Polymorphic distribution and phenotypic diversity of *Frankia* strains in nodule lobes of *Hippöphae salicifolia* D. Don

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***Frankia*, as a source of symbiotic N_2 fixation in non-leguminous actinorhizal plants has received tremendous importance in the last few decades. Studies pertaining to the determination of genetic diversity of *Frankia* strains in diversified groups of actinorhizal plants have been a subject of attention in recent times. We report here an attempt to isolate and identify consortia of *Frankia* strains from root nodules of *Hippöphae salicifolia* D. Don collected from the eastern Himalayas of north Sikkim. A total 31 *Frankia* axenic cultures were isolated from 27 root nodule lobes which differed in colouration and morphological pattern. The isolates were subjected to different formulation of carbon sources and pH variations with and without additional exogenous N_2 source. Finally a total of 11 single spore isolates were identified based on morphological and physiological differences. The present study reveals that there is an existence of a great deal of heterogeneity among the *Frankia* strains associated with *Hippöphae salicifolia* D. Don.**

Keywords: Diversity, *Frankia*, *Hippöphae salicifolia* D. Don, microscopy.

FRANKIA is a filamentous, microaerophillic, Gram-positive actinomycete bacteria with high G + C content, able to induce nitrogen-fixing root nodules in a number of non-leguminous actinorhizal plants[1]. In the last four decades a lot of research has been focused on the assessment of the nitrogen fixing ability of actinorhizal plants and in determining the genetic diversity of *Frankia* strains[2,3] associated with them. The study of *Frankia* and the biology of actinorhizal symbiosis have been hampered by the difficulty in obtaining these bacteria in pure cultures[1,4] and also by their slow growth rate under laboratory conditions[5,6]. Till date, hundreds of genetically distinct clonal cultures of *Frankia* have been isolated from a number of actinorhizal plant species[1,7–9], from a single species growing within a limited geographical area[7] or even in a single nodule from a host plant[8,10], which provides a sagacity of inquisitiveness regarding genetic stability of these filamentous actinomycete under both laboratory and natural conditions[1,11]. Although a number of *Frankia* strains from various actinorhizal plants have been isolated worldwide[1,12,13], no *Frankia* isolate from the actinorhizal woody shrub *Hippöphae salicifolia* D. Don has been reported so far from the Eastern Himalayan region of the Indian subcontinent[14] (Figure 1 *a*). The species *H. salicifolia* D. Don is a high-altitude, stress-tolerant, deciduous, spinescent, non-leguminous angiospermic plant that bears *Frankia* in its highly extensive root nodules (Figure 1 *b*), fixes atmospheric nitrogen and generally grows as a pioneer species during early ecological succession[14,15]. The species is of great significance not only in terms of restoration of soil fertility along the fragile mountain slopes thereby obstructing erosion and landslides[14–16] but also serve as a major source for meeting the increasing demand for fuelwood and fodder requirements[15], particularly of people dwelling along high-altitude regions of the Eastern Himalayas where natural resource is limited.

The aim of the present study was (i) isolation of *Frankia* strains associated with root nodules of nine *H. salicifolia* D. Don plants inhabiting different altitudinal variations within the same geographical location (North Sikkim and its adjoining areas) and (ii) identification of the isolated strains based on their phenotypic variability and morphological characteristics in culture by performing photomicrography. To conduct the studies, fresh root nodules, fruits and stem cuttings of *H. salicifolia* D. Don were collected from different elevation zones, surface-sterilized and planted into pots containing rhizospheric soil collected from study sites for root initiation (Figure 1 *c*). After 65 days, fresh, young nodules from emerging roots of the plants were collected (Figure 1 *d*) for isolation and culturing of *Frankia* strains[7]. Altogether 31 different *Frankia* axenic cultures were isolated from the root nodules according to the standard method[1,9,17,18]. The isolated *Frankia* strains that differed in coloration and morphology were grown and maintained in Qmod media[19] at 29 ± 0.5°C. For easy handling, smooth manipulation and proper identification of the isolated strains, these were provided with strain-code designations (HsIi1 to HsIi31)[20]. Also, to ascertain the identity of the isolated strains, five reference strains were obtained[21] (viz. strains AiPs1, Ag4b, HrI6, G2 and CpI2) which were grown in P2 liquid medium[21] to generate mass cultures for comparative analysis. Purity of *Frankia* strains was checked and maintained by repetitive light microscope observation and use of antibiotics such as cyclohexemide (50 µg/ml and nalidixic acid (10 µg/ml)[1,4,5,6,22]. The 31 isolated *Frankia* axenic cultures grew as small colonies with somewhat dissimilar morphology (round, oval or even filamentous outgrowths) and different colony colours ranging from dirty opaque white to red, pink and yellow. All the 31 isolates were obtained in purity (Figure 1 *j*) and were subjected to different formulations of carbon source[22] with and without additional exogenous nitrogen source by transferring the isolates to various liquid

