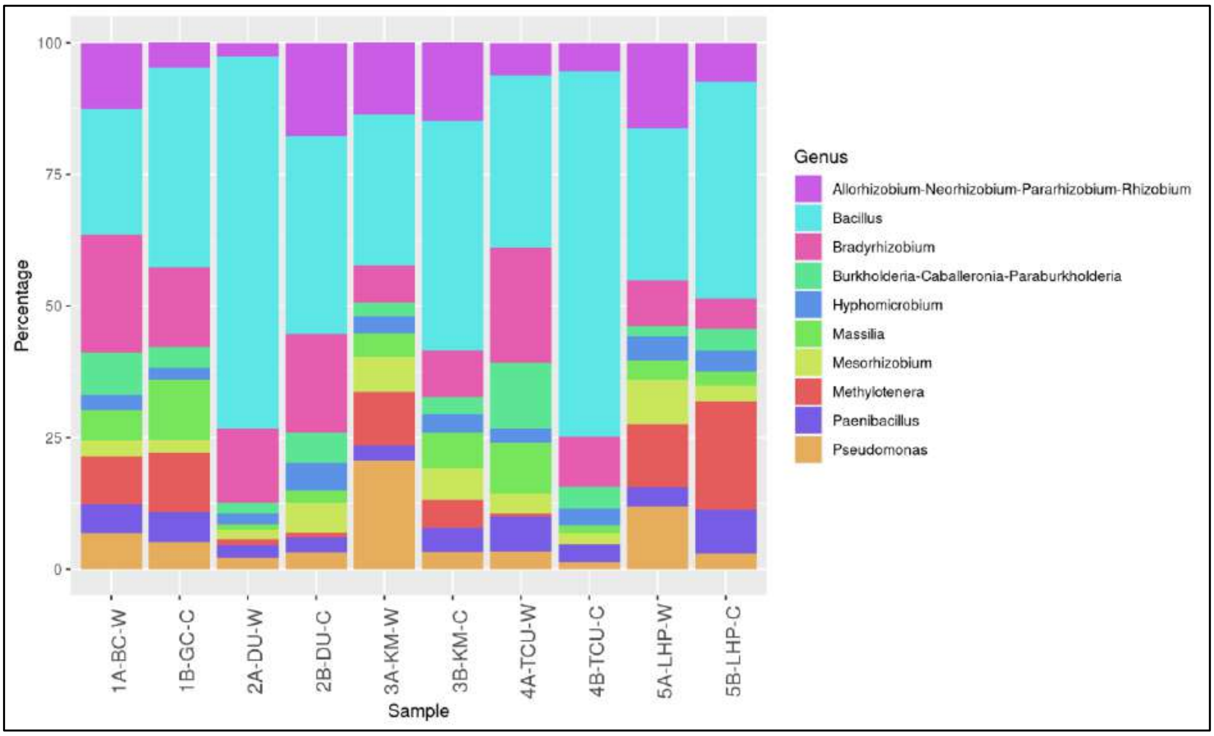

Figure 15b: Top 10 genus abundance distribution (SILVA)

Species abundance and distribution

Species abundance was reported by referring Greengenes database. Major species found in all 10 samples were *Bacillus flexus, Bacillus endophyticus, Bacillus longiquaesitum, Bacillus firmus, Bacillus muralis, Bradyrhizobium elkanii, Janthinobacterium lividum, Methylotenera mobilis, Rhizobium leguminosarum* and *Variovorax paradoxus*. The abundance of the species in different samples are shown in **figure 16**. Complete list of 1981 identified OUTs are attached as **Annexure 5**.

