

**MOLECULAR TYPING OF *FRANKIA* IN THE FIELD
COLLECTED ACTINORHIZAL ROOT NODULES FROM
DIFFERENT ALTITUDES IN SIKKIM**

By

ANINDITA KHAN



Dedicated to my parents

**THESIS SUBMITTED
IN FULFILMENT OF THE DEGREE OF
DOCTOR OF PHILOSOPHY IN BOTANY**

**NORTH-EASTERN HILL UNIVERSITY
SHILLONG -793022, INDIA**

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The North-Eastern Hill University

August, 2006

DECLARATION

I, Anindita Khan, hereby declare that the subject matter of this thesis entitled "Molecular typing of *Fusicladium* sp. the field collected Actinorrhizal root nodules from different altitudes in Sikkim" is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis is not submitted before for any research degree in any other University/Institute.

This is being submitted to the North-Eastern Hill University for the award of the degree of Doctor of Philosophy in Botany.

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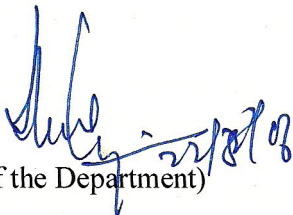
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2.3. Actinorhizal symbiosis	10
2.4. Application of molecular techniques on actinorhizal symbiosis	11
2.4.1. Molecular Phylogenetic studies in <i>Frankia</i>	12
2.4.2. PCR based studies on conserved regions	13
2.4.3. Studies on <i>nif</i> genes of <i>Frankia</i>	14
2.4.4. 16S-23S ITS region of <i>Frankia</i>	14
2.4.5. Other molecular approaches on the genetic diversity of <i>Frankia</i> and actinorhizal hosts	15
2.5. Molecular ecology of <i>Frankia</i>	16
3. MATERIALS AND METHODS	19
3.1. Study area	19
3.2. Collection sites	19
3.2.1. Collection of <i>Alnus nepalensis</i> nodules	21
3.2.1.1. Sites in east Sikkim district	21
3.2.1.2. Sites in north Sikkim	24
3.2.2. Collection of <i>Hippophae</i> nodules	26
3.3. Collection of material	26
3.3.1. Collection of root nodules	26
3.3.2. Collection of rhizospheric soil	27
3.4. Methods to study molecular diversity of <i>Frankia</i>	28

CONTENTS

1.	INTRODUCTION	1
2.	REVIEW OF LITERATURE	8
2.1	The Actinomycete: <i>Frankia</i>	8
2.2	Actinorhizal plants: The hosts	10
2.3	Actinorhizal symbiosis	10
2.4	Application of molecular techniques on actinorhizal symbiosis	11
2.4.1	Molecular Phylogenetic studies in <i>Frankia</i>	12
2.4.2	PCR based studies on conserved regions	13
2.4.3	Studies on <i>nif</i> genes of <i>Frankia</i>	14
2.4.4	16S-23S ITS region of <i>Frankia</i>	14
2.4.5	Other molecular approaches on the genetic diversity of <i>Frankia</i> and actinorhizal hosts	15
2.5	Molecular ecology of <i>Frankia</i>	16
3.	MATERIALS AND METHODS	19
3.1	Study area	19
3.2	Collection sites	19
3.2.1	Collection of <i>Alnus nepalensis</i> nodules	21
3.2.1.1	Sites in east Sikkim district	21
3.2.1.2	Sites in north Sikkim	24
3.2.2	Collection of <i>Hippophae</i> nodules	26
3.3	Collection of material	26
3.3.1	Collection of root nodules	26
3.3.2	Collection of rhizosphere soil	27
3.4	Methods to study molecular diversity of <i>Frankia</i>	28