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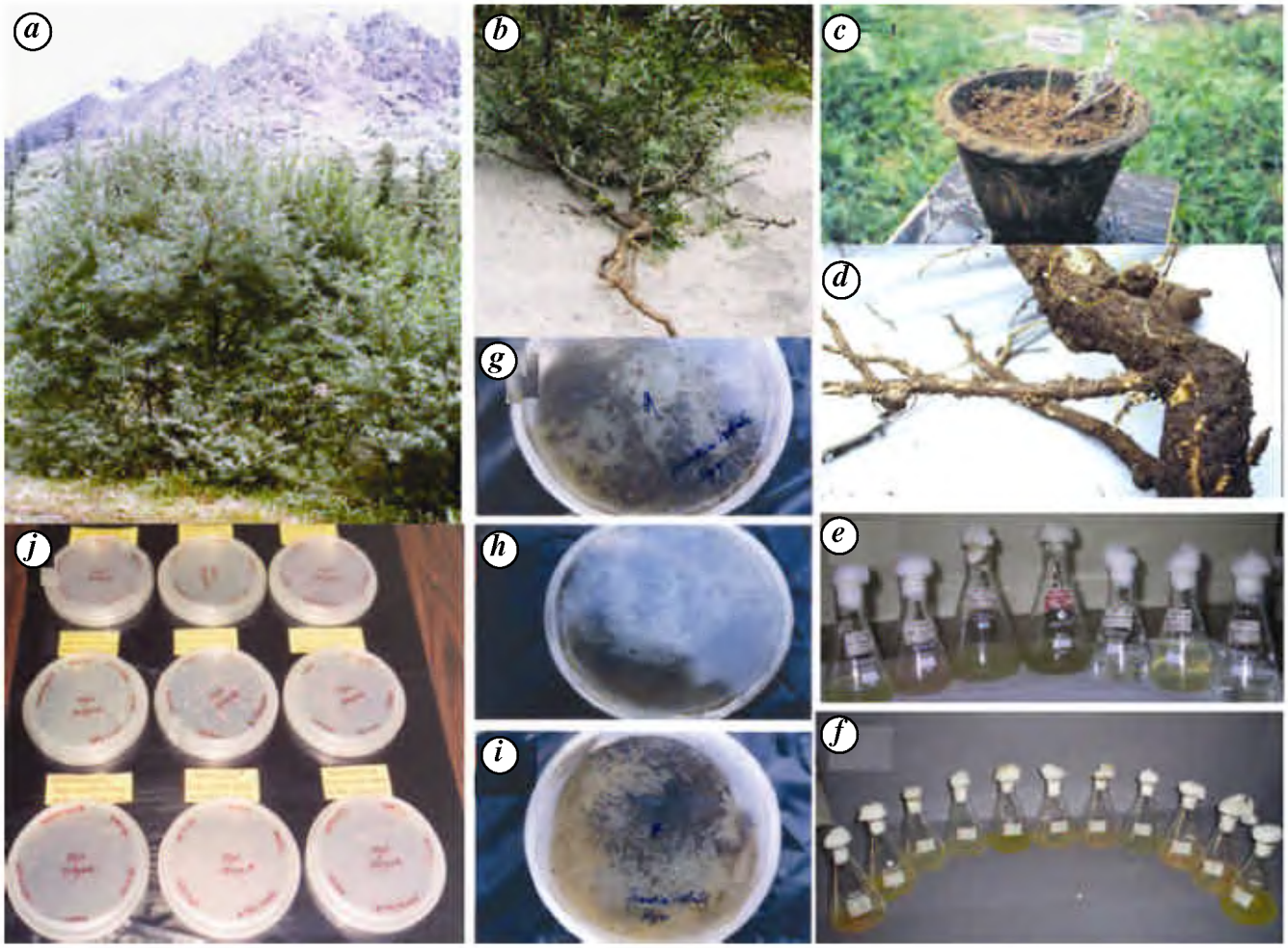