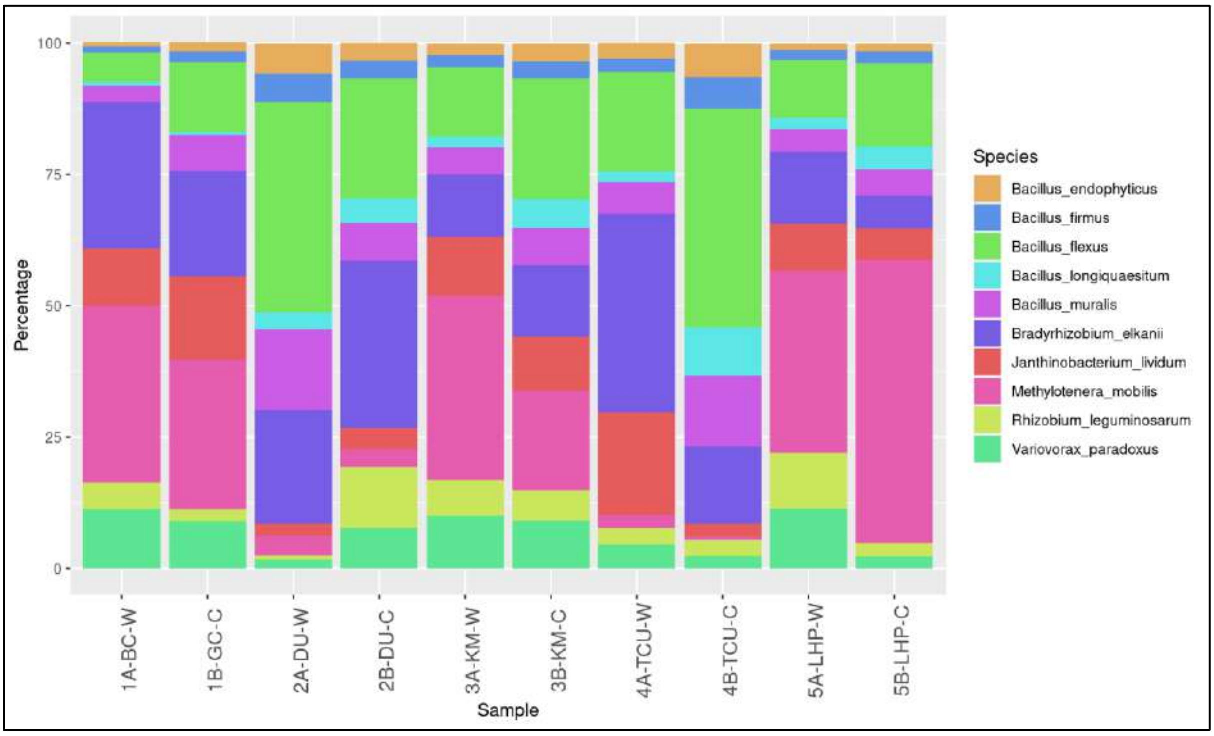

Figure 16: Top 10 species abundance distribution

Alpha diversity

Richness (number of taxonomic groups) and evenness (distribution of abundances of the groups) was depicted using different alpha diversity metrices. Indices Chao1 and ACE represented richness of the samples (**Figure 17a**). Compared to the wild sample from Chamoli-Uttarakhand (1A-BC-W), the cultivated sample (1A-GC-C) represented lower richness as low Chao1 and ACE coefficient was reported in this sample. However, the cultivated samples from Dayara-Uttarkashi (2B-DU-C), Khaliya-Munsyari (3B-KM-C) and Chandrashila-Uttarakhand (4B-TCU-C) reported higher species richness in terms of Chao1 and ACE as compared to their wild counterparts 2A-DU-W, 3A-KM-W and 4A-TCU-W respectively. But again, in the cultivated samples from Himachal Pradesh i.e., Tindi-Lahaul Spiti (5B-LHP-C), the richness was reported to be lower than the wild counterpart (5A-LHP-W).

The pattern of wild versus cultivated samples in terms of richness and relative abundance remained same to the richness when Shannon, Simpson, InvSimpson and Fisher indices (**Figure 17 a** and **b**) were studied, however with one exception. The cultivated sample from Chandrashila-Uttarakhand (4B-TCU-C) which reported higher richness than the wild counterpart (4A-TCU-W), was observed to represent lower diversity in terms of combined richness and relative abundance. Thus, cultivated samples- 1A-GC-C, 4B-TCU-C and 5B-LHP-C represented lower richness and relative abundance compared to their wild counterparts, while cultivated samples- 2B-DU-C and 3B-KM-C were observed to possess higher combined richness and relative abundance when compared to their wild counterparts.