3.4.1	Isolation of genomic DNA from nodules	28
3.4.2	Agarose gel electrophoresis	29
3.4.3	Quantification of DNA and estimation of band size	29
3.4.3.1	Quantification by direct observation	30
3.4.3.2	Quantification of DNA and estimation of band size by using Multi Analyst software	30
3.4.4	Amplification of genomic DNA by Polymerase Chain Reaction (PCR)	30
3.4.4.1	Primer stock solutions	32
3.4.4.2	dNTP solutions	32
3.4.4.3	<i>Taq</i> DNA polymerase and Assay Buffer	33
3.4.5	Agarose gel electrophoresis of the amplified products	33
3.4.6	Amplicon Length Polymorphism (ALP)	33
3.4.7	Phenol purification of DNA	33
3.4.8	Amplicon Restriction Pattern (ARP) or PCR/RFLP (Restriction fragment length profile) analysis of PCR products	35
3.4.9	Nucleotide Sequencing	35
3.5	Acetylene Reduction Assay (ARA)	36
3.6	Methods used for soil analysis	37
3.7	Computer analysis of data	39
4.	RESULTS AND DISCUSSION	41
4.1	Collection of nodules	41
4.2	DNA extraction	42
4.3	PCR Amplification of 16S-23S rRNA ITS region	42

4.4	Amplified Fragment Length Profile (AFLP)	
	or Amplicon Length Profile (ALP)	50
4.5	Restriction Digestion of Amplified Products	50
4.5.1	Patterns of high altitude, Site 1	53
4.5.2	Patterns of high altitude, Site 2	58
4.5.3	Patterns of high altitude, Site 3	58
4.5.4	Patterns of middle altitude, Site 1	58
4.5.5	Patterns of middle altitude, Site 2	58
4.5.6	Patterns of middle altitude, Site 3	58
4.5.7	Patterns of low altitude, Site 1	59
4.5.8	Patterns of low altitude, Site 2	59
4.5.9	Patterns of Low altitude, Site 3	59
4.5.10	Patterns of microsymbionts associated with <i>Hippophae</i> sp	59
4.6	Analysis of the banding patterns on the basis of ARP / PCR-RFLP	60
4.7	Cluster Analysis	61
4.8	Nucleotide sequence analysis	64
4.8.1	High altitude, Site 1	66
4.8.2	High altitude, Site 2	73
4.8.3	High altitude, Site 3	78
4.8.4	Middle altitude, Site 1	82
4.8.5	Middle altitude, Site 2	86
4.8.6	Middle altitude, Site 3	91

4.8.7	Low altitude, Site 1 and Site 2	91
4.8.8	Low altitude, Site 3	92
4.8.9	Samples of <i>Hippophae</i> sp.	92
4.9	Phylogenetic Analysis	96
4.10	Acetylene Reduction Assay (ARA)	99
4.11	Soil Analysis	99
4.12	Analysis of Variance: ANOVA	106
4.13	Student-Newman-Keuls test (SNK)	109
4.14	Principal Components Analysis (PCA)	109
4.15	Multiple Regression Analysis (MRA)	117
5.	CONCLUSION	118
6.	REFERENCES	119
7.	APPENDIX 1	132
8.	APPENDIX 2	133
9.	APPENDIX 3	134

LIST OF FIGURES

Fig No.	Page
1. Geographic locations of the collection sites	20
2. <i>Alnus nepalensis</i> (Near Hanumantok, Gangtok, East Sikkim)	22
3. Roots of <i>Alnus nepalensis</i> showing nodule clusters	22
4. Alder stands on the banks of river Teesta, North Sikkim	23
5. Alder stands on the mountain slopes, Gangtok, East Sikkim	23
6. <i>Hippophae</i> sp.	25
7. <i>Hippophae</i> (with berries), near Lachen, North Sikkim	25
8. Isolation of genomic DNA of <i>Frankia</i> (a to e)	43
9-a. Diagrammatic map showing organization of <i>rrn</i> operon in prokaryotes	44
9-b. Primer pair used for amplification	44
10. Diagrammatic representation of the amplification reaction	45
11. PCR amplification of high altitude samples (a to c)	46
12. PCR amplification of middle altitude samples (a to c)	47
13. PCR amplification of low altitude samples (a to c)	48
14. PCR amplification of <i>Hippophae</i> samples	49
15. Gel photograph of amplicon length profile	49
16. Restriction fragment length polymorphism of high altitude samples	54
17. Restriction fragment length polymorphism of middle altitude samples	55
18. Restriction fragment length polymorphism of low altitude samples	56
19. Restriction fragment length polymorphism of <i>Hippophae</i> samples	57
20. Cluster dendrogram	63
21. Diagrammatic representation of high altitude, site 1 sequences	67
22. Diagrammatic representation of high altitude, site 2 sequences	74