Figure 1. ***a***, Distribution of actinorhizal plant *Hippöphae salicifolia* D. Don in North Sikkim. ***b***, Root system of a *H. salicifolia* D. Don plant in the study site. ***c***, Nursery propagation of stem cutting under greenhouse conditions. ***d***, Root nodules of *H. salicifolia* D. Don collected for *Frankia* isolation. ***e***, *In vitro* growth of *Frankia* strains supplemented with various carbon sources. ***f***, *In vitro* growth of *Frankia* strains in BAP medium with pyruvate. ***g–i***, Axenic cultures of *Frankia* in Qmod-agar petri dishes ***j***, Purified strains of *Frankia* in Qmod-agar petri dishes.

media (Figure 1 *e*) supplemented with carbon sources, including both simple sugars (viz. glucose, fructose, maltose, xylose, madurose, trehalose, mannitol, mannose, lactose and sucrose) as well as organic acids[7,9] (viz. pyruvate, propionate, succinate, acetate and tween 80). All the 31 strains depicted concomitant growth behaviour to the simple sugars, except madurose and xylose, although the two sugars form the basic constituents of *Frankia* cell membrane *in vivo* nodules[23]. On the other hand, among the organic acids tested, only pyruvate supported growth of all the isolates, while propionate supported growth of only 15 isolates. Sodium succinate supported growth of seven isolates and none of the isolates grew in acetate or tween 80. It was also observed that exogenous nitrogen source was inevitable for growth of isolates under *in vitro* conditions[4,24]. To acquire a vivid physiognomy concerning the identity of the isolated strains, these were subjected to growth at different pH regimes starting from moderately acidic (pH 5.5), to neutral (pH 7.0) taking into consideration previous findings[1,7,25], whereby optimum pH levels for maximum growth of the reference strains used in the present studies were reported to be in the range 6.0–6.8 under *in vitro* conditions[25]. Results obtained confirmed that all the isolates (excluding isolate HsIi14) portrayed the characteristics of typical *Frankia* strain, since maximum growth could be observed at neutral or near-neutral pH. Isolate HsIi14 grew at a rather acidic pH and interestingly showed a concomitant decrease in growth with an increase in basicity of the medium. Such an extraordinary character was earlier reported for the *Frankia* strain CpI1, which showed maximum growth at 33°C, pH 6.0 in batch culture[5]. As the thermotolerance pattern of the isolated strains was unknown to us, the strains were therefore subjected to different temperatures ranging from 28 to 33°C, taking into account the optimum growth conditions tested for the reference strains earlier[21,25]. It was observed that all the isolated strains showed maximum growth at 29°C, which henceforth has been regarded as the optimum temperature for studies reported herewith. As the identity of the isolated strains was still unclear and since it is well-known