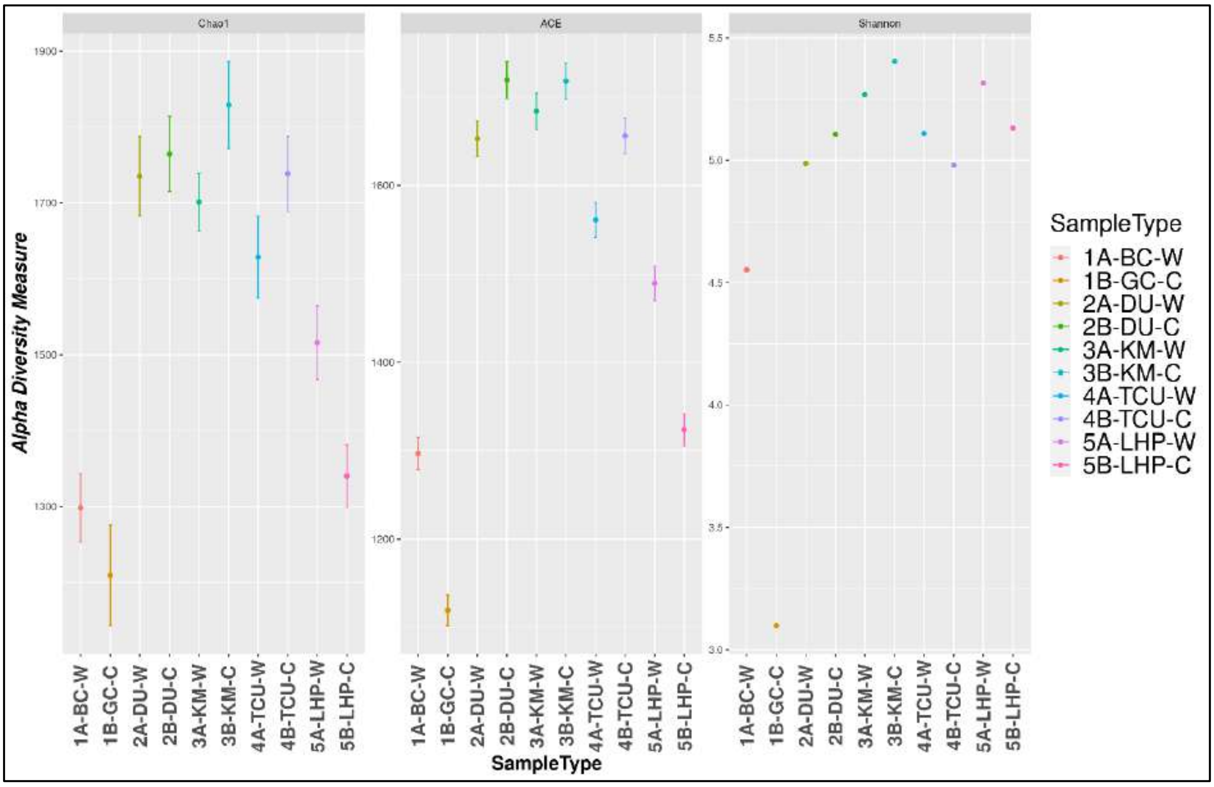

Figure 17a: Alpha diversity indices- Chao1, ACE and Shannon

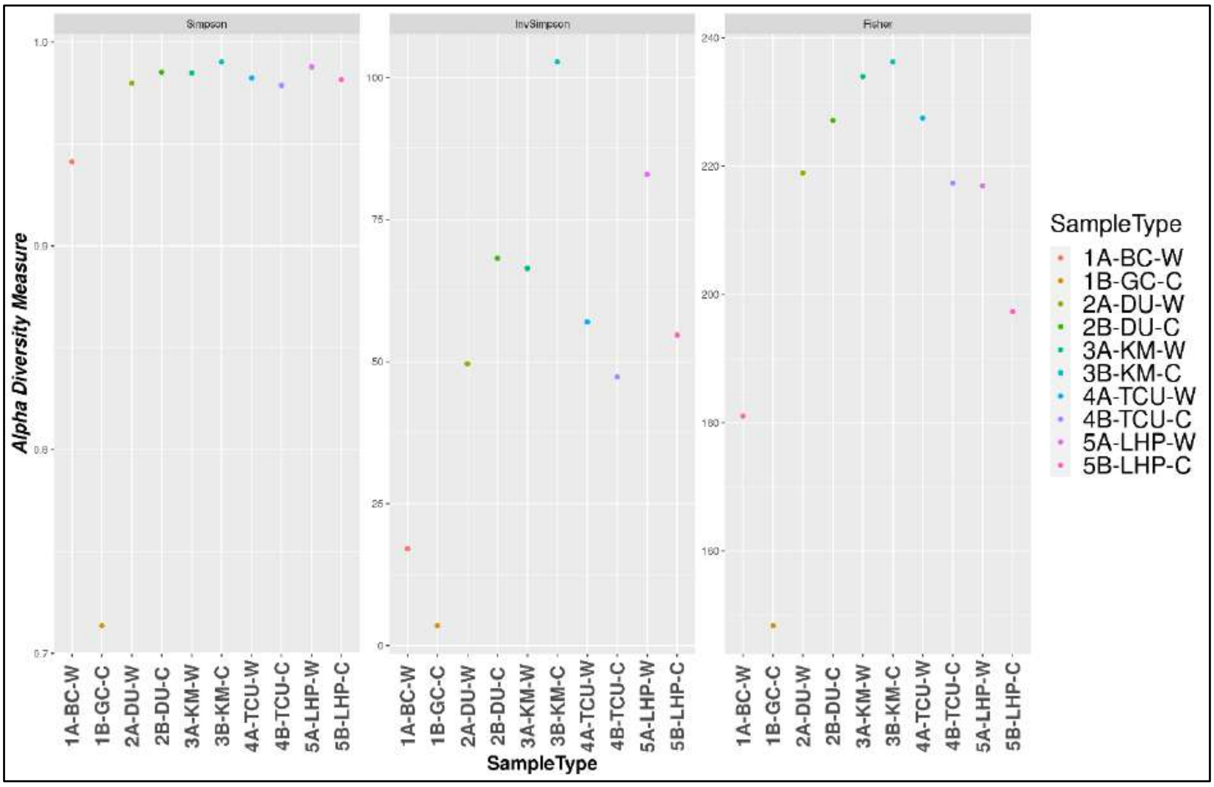

Figure 17b: Alpha diversity indices- Simpson, InvSimpson and Fisher

Beta diversity

The Diversity was derived using PCoA (Principal Coordinate Analysis) and is plotted in **figure 18**. Overall, the samples were grouped in three clusters:

Cluster-1: Wild and cultivated samples Chamoli-Uttarakhand (1A-BC-W and 1B-GC-W).

Cluster-2: Wild and cultivated accessions from Khaliya-Munsyari (3A-KM-W and 3B-KM-C) and Tindi-Lahaul Spiti (5A-LHP-W and 5B-LHP-C).

Cluster-3: Wild and cultivated accessions from Dayara-Uttarkashi (2A-DU-W and 2B-DU-C) and Chandrashila-Uttarakhand (4A-TCU-W and 4B-TCU-C).

As wild and cultivated counterparts of respective diverse populations were clustered together, low wild versus cultivated diversity is concluded.

