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ORIGINAL ARTICLE

Molecular diversity of *Frankia* from root nodules of *Hippophae salicifolia* D.Don found in Sikkim

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Abstract Molecular diversity of *Frankia* was assessed directly from the root nodules of *Hippophae salicifolia* naturally occurring in North Sikkim. Amplicon restriction patterns (ARPs) were developed by digesting 16S-ITS-23S amplicons with *Rsa*I. Three ARPs were detected, showing diversity among strains of *Frankia* that nodulate *Hippophae*. This was confirmed by sequencing one amplicon each for the three ARPs. Therefore, ARP can be used as a tool for screening amplicons for nucleotide sequencing.

Keywords Amplicon restriction pattern · *Frankia* · *Hippophae* · ITS

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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. Nitrogen must be converted into ammonium (NH_4^+) or nitrate (NO_3^-) ions before it can be used by plants and animals. Conversion of molecular nitrogen to NH_4^+ forms is also called nitrogen fixation. Only a few microorganisms can 'fix' atmospheric nitrogen, making all other living organisms dependent on them for their requirements of 'fixed' nitrogen. Microorganisms such as *Rhizobium* and *Frankia* form symbiotic associations with host plants and utilize fixed carbon supplied by host for fixing atmospheric di-nitrogen (N_2). 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 [1].

Because the *Frankia* symbiosis results from an actinomycetic invasion of plant roots, it has been termed as "actinorhizal symbiosis" [2]. 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 [3]. Actinorhizal plants belong to four subclasses, eight families, 25 genera and more than 220 species [4]. Well known genera are *Alnus* (Betulaceae), *Myrica* (Myricaceae), *Casuarina* (Casuarinaceae), *Elaeagnus* and *Hippophae* (Elaeagnaceae). *Frankia* have attracted

attention because they form root nodules on a broad range of non-leguminous plants and because such nodules fix N_2 as effectively as rhizobial nodules.

The genus *Hippophae*, commonly known as sea buckthorn, is an actinorhizal plant that forms a symbiotic association with *Frankia*. It is a very attractive ornamental shrub with silvery deciduous leaves and colorful orange berries that persist through most of the winter. It is a native of Eurasia and has been used by humans for centuries [5, 6]. 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 [7]. The unusually hairy *H. gyantsensis* occurs only in a restricted part of Tibet adjacent to Sikkim [7]. *H. salicifolia* is widespread in the eastern Himalayas. Sea buckthorn is useful as a soil enhancer, pollution reducer, source of firewood and as a landscape management tool [8].

In actinorhizal symbiosis, both the host and the microbe have very important roles to play. Therefore, a superior and efficient host microbe relationship could positively effect the nitrogen fixing capacity of the *Frankia* strain [9]. And for establishing this superior and efficient relationship, the most infective, effective and competitive *Frankia* strains have to be selected, which in turn require investigations on diversity existing within the genus.

The present investigation was designed to assess the molecular diversity existing in the natural population of *Frankia* nodulating *Hippophae salicifolia* found in the Sikkim Himalayas.

Materials and methods

The sample collection site for the present study was located in Sikkim, which is a small mountainous state in the Eastern Himalayan region of India, extending approximately 114 km from North to South and 64 km from East to West, having a total geographical area of 7096 sq km. The state is situated between 88°00'58" and 88°55'25" East longitude and 27°04' and 28°07'48" North latitude. It is surrounded by vast stretches of Tibetan plateau to the North; Kingdom of Bhutan in the East; Darjeeling district of West Bengal to the South and Kingdom of Nepal in the West. The state has four districts namely East, West, North and South. Sikkim is bestowed with abundant natural resources. Although Sikkim constitutes only 0.2% of the geographical area of India, in terms of species richness it ranks very high. It has been identified as part of Indo-Burma Biodiversity Hot Spot [10].

A site in the North Sikkim district was selected for the present study. It was located approximately 1 km from Lachen at 2672 m above msl. Root nodules were collected from a big *Hippophae salicifolia* stand adjacent to a stream. The age of the trees was between 10 and 12 years. Soil was tight, compact, sandy and brownish in color.

Collections of root nodules were carried out in the month of October based on the observation of Varghese [11] that the nodule growth was best soon after the monsoon rains. Ten plants were randomly selected and the lateral roots were traced to the plant. After digging the surrounding soil to a depth of six inches, many nodule clusters were exposed. The collected nodules were kept in fresh polythene bags and brought to the laboratory. They were stored after cleaning and used for isolation of DNA.

Isolation of genomic DNA from nodules

Extraction of total genomic DNA from individual nodule lobes was carried out following the methodology given by Rouvier et al. [12] with minor modifications [13, 14].