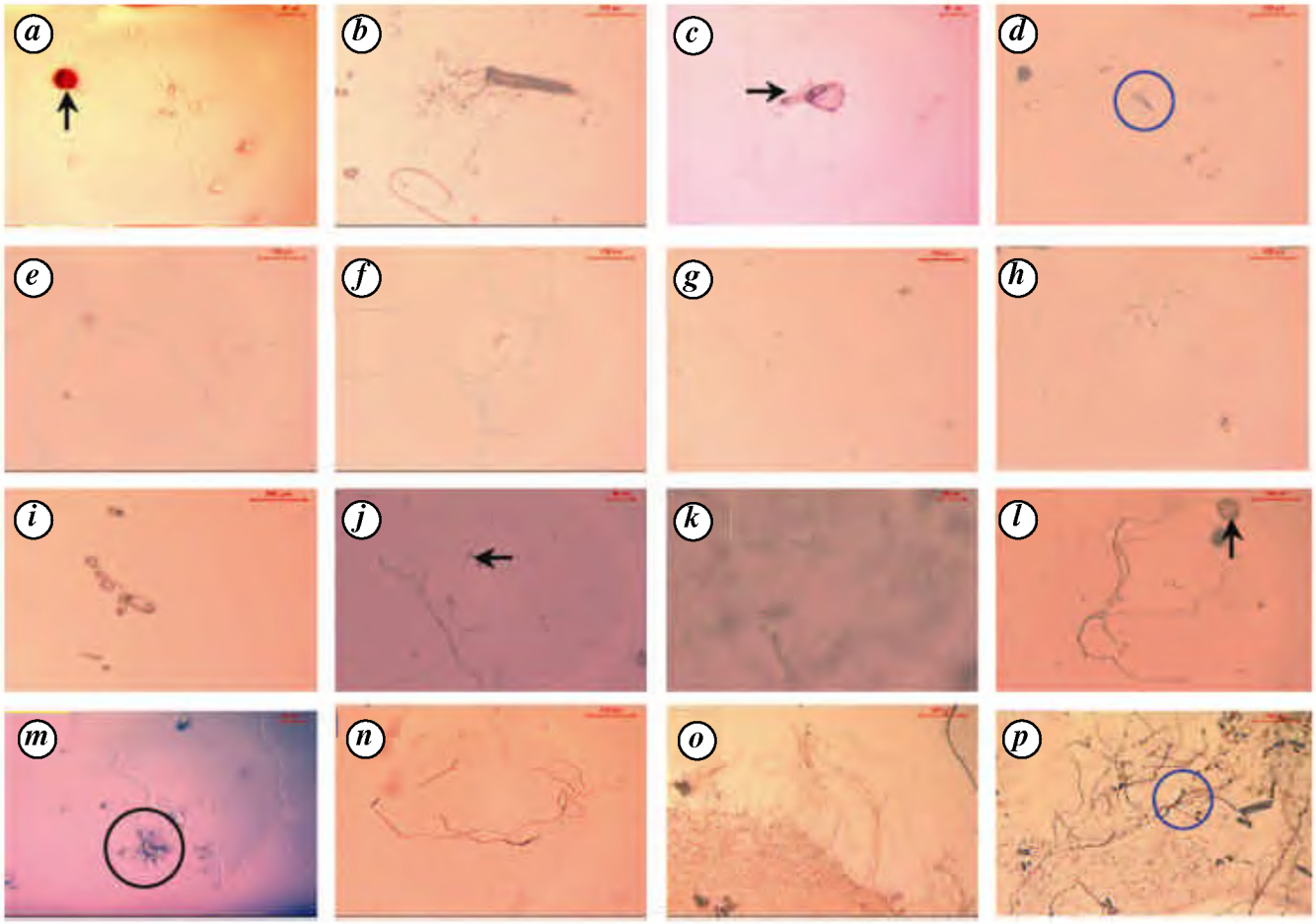

Figure 2. Phase contrast photomicrography of *Frankia*. ***a***, Single-spore isolate HsIi2 (arrow mark); ***b***, Isolate HsIi4; ***c***, Single germinating spore of isolate HsIi4; ***d***, Isolate HsIi5 with multilocular sporangia (circled blue); ***e***, Isolate HsIi7; ***f***, Isolate HsIi8; ***g***, Isolate HsIi9 ***h***, Isolate HsIi10; ***i***, Isolate HsIi10 with large stalked sporangiophores; ***j***, Isolate HsIi11 with germinating single spore (arrow mark); ***k***, Isolate HsIi12 with sinuate hyphae ***l***, Isolate HsIi13 with large round sporangiophore (arrow mark); ***m***, Isolate HsIi14 with aggregated spores and hyphae (circled black); ***n***, Reference strain Ag4b depicting ramified branching of filaments; ***o***, Reference strain G2 with interwoven meshwork of filaments in culture; ***p***, Reference strain HrI6 with branched filaments and dermatophilus-type multilocular sporangia in P2 media culture (circled blue). Photographs were treated with false colour to obtain clarity and better contrast of the bright field images. Magnification bar = 200 µm (***b***, ***g***, ***i***, ***o***) 100 µm (***d***, ***e***, ***f***, ***h***, ***l***, ***n***, ***p***) and 30 µm (***a***, ***c***, ***j***, ***k***, ***m***).

that the system of isolating *Frankia* from crushed nodule lobes does not necessarily ensure the isolation of genetically pure strains[1,3,8], it was therefore decided to generate genetically pure single-spore cultures of all the 31 isolates and the reference strains by entrapment in Na^{+2} alginate beads through the plating method[26]. The plates were incubated at 29 ± 0.5°C in the dark. Single-spore bacterial colonies were marked individually, allowed to develop for eight weeks[8,26] and the