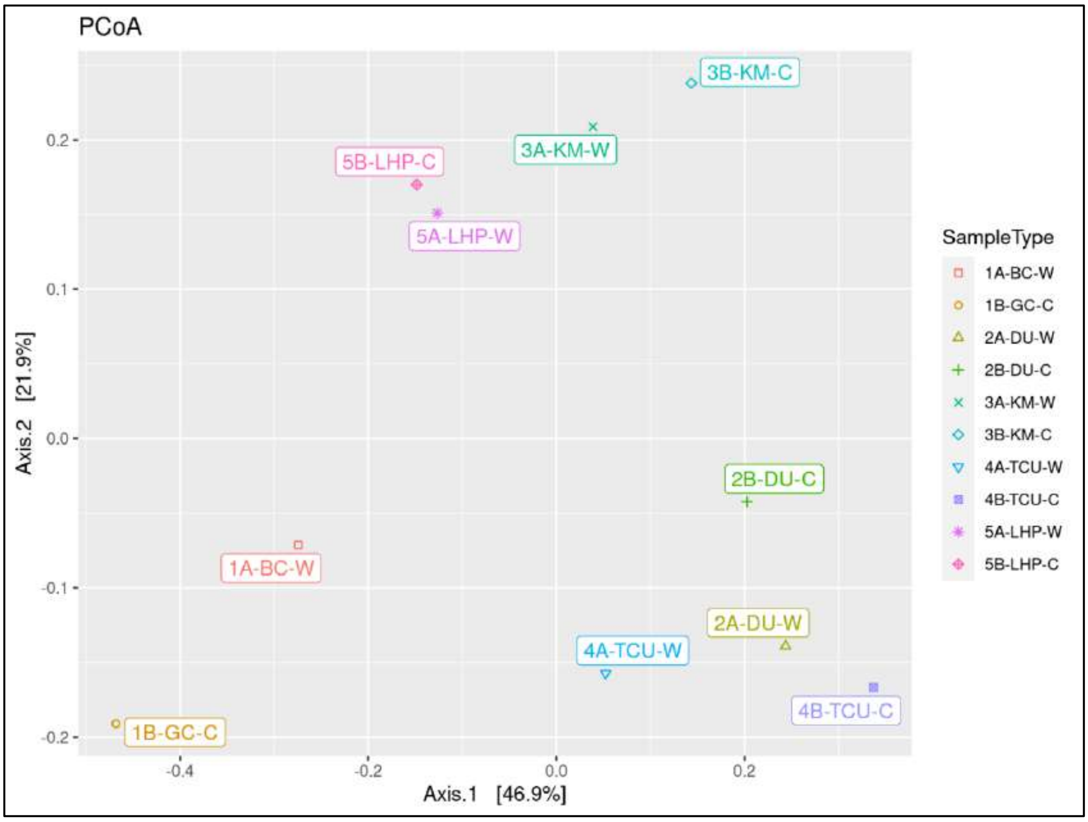

Figure 18: Diversity using PCoA (Principal Coordinate Analysis) plot.

Unique OTUs in wild versus the cultivated populations of Chamoli-Uttarakhand (1A-BC-W vs 1B-GC-C)

Fisher's exact test statistically reported significant difference among OTUs abundance between the wild and cultivated samples. 316 unique OTUs in total were reported from the wild sample (1A-BC-W) versus the cultivated sample (1B-GC-C) of Chamoli-Uttarakhand. Top 10 of these unique OTUs are shown in **table 16**. Some of the notable species found associated only with the wild samples are *Azorhizobium Doebereiner*, *Acidovorax facilis*, *Terriglobus***,** *Agrobacterium vitis* and *Comamonas terrigena*.

Unique OTUs in wild versus the cultivated populations of Dayara-Uttarkashi-Uttarakhand (2A-DU-W vs 2B-DU-C)

A total of 195 unique OTUs were reported by Fisher's exact test in the wild sample (2A-DU-W) versus the cultivated sample (2B-DU-C) of Dayara-Uttarkashi-Uttarakhand. Top 10 of these unique OTUs are shown in **table 17**. Some of the notable species found associated only with the wild samples are *Nitrosovibrio tenuis*, *Alkaliphilus*, *Geobacter lovleyi*, *Virgisporangium ochraceum* and *Actinoallomurus iriomotensis*.

Unique OTUs in wild versus the cultivated populations of Khaliya-Munsyari-Uttarakhand (3A-KM-W vs 3B-KM-C)

A total of 211 unique OTUs were reported by Fisher's exact test in the wild sample (3A-KM-W) versus the cultivated sample (3B-KM-C) of Khaliya-Munsyari-Uttarakhand. Top 10 of these unique OTUs are shown in **table 18**. Some of the notable species found associated only with the wild samples are *Planifilum, Alkaliphilus, Frigoribacterium, Pleomorphomonas oryzae* and *Dactylosporangium*.

Unique OTUs in wild versus the cultivated populations of Chandrashila-Uttarakhand (4A-TCU-W vs 4B-TCU-C)

A total of 163 unique OTUs were reported by Fisher's exact test in the wild sample (4A-TCU-W) versus the cultivated sample (4B-TCU-C) of Chandrashila-Uttarakhand. Top 10 of these unique OTUs are shown in **table 19**. Some of the notable species found associated only with the wild samples are *Microbacterium aurum*, *Streptococcus luteciae, Perlucidibaca, Dactylosporangium, Syntrophus, Halorhodospira* and *Saccharothrix*.

Unique OTUs in wild versus the cultivated populations of Tindi-Lahaul Spiti-Himachal (5A-LHP-W vs 5B-LHP-C)

A total of 270 unique OTUs were reported by Fisher's exact test in the wild sample (5A-LHP-W) versus the cultivated sample (5B-LHP-C) of Tindi-Lahaul Spiti-Himachal. Top 10 of these unique OTUs are shown in **table 20**. Some of the notable species found associated only with the wild samples are *Acinetobacter rhizosphaerae, Uliginosibacterium, Gluconacetobacter, Methylobacterium hispanicum, Pseudomonas citronellolis, Luteibacter* and *Sporosarcina aquimarina*.