Amplification of genomic DNA by polymerase chain reaction (PCR)

DNA extracts were subjected to PCR amplification of the 16S-ITS-23S rRNA region using specific primers described by Bosco et al. [15]. The reaction mix contained 2.5 µl of 10 µM primer (Microsynth, Switzerland), 2.5 µl of 10X PCR buffer (Bangalore Genei, India), 2.5 µl of 25 µM $MgCl_2$ (Bangalore Genei, India), 10 µl of 5 mM deoxynucleotide triphosphate mix (Bangalore Genei, India), 0.75 µl of *Taq* polymerase (3 units μl^{-1}) (Bangalore Genei, India), 1 µl of template DNA and ultrapure water to make the total volume to 25 µl per tube. Each amplification reaction was carried out for 35 cycles using a thermal cycler (Gene Amp PCR 2400, Perkin-Elmer). Each cycle comprised 1 min denaturation at 94°C, 1 minute annealing at 49°C and 1 min of elongation at 72°C. A hot start was given for 5 min at 94°C and at the end of the run an additional 7 min extension time at 72°C was added to allow complete extension of all DNA double strands [14]. The annealing temperature was standardized for securing amplification of *Hippophae* compatible *Frankia* DNA. Amplified DNA samples were run in 0.8% agarose gel at 70 volts for 90 min. The gel was stained in ethidium bromide for about 15 min and scanned and photographed using BioRad GelDoc1000 and the band sizes were calculated using the Multi Analyst® software (version 1.1).

Amplicon restriction pattern (ARP) analysis of PCR products

The amplified 16S-ITS-23S region was subjected to restriction digestion overnight at 37°C using *Rsa*I (Roche Phar-

maceuticals). *Rsa*I was selected based on computer simulated mock digestion of the DNA sequence of appropriate region of *Frankia*. The restriction digestion mixture was prepared in a 0.5 ml tube in which 10 µl (approx. 40 to 50 ng of DNA) of the amplicon, 5 units of the restriction enzyme, and 2 µl of appropriate 10X buffer (buffer B) were mixed. Pure distilled water was added to make the final volume to 20 µl. After digestion the samples were run in ethidium bromide stained 4% agarose gel using horizontal electrophoresis in 1X TBE buffer at 50 volt (2–3 V/cm) for 5–6 hrs. The gel was then scanned and photographed using BioRad GelDoc1000 and the profiles were analyzed using the Multi Analyst® software. These patterns were compared with patterns of similar digestion carried out on alder compatible *Frankia* [14]. NTSYS (version 2.1) was used for cluster analysis of the banding patterns. The cluster dendrogram was constructed using sequential agglomerative hierarchical nested cluster analysis (SAHN) and it was based on a similarity matrix which in turn was based on Jaccard's coefficients.

Amplicon DNA sequencing

Sequences of selected amplicons were done at the Center for Genome Research and Biocomputing at Oregon State University (Corvallis, Oregon, USA).

Results and discussion

The amplicons were found to be approximately 1200 bp. This included the distal part of the 16S rDNA, the ITS and the initial part of the 23S rDNA.

Amplicon restriction patterns

Interestingly, these samples showed considerable variation in banding patterns even within a very small population (Fig. 1). Three restriction banding patterns were observed: samples H1, H3, H5, and H7 were found similar with five faint bands (P1) whereas H2 and H4 were grouped as P2 with five comparatively strong bands and H6 was found to be different from others and was considered as separate pattern P3. The cluster analysis of amplicons based on their banding patterns showed three broad clusters, I, II and III. Cluster I grouped 33 samples representing eight different patterns into four sub clusters IA, IB, IC and ID, which were further divided into minor clusters (Fig. 2). Cluster II included 50 samples representing nine patterns into two major sub clusters IIA and IIB, which were again divided into minor clusters. Cluster III represented *Frankia* nodulating *Hippophae salicifolia.* The three patterns were found to be very diverse with a Jaccard's coefficient of just about 0.23 for similarity of pattern 2 with patterns 1 and 3. The Jaccard's coefficient for the similarity of patterns 1 and 3 was approximately 0.77.

Nucleotide sequencing and analysis

One sample representing each banding pattern was selected for sequencing. H1, H2 and H6 were sequenced. The closest match for each sequence in a BLAST search was *Frankia*. Sample H2 could be sequenced up to a length of 1040 bp, whereas the sequence lengths of samples H1 and H6 were relatively short, being 418 bp and 566 bp respectively (GeneBank accession numbers DQ988980, DQ988981 and DQ988982).

When these three sequences were aligned together for the overlapping region, they showed many differences.

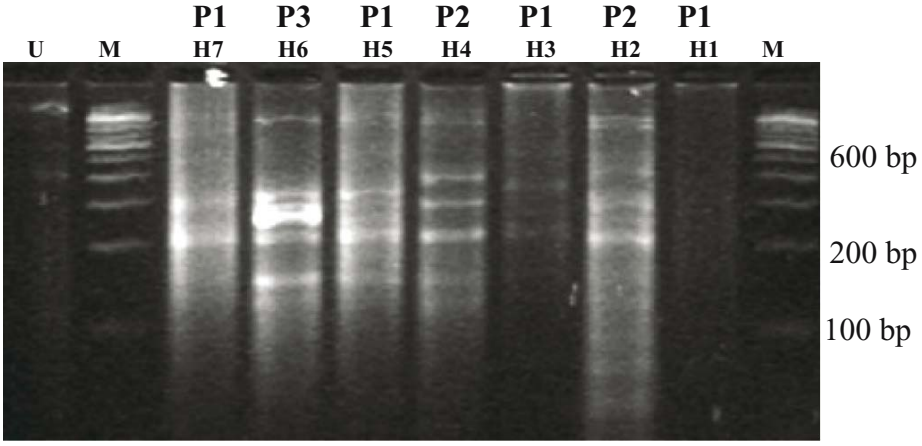

Fig. 1 Amplicon restriction patterns obtained by digestion with *Rsa*I. (U – undigested amplicon, M – molecular weight marker, H1 to H7 – sample number, P1 to P3 – ARP patterns)