23. Diagrammatic representation of high altitude, site 3 sequences	79
24. Diagrammatic representation of middle altitude, site1 sequences	83
25. Diagrammatic representation of middle altitude, site2 sequences	87
26. Phylogenetic tree	98
27. (a to c) Graphical representation of soil analyses – pH, organic carbon, and total nitrogen	103
(d to f) Graphical representation of soil analyses – available phosphorus, available potassium and exchangeable calcium	104
(g and h) Graphical representation of soil analyses – exchangeable magnesium and electrical conductivity	105
28. (a to c) Graphical representation of PCA	114
(d and e) Graphical representation of PCA	115

LIST OF TABLES

Table No.	Page
1. Sample numbers representing different restriction patterns	51
2. Banding patterns with <i>RsaI</i>	52
3. Sequenced samples	65
4. Comparison between RFLP and webcutter generated banding patterns of High altitude, site 1	67
5. Comparison between RFLP and webcutter generated banding patterns of High altitude, site 2	74
6. Comparison between RFLP and webcutter generated banding patterns of High altitude, site 3	79
7. Comparison between RFLP and webcutter generated banding patterns of Middle altitude, site 1	83
8. Comparison between RFLP and webcutter generated banding patterns of Middle altitude, site 2	87
9. Acetylene Reduction Assay of nodule samples from different sites	100
10. Mean and standard deviation values of different soil parameters	102
11. Analysis of Variance (ANOVA)	107
12. SNK (Student Newman-Keuls Test) grouping	110
13. Principal components for all parameters with eigenvectors	112
14. Eigenvalues, total variance and cumulative percentage	112
15. Multiple Regression Analysis	116

CHAPTER 1

INTRODUCTION

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen which is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. Though there is an abundant supply of nitrogen in the atmosphere, molecular nitrogen is metabolically unavailable directly to higher plants and animals. The presence of a triple bond between the two nitrogen atoms of a molecule makes it almost inert. Nitrogen must be converted into ammonium (NH_4^+) or nitrate (NO_3^-) ions before it could be used by plants and animals. Conversion of molecular nitrogen to ammonium or nitrate forms is also called as nitrogen fixation. Only few microorganisms can 'fix' atmospheric nitrogen, making all other living organisms dependent on them for their requirements of 'fixed' nitrogen.

Micro-organisms that fix nitrogen are called diazotrophs. Biological nitrogen fixation is brought about by two types of micro-organisms – non-symbiotic or free living and symbiotic. The free living non-photosynthetic diazotrophs require a chemical energy source for bringing about nitrogen fixation, whereas the free living photosynthetic diazotrophs utilize light energy for the purpose. Free living micro-organisms like *Scytonema*, *Rivularia* or *Klebsiella* fix nitrogen utilizing fixed carbon as energy source. They therefore contribute very little fixed nitrogen to agricultural crops.

On the other hand diazotrophs that live in close proximity to plant roots and obtain fixed carbon from the plants are symbiotic or associative nitrogen-fixing microorganisms. Microorganisms like *Rhizobium* and *Frankia* form symbiotic association with host plant(s) and utilize fixed carbon supply of the host for fixing atmospheric di-nitrogen. These microorganisms make a substantial contribution of fixed

nitrogen to agriculture and forestry. It has been estimated that *Frankia* contributes about 2-362 kg N/ha/yr while the estimated contribution of Rhizobium-Legume symbiosis is about 24-584 kg N/ha/yr. (Shantharam and Mattoo, 1997).

Since the *Frankia* symbiosis results from an actinomycetic invasion of plant roots, it has been termed as “actinorhizal symbiosis” (Benson, 1988). Accordingly, the plants nodulated by *Frankia* are called “actinorhizal plants”. Although these plants are taxonomically diverse, they have some common features. For example, all of them are dicotyledonous and perennial angiosperms (Baker and Schwintzer, 1990). Actinorhizal plants belong to four subclasses, eight families, twenty-five genera and more than 220 species (Wall, 2000). Well known genera are *Alnus* (Betulaceae), *Myrica* (Myricaceae), *Casuarina* (Casuarinaceae), *Elaeagnus*, *Hippophae* (Elaeagnaceae), etc. *Frankia* have attracted attention recently because they form root nodules on a broad range of non-leguminous plants and because such nodules fix N₂ as effectively as rhizobial nodules.