Table 16: Representing Top 10 unique OTUs in wild samples from Chamoli-Uttarakhand (1A-BC-W) vs 1B-GC-C comparison along with relative frequency

OTUs	**Taxonomy**	**1A.BC.W relative frequency (%)**
Otu720	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Dialister;s__Dialister;	0.01779961
Otu170	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae;g__Azorhizobium;s__Azorhizobium_doebereinerae;	0.01779961
Otu250	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Rickettsiaceae;g__Unclassified;s__Unclassified;	0.01579965
Otu021	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Acidovorax;s__Acidovorax_facilis;	0.01579965
Otu519	k__Bacteria;p__Acidobacteria;c__Acidobacteriia;o__Acidobacteriales;f__Acidobacteriaceae;g__Terriglobus;s__Terriglobus;	0.0137997
Otu512	k__Bacteria;p__Acidobacteria;c__Solibacteres;o__Solibacterales;f__PK34;g__Unclassified;s__Unclassified;	0.0137997
Otu982	k__Bacteria;p__Chloroflexi;c__Chloroflexi;o__[Roseiflexales];f__[Kouleothrixaceae];g__Unclassified;s__Unclassified;	0.0137997
Otu732	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__Pelotomaculum;s__Pelotomaculum;	0.01179974
Otu151	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Agrobacterium;s__Agrobacterium_vitis;	0.01179974
Otu012	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Comamonas;s__Comamonas_terrigena;	0.01179974

Table 17: Representing Top 10 unique OTUs in wild samples from Dayara-Uttarkashi-Uttarakhand (2A-DU-W) vs 2B-DU-C comparison along with relative frequency

OTUs	Taxonomy	2A.DU.W relative frequency (%)
Otu117	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Nitrosomonadales;f__Nitrosomonadaceae;g__Nitrosovibrio;s__Nitrosovibrio_tenuis;	0.011399008
Otu1079	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Alkaliphilus;s__Alkaliphilus;	0.009099208
Otu875	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Pseudonocardiaceae;g__Unclassified;s__Unclassified;	0.008399269
Otu1044	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfuromonadales;f__Geobacteraceae;g__Geobacter;s__Geobacter_lovleyi;	0.0068994
Otu1433	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Thermomonosporaceae;g__Unclassified;s__Unclassified;	0.0045996
Otu1067	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Anaerobacillus;s__Anaerobacillus;	0.0045996
Otu725	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Anaerovibrio;s__Anaerovibrio;	0.003799669
Otu1223	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Syntrophobacterales;f__Syntrophaceae;g__Desulfobacca;s__Desulfobacca;	0.003799669
Otu1427	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__Virgisporangium;s__Virgisporangium_ochraceum;	0.002999739
Otu1434	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Thermomonosporaceae;g__Actinoallomurus;s__Actinoallomurus_iriomotensis	0.002999739

Table 18: Representing Top 10 unique OTUs in wild samples from Khaliya-Munsyari-Uttarakhand (3A-KM-W) vs 3B-KM-C comparison along with relative frequency

OTUs	**Taxonomy**	**3A.KM.W relative frequency (%)**
Otu985	k__Bacteria;p__Chloroflexi;c__Chloroflexi;o__Herpetosiphonales;f__Unclassified;g__Unclassified;s__Unclassified;	0.008201386
Otu1270	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Thermoactinomycetaceae;g__Planifilum;s__Planifilum;	0.007401251
Otu1314	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Tissierellaceae];g__GW-34;s__GW-34;	0.006601116
Otu1079	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae;g__Alkaliphilus;s__Alkaliphilus;	0.006601116
Otu1429	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__Dactylosporangium;s__Dactylosporangium;	0.00580098
Otu734	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptococcaceae;g__Desulfurispora;s__Desulfurispora;	0.00580098
Otu1421	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Frigoribacterium;s__Frigoribacterium;	0.004900828
Otu1329	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae;g__Garciella;s__Garciella;	0.004900828
Otu879	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Williamsiaceae;g__Williamsia;s__Williamsia;	0.004100693
Otu185	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylocystaceae;g__Pleomorphomonas;s__Pleomorphomonas_oryzae;	0.004100693

Table 19: Representing Top 10 unique OTUs in wild samples from Chandrashila-Uttarakhand (4A-TCU-W) vs 4B-TCU-C comparison along with relative frequency