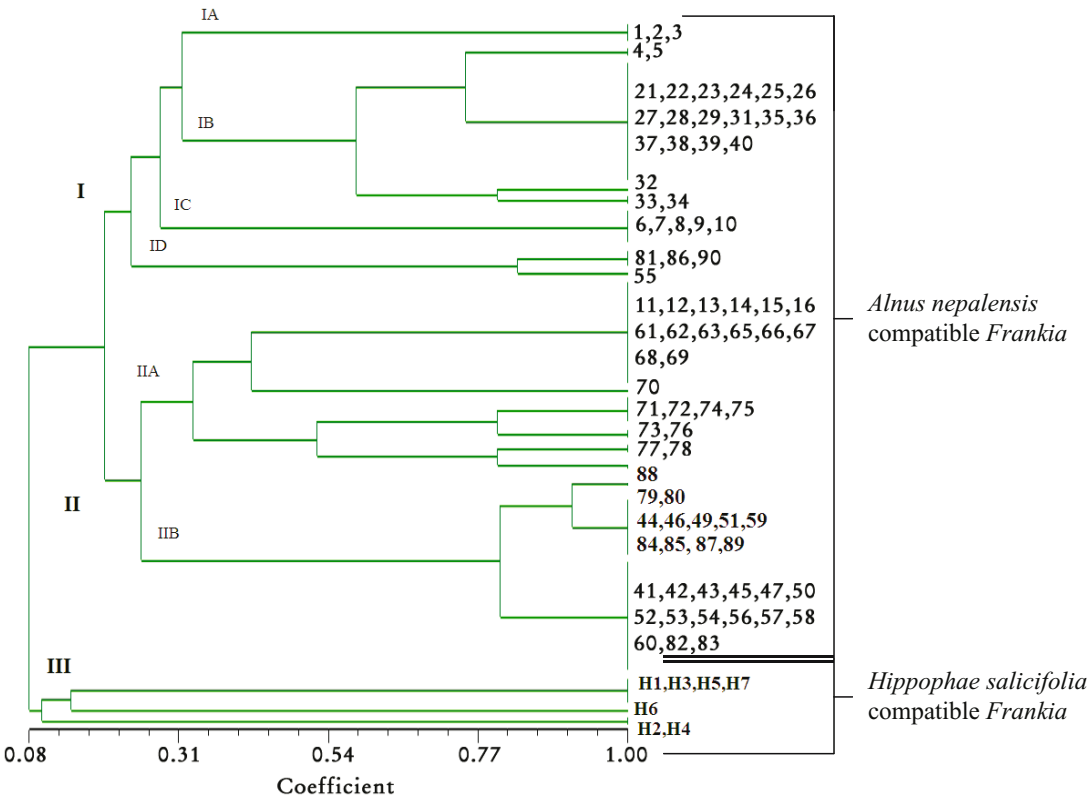

Fig. 2 Cluster analysis of ARPs of *Frankia* nodules collected from North Sikkim. *Alnus* compatible *Frankia* sequences have been discussed by Khan et al. [14]. Coefficient represents Jaccard's coefficient.

These sequences were separately subjected to computer simulated digestion using Webcutter®. Four cutting sites were identified along the sequence of sample H2, producing five bands. Four of these bands were actually seen in the gel. In sample H6, Webcutter® could identify three cutting sites; however because only partial sequence was available for simulated digestion, it could not be compared with the actual patterns observed. Similarly, the 418 bp sequence of sample H1 available for mock digestion showed no cutting sites along its length.

We found a surprisingly high level of diversity in the ARPs obtained from the small number of samples studied. This diversity was confirmed by the nucleotide sequencing. In a similar study we found two different ARPs for ten *Alnus nepalensis* root nodule samples collected from North Sikkim [14]. This is indicative of a larger diversity for *Frankia* nodulating *Hippophae salicifolia* compared to that nodulating alder. *Elaeagnus* compatible *Frankia* isolated from soils collected from Tunisia too showed large diversity [16]. On the other hand, Clawson et al. [17] found a shallow level of diversity in the 16S rDNA analysis of family Elaeagnaceae. However, they did not include the genus *Hippophae*. Our report is the first to study the diversity of *Frankia* nodulating *Hippophae salicifolia*. We are not sure if the diversity would increase or decrease if more samples are investigated. Nevertheless there is a need to investigate more samples from *Hippophae* root nodules for confirming our preliminary results. We suggest that our approach of using analysis of ARPs for selecting samples for nucleotide sequencing may be used for further studies.

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