well-developed colonies were finally transferred to liquid BAP medium[24] supplemented with 10 mM sodium pyruvate and 5 mM ammonium chloride. From the 31 isolates subjected to single-spore isolation, 27 different single-spore isolates were identified based on microscopic observations[8,27], a few of which were photographed (Figure 2 *a*, *c*, *j*). Reference strains did not yield more than one single-spore colony type[21,25]. Bright field microscopic examination of the single-spore isolates and three reference strains was performed[33] by Phase Contrast LEICA DMRX image processing system at different magnifications. Measurements of morphological contrariety like colony colour, hyphal diameter, presence/absence of spores, vesicles and sporangia forms were recorded with 'Leica Qwin' image analysing software supplied with the microscopic system (Table 1). Eleven out of 27 single-spore isolates from *H. salicifolia* D. Don appeared dissimilar when studied using photomicrography. The mean diameter of the filaments was observed to be largest in isolate HsIi10 (18.64 µm m^{-2}) and the smallest in isolates HsIi11 and HsIi14 (5.83 µm m^{-2}). The reference strain isolate G2 (3.16 µm m^{-2}) exhibited the smallest filamentous forms compared to all the strains tested. A great deal of heterogeneity in the structure and diameter of sub-hyphae arising out of the filaments and sporangia forms could also be observed in almost all the isolates (Table 1). In addition, a variety of sporangia type (Table 2) and a large amount of variation in size and diameter of sporangia forms (Table 1) were observed in the isolates during photomicrographic studies. Isolate HsIi10 dis-

Table 1. Morphological characteristics of *Frankia* axenic cultures observed through phase contrast photomicrography