OTUs	**Taxonomy**	**4A.TCU.W relative frequency (%)**
Otu1026	k__Bacteria;p__Cyanobacteria;c__Nostocophycideae;o__Nostocales;f__Nostocaceae;g__Nodularia;s__Nodularia;	0.01669985
Otu1407	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__Microbacterium;s__Microbacterium_aurum;	0.014999865
Otu1492	k__Bacteria;p__Chloroflexi;c__Thermomicrobia;o__Ellin6537;f__Unclassified;g__Unclassified;s__Unclassified;	0.014999865
Otu655	k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;s__Streptococcus_luteciae;	0.011699895
Otu1560	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Perlucidibaca;s__Perlucidibac;	0.008299925
Otu1429	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micromonosporaceae;g__Dactylosporangium;s__Dactylosporangium;	0.00669994
Otu1498	k__Bacteria;p__Nitrospirae;c__Nitrospira;o__Nitrospirales;f__[Thermodesulfovibrionaceae];g__Unclassified;s__Unclassified;	0.00669994
Otu1589	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Syntrophobacterales;f__Syntrophaceae;g__Syntrophus;s__Syntrophus;	0.00669994
Otu1201	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__Ectothiorhodospiraceae;g__Halorhodospira;s__Halorhodospira;	0.00669994
Otu1780	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Actinosynnemataceae;g__Saccharothrix;s__Saccharothri;	0.004999955

Table 20: Representing Top 10 unique OTUs in wild samples from Tindi-Lahaul Spiti-Himachal (5A-LHP-W) vs 5B-LHP-C comparison along with relative frequency

OTUs	Taxonomy	5A.LHP.W relative frequency (%)
Otu1474	k__Bacteria;p__Actinobacteria;c__Rubrobacteria;o__Rubrobacterales;f__Rubrobacteraceae;g__Rubrobacter;s__Rubrobacter;	0.05500231
Otu382	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter;s__Acinetobacter_rhizosphaerae;	0.049502079
Otu087	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;f__Rhodocyclaceae;g__Uliginosibacterium;s__Uliginosibacterium;	0.018300769
Otu408	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Gluconacetobacter;s__Gluconacetobacter;	0.018300769
Otu180	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae;g__Methylobacterium;s__Methylobacterium_hispanicum;	0.016500693
Otu1167	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__Pseudomonas_citronellolis;	0.016500693
Otu1165	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Luteibacter;s__Luteibacter;	0.016500693
Otu629	k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Planococcaceae;g__Sporosarcina;s__Sporosarcina_aquimarina;	0.011000462
Otu401	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Erwinia;s__Erwinia_soli;	0.011000462
Otu508	k__Bacteria;p__Acidobacteria;c__Solibacteres;o__Solibacterales;f__[Bryobacteraceae];g__Bryobacter;s__Bryobacter;	0.009200386

Overview of Unique OTUs having role in plant growth in wild versus the cultivated populations

Significant number of unique OTUs were reported in the wild samples when compared to the cultivated counterparts. Further, previous researches shows that many of these species were reported to have significant roles in plant growth and metabolism. For instance, *Azorhizobium Doebereiner* found in wild populations from Chamoli-Uttarakhand (1A-BC-W) was reported to be found in the root nodules of the fast-growing woody species Sesbania virgata (Maria et al. 2006). This was among thirty-four rhizobium strains isolated from the root nodules and was reported to have cultural characteristics like that of *Azorhizobium caulinodans*. In a study (Chen et al. 1993) if was found that the para-nodules of wheat plant when infected with *A. caulincdans* results in massive proliferation of bacterial cells and this results in transferring of fixed nitrogen to the plant, contributing up to 16–23% of the nitrogen budget of infected wheat. Another species found associated with the wild populations from Chamoli-Uttarakhand (1A-BC-W), reported to have significant role in plant growth is *Acidovorax facilis*. It is an aerobic bacterium used as a soil inoculant in agriculture to improve plant growth (Willems et al. 1990). Next, Nitrosovibrio tenuis observed in the wild populations from Dayara-Uttarkashi-Uttarakhand (2A-DU-W) is an ammonia-oxidizing bacterium and is distributed in oligotrophic soils, such as grasslands, forest soils and in mountainous environment (Harms et al. 1976). Another bacterium *Actinoallomurus iriomotensis* observed in the wild populations (2A-DU-W) belongs to the Actinomycetes family. Actinomycetes, as saprophytic organisms, play an important role in decomposing organic matter to nutrients those can be easily assimilated (Janso and Carter, 2010).

Pleomorphomonas oryzae reported from the wild populations of Khaliya-Munsyari-Uttarakhand (3A-KM-W) is a nitrogen-fixing bacterium earlier identified in roots of *Oryza sativa* (Xie and Yokota, 2010). It is a mesophilic, Gram-negative, rod-shaped bacterium. An Actinomycetes, *Dactylosporangium* was also reported from the wild population of Khaliya-Munsyari-Uttarakhand (3A-KM-W) and also in the wild populations of Chandrashila-Uttarakhand (4A-TCU-W).