Among actinorhizal plants, the two genera *Alnus* and *Casuarina* exhibit highest nitrogen fixing potential (Dommergues, 1996) and *Alnus* is the most extensively studied among them. *Alnus* species are important among actinorhizal plants as pioneers in ecological succession of skeletal soils (Lawrence, 1951; Crocker and Major, 1955; Damière *et al.*, 1986). They are widely used for intense forest management (Tarrant and Trappe, 1971; Borman and Gordon, 1984), biomass production (Zavitkovski *et al.*, 1979) and regeneration of disturbed lands (Heilman, 1982). It is thought to have originated in Indo-China region (Furlow, 1979). About 47 species of *Alnus* are known (Swensen and Mullin, 1997). In India, only two species are found (*A. nepalensis* and *A. nitida*) and they are naturally distributed throughout the temperate Himalaya. *A. nepalensis* is confined to the higher elevations of Meghalaya and Arunachal Pradesh, and *A. nitida* is found in

Himachal Pradesh. Trees of *A. nepalensis* are also found in some locations of Nagaland and Tamil Nadu (Varghese, 2000).

The genus *Hippophae*, commonly known as sea buckthorn is another actinorrhizal plant which forms symbiotic association with *Frankia*. A fascinating plant species, it is a very attractive ornamental shrub. It has silvery deciduous leaves and colourful orange berries that persist through most of the winter. It is a native of Eurasia and used by humans for centuries (Akulinin, 1958; Ge *et al.*, 1985). Among all the species of this genus, *Hippophae rhamnoides* is the most widespread. It has been divided into approximately eight geographically separated subspecies, but some scientists think that some of these deserve the rank of species (Small *et al.*, 2000). The unusually hairy *H. gyantsensis* occurs only in a restricted part of Tibet adjacent to Sikkim (Small *et al.*, 2000).

Hippophae is believed to be a colonizer of open habitats. In India their distribution is strictly restricted to the higher altitudes of the Himalayas. The highly efficient relationship with *Frankia* results in the improved root growth which enhances the entire soil ecosystem.

Sea buckthorns have great economic value. The berries, leaves and seeds have tremendous nutritional and medicinal value. It has three to sixteen times as much vitamin C as Kiwi fruit. Its superoxide dismutase content of the fruit juice is four times higher than that found in Ginseng. It contains twenty four minerals, eighteen amino acids and eight vitamins (Small *et al.*, 2000). Sea buckthorn is also useful as soil enhancer, pollution reducer, source of firewood, and as a landscape management tool (Li and Schroeder, 1996).

The actinorrhizal plants are found primarily in the temperate zone. Only some species of the Casurinaceae and the Myricaceae can be considered truly tropical. Some species of

Alnus and *Elaeagnus* are also found in the tropical zone but these species are restricted to the higher elevations of mountain regions, where the climate is essentially temperate. In high latitude countries such as Scandinavia, Canada and New Zealand conditions are not favourable for legumes to thrive. However, actinorhizal plants are abundant and capable of vigorous growth (Silvester, 1977).

These plants involved in symbiosis with *Frankia* are important pioneer species in nitrogen poor soils or disturbed environments (Benson *et al.*, 1984). They are extremely useful for rehabilitation of degraded and salt affected soils, where other plants may fail to grow. The high nitrogen content of the leaf litter of actinorhizal trees increases soil fertility and can pave the way for diversification of species within the ecosystem. Recent genetic studies show that possibly the eight different tree families of actinorhizal host plants developed the capacity for symbiosis at separate stages during evolution (Swensen, 1996; Myrold *et al.*, 1999).