Diameter ($\mu m/m^2$)	*Frankia* isolate from *Hippöphae salicifolia* D.Don											Reference strain		
	HsIi2	HsIi4	HsIi5	HsIi7	HsIi8	HsIi9	HsIi10	HsIi11	HsIi12	HsIi13	HsIi14	HrI6	Ag4b	G2
Filament-1	7.24 ±	13.38 ±	9.98 ±	9.42 ±	9.99 ±	6.90 ±	16.94 ±	5.02 ±	10.12 ±	11.82 ±	5.02 ±	9.81 ±	12.76 ±	2.98 ±
	0.21	0.26	0.46	0.66	0.38	0.30	0.89	0.22	0.42	0.59	0.22	0.32	0.48	0.12
Filament-2	8.73 ±	18.40 ±	12.24 ±	8.39 ±	9.81 ±	6.60 ±	19.00 ±	6.46 ±	9.88 ±	12.46 ±	6.46 ±	10.22 ±	9.88 ±	3.05 ±
	0.39	0.42	0.80	0.64	0.32	0.34	1.03	0.20	0.33	0.56	0.20	0.51	0.33	0.17
Filament-3	10.46 ±	10.22 ±	11.62 ±	8.10 ±	9.58 ±	7.35 ±	20.00 ±	6.01 ±	9.54 ±	12.76 ±	6.01 ±	12.46 ±	10.76 ±	3.45 ±
	0.52	0.51	0.57	0.78	0.28	0.49	0.76	0.30	0.32	0.49	0.30	0.56	0.33	0.14
Sub-hyphae-1	4.91 ±	8.61 ±	8.63 ±	6.64 ±	5.36 ±	2.98 ±	10.05 ±	3.08 ±	7.40 ±	4.14 ±	3.13 ±	3.90 ±	3.66 ±	1.35 ±
	0.18	0.24	0.19	0.88	0.21	0.17	0.48	0.11	0.32	0.31	0.12	0.12	0.30	0.17
Sub-hyphae-2	3.45 ±	10.74 ±	8.28 ±	6.55 ±	7.09 ±	3.28 ±	10.16 ±	3.05 ±	6.54 ±	4.57 ±	2.37 ±	3.95 ±	4.27 ±	1.34 ±
	0.14	0.56	0.51	0.49	0.18	0.23	0.66	0.17	0.38	0.18	0.20	0.11	0.18	0.17
Sub-hyphae-3	3.86 ±	9.22 ±	9.28 ±	6.49 ±	7.24 ±	3.40 ±	6.30 ±	2.53 ±	7.24 ±	4.55 ±	2.19 ±	4.57 ±	3.18 ±	1.34 ±
	0.10	0.35	0.29	0.55	0.26	0.23	0.31	0.19	0.26	0.19	0.15	0.18	0.25	0.17
Sub-hyphae-4	3.95 ±	9.77 ±	8.77 ±	6.83 ±	7.40 ±	3.90 ±	9.77 ±	2.98 ±	7.09 ±	4.27 ±	2.52 ±	5.36 ±	2.62 ±	1.63 ±
	0.11	0.47	0.25	0.47	0.32	0.12	0.46	0.12	0.18	0.18	0.20	0.21	0.28	0.24
Sub-hyphae-5	3.21 ±	8.71 ±	9.30 ±	7.49 ±	6.54 ±	3.24 ±	8.62 ±	2.42 ±	5.36 ±	5.25 ±	2.14 ±	3.05 ±	3.51 ±	1.61 ±
	0.13	0.58	0.28	0.78	0.38	0.19	0.49	0.21	0.21	0.24	0.18	0.17	0.17	0.20

Mean ± = SE (n = 6).

Table 2. Physiological characteristics of *Frankia* axenic cultures under *in vitro* cultured condition

