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

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Received: 1 November 2007 / Accepted: 31 December 2007

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 Rsal. 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

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 N₂ 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 affect 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 to 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 MgCl₂ (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 *RsaI* (Roche Phar-