Though the actinorhizal association is analogous to the much better studied *Rhizobium*-legume symbiosis, very little is known about *Frankia*, especially its ecology and genetics. Thus there is a need for obtaining basic information about the population sizes and diversity of *Frankia*. It may be noted that the first successful isolation of *Frankia* was reported only in 1978, when strain Cp11 was obtained from *Comptonia peregrina* by Callaham *et al.* (1978).

Over the past few decades, agriculture in the developed world has become increasingly dependant on industrially fixed chemical fertilizers and pesticides for achievement and maintenance of the high yields possible with modern crop types. In recent years, the adverse effects of these chemicals on the rural population, the environment and on food safety, has urged the need for changes in agricultural production methods, with the objectives of sustainability, economic production,

conservation of natural resources, and reduction in the use of synthetic chemicals. The challenge is to institute sustainable methods without compromising food-production levels, particularly in view of the need to increase agricultural yields worldwide to accommodate population growth.

There are several significant environmental reasons to seek alternative to chemically fixed nitrogen fertilizer. Chemical fixation of atmospheric nitrogen affects the balance of the global N₂ cycle, pollutes ground water, increases the risk of chemical spills and increases atmospheric nitrous oxide- a potent “green house” gas. On the other hand, biological nitrogen fixation offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving the quality and quantity of internal resources.

Forest trees are essential for human society. They provide fuel, fiber, building materials, food and medicines among other things. Forests stabilize environments and trees are cornerstones of ecosystems. The importance of nitrogen-fixing actinorhizal plants in forestry has been recognized (Montpetit and Lalonde, 1988). These plants are widely used in such practices as reforestation, land reclamation and biomass production (Fessenden 1979; Gordon *et al.*, 1979; Ponder, 1983; Tarrant *et al.*, 1971).

The genetic resources of forest trees must be conserved to maintain and improve our forests. Various classification systems have been used to categorize modern biotechnologies used in forestry sector, mainly because of the potential genetic gains they could confer. This can be categorized as – biotechnology employing molecular markers, biotechnology aimed at enhancing plant propagation and biotechnology for modifying the genome of forest tree species. Application of biotechnologies in forests has been seen as a unique opportunity for obtaining new information on the extent, patterns and functioning of tree genetic diversity and for providing new tree varieties and

reproductive materials adapted to changing environmental, social and economic environments (Fenning and Gershenzon, 2002).

Biotechnology can provide tools for the identification of superior genotypes through the characterization of DNA markers. The same tools are used to study the genetic diversity of tree populations which is a component of biodiversity and is important in ensuring the sustainability of the forest resource.

Frankia-actinorhizal symbiosis has attracted a great attention in Biological Nitrogen Fixation research recently. Prior to this our understanding of the molecular genetics of *Frankia* was restricted because of the fact that frankiae have slow growth rates, and there was lack of availability of a suitable vector for introduction of alien DNA into *Frankia* cells. *Frankia* strains have tremendous diversity at molecular level as shown by DNA-DNA hybridization of total cellular DNAs. Moreover, plasmids have been found in some frankiae. Several of the *nif* genes have been identified in *Frankia* but progress in identifying *nod* genes has been limited.

Actinorhizal symbiosis is such an association where both the host and the microbe have very important roles to play. Therefore, a superior and efficient host microbe relationship could positively affect nitrogen-fixing capacity of the *Frankia* strain (Chauhan, 2000). And for establishing this superior and efficient relationship, most infective, effective and competitive *Frankia* strains have to be selected, which in turn require investigations on diversity existing within the species.

Study of genetic diversity of *Frankia* would become useful if this information is used in enhancing its natural abilities. There exists tremendous potential if we are able to enhance the nitrogen fixing ability of *Frankia* and are able to extend its host range to include some horticultural trees. However, we must understand the effect of ecological

parameters on molecular constitution and behaviour before we set out to achieve the above. It is in this context that the present study was taken up.

Objectives

The present study was taken up to study the impact of certain ecological parameters on distribution of different strains at different sites. To achieve this broad objective, the following specific tasks were laid out:

1. Molecular typing of *Frankia* strains present in the field collected nodules of some genera of actinorhizal trees from Sikkim.
2. To study relationship, if any, between ecological parameters (different soil characteristics, altitude etc.) and molecular diversity of *Frankia*.

Review of Literature