Strain type	Generation time (h)	Specific growth rate	Packed cell volume	Colony type	Pigment production	Sporangia type
HsIi2	166.73 ± 2.44	0.034 ± 0.001	439.00 ± 10.59	Pinkish	None	Oval
HsIi4	132.55 ± 2.44	0.056 ± 0.002	530.00 ± 6.08	Pinkish	Yellowish	Oval
HsIi5	169.92 ± 3.22	0.030 ± 0.001	611.33 ± 5.54	Dirty white	Dirty-opaque	Calabash
HsIi7	116.72 ± 2.85	0.065 ± 0.0006	396.33 ± 6.98	Dirty white	Dirty-opaque	Dermatophilus
HsIi8	138.94 ± 2.90	0.051 ± 0.001	662.00 ± 20.52	Yellowish	Yellowish	Sarcina
HsIi9	115.00 ± 3.01	0.065 ± 0.0003	630.66 ± 10.08	Pinkish-white	Dirty-opaque	Oval
HsIi10	133.25 ± 1.97	0.056 ± 0.002	652.00 ± 15.88	Pinkish	Dirty-opaque	Stalked
HsIi11	109.56 ± 6.61	0.074 ± 0.001	396.66 ± 6.88	Dirty white	None	Stalked
HsIi12	175.88 ± 3.59	0.025 ± 0.002	532.33 ± 9.20	Pinkish	None	Dermatophilus
HsIi13	162.26 ± 4.55	0.038 ± 0.001	537.66 ± 6.33	Reddish	None	Round
HsIi14	131.00 ± 3.16	0.056 ± 0.002	702.66 ± 29.36	Yellowish	Yellowish	Oval
HrI6*	148.22 ± 4.86	0.056 ± 0.002	131.00 ± 1.52	White	None	Dermatophilus
Ag4b*	118.38 ± 3.46	0.062 ± 0.001	145.33 ± 2.60	White	None	Not available
G2*	194.73 ± 4.23	0.015 ± 0.001	136.00 ± 8.71	Reddish	Reddish	Oval

*Reference strains.
Mean ± = SE (n = 3).
Packed cell volume of all strains was calculated with a hemocytometer.
Pigment production was recorded based on morpholgical observations during growth in liquid BAP medium supplemented with 10 mM sodium pyruvate as carbon source and 5 mM ammonium chloride as sole nitrogen source.
Sporangia types were recorded from microscopical observation in DMRX phase contrast photomicrography.

played large sporangia forms with an average diameter of 51.36 $\mu m\ m^{-2}$, while isolates HsIi14 and HsIi11 had the smallest sporangia forms with diameter 11.20 and 10.65 $\mu m\ m^{-2}$ respectively. Presence of vesicles and spores could be observed in almost all isolates, but variation in frequency of occurrence was noticed amongst them. Isolate HsIi5 demonstrated typical appearance of multilocular sporangia with a flower like appearance in culture under *in vitro* conditions (Figure 2 *d*), while isolate HsIi14 showed unusual aggregation of spores and hyphae (Figure 2 *m*). After confirmation of all the 27 single-spore isolates as 11 distinct *Frankia* strains, these were subsequently subjected to various physiological tests to evaluate species identity amongst the strains by comparing them to the reference strains tested (Table 2). The generation time of all the isolates was found to be much higher, a prerequisite criterion for *Frankia* strain determination[1,11]. Amongst the isolated strains, slowest growth was observed for strain HsIi12,

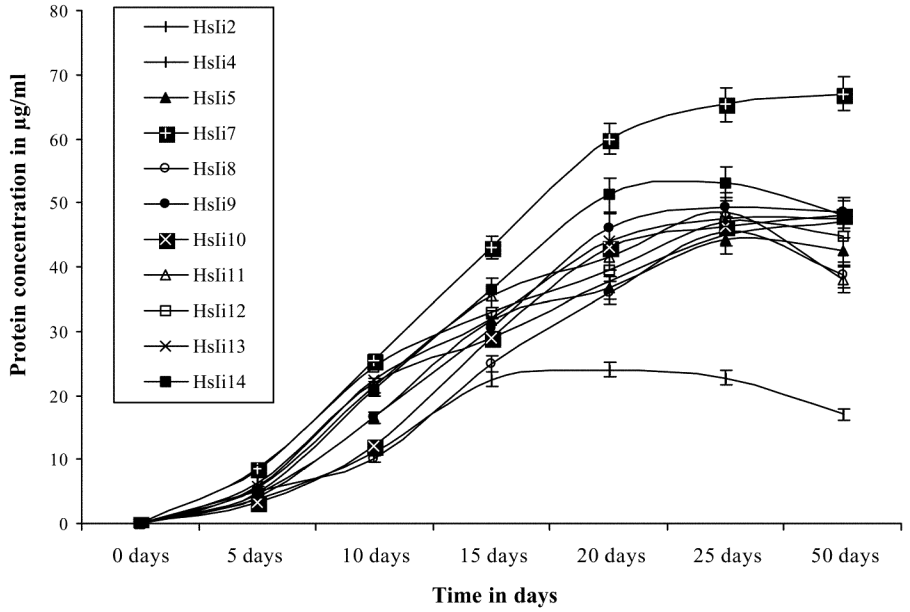

Figure 3. Growth characteristics of isolated *Frankia* strains in liquid BAP medium supplemented with sodium pyruvate as sole carbon source. Bars represent ± SD.