Further, bacterium *Acinetobacter rhizosphaerae* was observed in the wild populations of Tindi-Lahaul Spiti-Himachal (5A-LHP-W). *A. Rhizosphaerae* stands out as a beneficial microorganism within the Acinetobacter genus, recognized for its properties that support and enhance plant growth (Gulati et al. 2009). This plant growth-promoting bacteria has ability to enhance the salt stress resistance of rice seedlings (Sun et al. 2023) and is also known to degrade organophosphorus pesticides (Chanika et al. 2011).

CHAPTER 5
Conclusion

Aconitum heterophyllum is a medicinally important species that is critically endangered because of over-exploitation of its roots for indigenous medicine. The present unsustainable harvesting has resulted in a drastic reduction of its wild populations. Conservation of *A. heterophyllum* requires understanding and maintenance of its genetic diversity.

Genetic diversity is the variation and variability of genes in a population, species, or an ecosystem. Genetic diversity is a very important component of biodiversity that determines the adaptability and robustness of creatures to environmental change. Investigating the genetic diversity carries vital importance for numerous reasons. To begin with, genetic diversity is the basis of a species adaptability to environmental change, disease resistance, and resistance to various stress factors. When it comes to endangered plant species, a thorough understanding of their genetic composition is important to develop an effective conservation strategy. By studying genetic diversity, scientists can identify genetical characteristics those are crucial to the survival of the species. Moreover, a clear understanding of genetic diversity is crucial in breeding program development, with the purpose of improving the species resistance and guaranteeing its long-term sustainability. Assessment of genetic diversity offers insights into the evolutionary history of endangered plants, guiding conservationists to make informed decisions for habitat restoration, population management, and reintroduction initiatives.

Genetic diversity in *A. heterophyllum* was studied using characterized microsatellite markers. Microsatellite markers, also known as simple sequence repeats (SSRs), are short, repetitive DNA sequences found in the genomes of organisms. These markers are valuable in genetic studies for assessing the genetic diversity, structure of the population, and relatedness among individuals. Microsatellites in the targeted species was characterized by exploring cross species transferability and by using Anchored microsatellite-primed PCR (AMP-PCR). The study reports successful cross-species transferability of microsatellite markers from different species of the genus *Aconitum* to *A. heterophyllum* species. Further, polymorphism in *A. heterophyllum* was successfully detected using AMP-PCR and markers were characterized.

Present study found only a few isolated populations of *A. heterophyllum*, and most of them consisted of only a small number of individuals. The study also reported that the studied populations have lower genetic diversity, considerable genetic differentiation, and limited flow of gene among them.

Strategies are needed urgently for Conservation to protect and restore *A. heterophyllum*. Introducing genetic diversity through seed dispersal and artificial pollination, collecting seeds for germplasm preservation, and establishing conservation plots are recommended strategies. Additionally, propagating the species for ex-situ conservation and reintroducing individuals into natural habitats are crucial steps for its survival.

Conservation plots or designated areas should be established to safeguard populations with notable genetic variation, ensuring the protection of habitats of *A. heterophyllum*. This holistic approach will contribute to the long-term survival and sustainability of this critically endangered medicinal plant.

Further, the rhizospheric root microbiome of the targets species was explored using metagenomic approach. The rhizosphere is an active zone in plant's and microorganism's interaction, which has direct impact on the plant health, fitness, development and for nutrient cycling. Unique microbes were identified by comparison of wild and cultivated root microbiome of *A. heterophyllum*. Several rhizospheric bacteria with reported beneficial effects on the plant, the Plant Growth Promoting Rhizobacteria (PGPR), linked with the wild populations are reported in the present study. The identified microbes can be utilized for promoting sustainable and efficient cultivation of the species, fostering a balanced and mutually beneficial relationship between plants and beneficial microorganisms in the soil. These PGPR can also stimulate the development of a well-branched and robust root system. Improved root architecture enhances nutrient and water absorption, leading to better overall plant performance and better yield of Ativisa- the medicinal important part of the targeted species.

A holistic approach, integrating genetic diversity conservation and microbial interactions, will contribute to the long-term survival and sustainability of *A. heterophyllum*, ensuring that this valuable medicinal plant can continue to thrive and benefit future generations.

Chapter 6
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