while strains HsIi7 and HsIi9 showed faster growth behaviour compared to others[25]. The specific growth rate of all the strains was observed to be low, while the packed cell volume was found to be much higher in the isolated strains compared to the reference strains (Table 2). Colour of the colony morphology was determined from growth of the isolates in solid Qmod media[19], while that of the reference strains was taken from the literature[21]. Pigment production is another important criterion for *Frankia* strain determination[1,4,18]. This was conspicuous from the growth of the strains in liquid BAP media supplemented with pyruvate and ammonia as sole carbon and nitrogen sources, which was evident from the physiological tests conducted, whereby pyruvate favoured maximum growth of the isolated strains compared to all other carbon sources tested (Figure 1*f*). With a final intention to ascertain the identity of the 11 single-spore isolates, the growth characteristics of the strains in BAP medium supplemented with pyruvate as carbon source were observed by determining the total soluble protein content in culture[28]. It was found to be the highest for strain HsIi7 and lowest for strain HsIi2 respectively (Figure 3).

The present study reveals that there exists a great deal of diversity among the *Frankia* strains harbouring the actinorhizal plant *H. salicifolia* D. Don, along different altitudinal variations of the Eastern Himalayan region in North Sikkim. The most intrusive findings in the present study were the presence of typical multilocular sporangia in isolate HsIi5, different sporangia types in all the isolates, aggregation of spores in isolate HsIi14 and low frequency of spores/vesicles in isolates HsIi7 and HsIi8 respectively. Other interesting findings in the present investigation are the similarities in filament diameter of growing hyphae and presence of multilocular sporangiophores observed for isolates from *H. salicifolia* D. Don and the reference strain HrI6 (isolated from *H. rhamnoides* stands in Finland)[25]. This probably suggests the solitary origin of the endosymbiont actinomycete associated with actinorhizal plants, with a predisposition for symbiotic nitrogen fixation that probably evolved during angiosperm evolution[1,11], since both the host species are different and are symbiotically associated to *Frankia* strains at different geographical locations separated by a landmass exposed to different climatic regimes. The importance of evaluating molecular phylogeny of the isolated and identified *Frankia* strains from the host plant *H. salicifolia* D. Don and their comparison to reference strains isolated from other host plant species elsewhere may provide enormous information[2,25] regarding their probable clade of origin.

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Diversity of *phlD* alleles in the rhizosphere of wheat cropped under annual rice–wheat rotation in fields of the Indo-Gangetic plains: influence of cultivation conditions

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The antibiotic 2,4-diacetyphloroglucinol is a major determinant in the biocontrol of plant growth promoting rhizobacteria associated with crops of agronomic relevance. The *phlD* gene is a useful marker of genetic and phenotypic diversity of 2,4-DAPG-producing rhizobacteria. A two-step amplification procedure was developed in order to assess directly the presence of *phlD* in environmental DNA, avoiding the tedious procedure of *phlD*-positive strain screening and isolation. We found a predominance of one or two *phlD* alleles in wheat fields cultivated in rice–wheat rotations for twenty years, suggesting that continuous rice–wheat cropping would lead to an enrichment of particular *phlD* genotypes. We also recovered new sequences with no close relative among known *phlD* sequences, indicating that part of the *phlD* allelic diversity might have been missed using standard media culture conditions.

Keywords: 2,4-DAPG, wheat rhizosphere, rice–wheat, *phlD*.

THE limited incidence of soil-borne pathogens in the rice–wheat systems, is probably due to the repeated transitions

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