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Evolutionary implications of nucleotide sequence relatedness between *Alnus nepalensis* and *Alnus glutinosa* and also between corresponding *Frankia* microsymbionts

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Abstract

Frankia DNAs were isolated directly from root nodules of *Alnus nepalensis* and *Alnus nitida* collected from various natural sites in India. For comparison, a nodule sample from *Alnus glutinosa* was also collected from Tuebingen, Germany. Nucleotide sequence analyses of amplified 16S–23S ITS region revealed that one of the microsymbionts from *Alnus nepalensis* was closely related to the microsymbiont from *Alnus glutinosa*. A similar exercise on the host was also carried out. It was found that one sample of *Alnus nepalensis* was closely related to *Alnus glutinosa* sequence from Europe. Since both *Frankia* and the host sequences studied revealed proximity between *Alnus glutinosa* and *Alnus nepalensis*, it is hypothesised that the common progenitor of all the alders first entered into an association with *Frankia*, and the symbiotic association has evolved since.

Introduction

Alnus, commonly known as alder, belongs to the family Betulaceae comprising of six genera (*Alnus, Betula, Carpinus, Corylus, Ostrya* and *Ostrypsis*) (Lawrence, 1967) and about 130 species (Chen *et al*., 1999). Of these six genera, only *Alnus* is reported to symbiotically associate with the actinomycete *Frankia*. Forty-seven species of *Alnus* have been identified so far (Baker and Schwintzer, 1990) and only two of them (*nepalensis* and *nitida*) are found in India.

It was hypothesised that if the progenitor of genus *Alnus* had entered into an association with *Frankia* prior to diversification, the evolutionary pattern of *Frankia* and alders should have some similarity. That means that the two species of *Alnus* showing close proximity to each other should harbour closely related *Frankia* strains as well. The phylogenetic information generated for alders and *Frankia* was therefore used to determine if this was true.

Earlier investigations had shown genetic diversity in strains of *Frankia* isolated from nodules of *Alnus nepalensis* growing in India (Ganesh et al., 1994). The present work was initiated to investigate the differences at molecular level among some *Frankia* strains present in the nodules of Indian alders. Further, to establish the relationship between *Frankia* found in India and that found in Europe, a comparison was also made between Indian frankiae with one strain present in the nodules of *Alnus glutinosa* from Europe. We isolated *Frankia* DNAs from field-collected nodules and sequenced amplified ribosomal DNA sequences to establish molecular diversity and phylogenetic relationship.

To reconstruct phylogenetic relationships between the alder hosts, we used sequences of the 18S–28S Internally Transcribed Spacer (ITS) region, primarily because earlier workers had emphasised the utility of this region for inferring phylogenetic relationships at lower taxonomic levels (Chen et al., 1999; Savard et al., 1993). One hundred trees of *Alnus nepalensis* were investigated and a lot of variability was detec-

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ted on the basis of PCR-RFLP studies (Chauhan and Misra, 2002). Consequently, taking PCR-RFLP as a molecular criterion (Verghese and Misra, 2000) we chose three trees for the purpose of comparing with *Alnus glutinosa*. The 18S–28S ITS region for these three samples was sequenced, aligned with the sequences of other members of Betulaceae and analysed for phylogenetic inference.

Materials and Methods

Sample collection

Nodule samples were collected from different locations listed in Table 1. Trees were randomly selected and nodule sampling was done in three replicates from each location. Each nodule cluster comprised of more than 10 visibly active nodule lobes. With a view to include at least one other species of *Alnus* for a comparative study, nodules were also collected from *Alnus glutinosa* from Tuebingen, Germany. All samples were brought to the lab on ice. They were thoroughly washed with mild detergent and water, followed by several washes of double distilled water. Nodule surfaces were sterilised using H_2O_2 (5%) and nodules were stored at −20 °C.

For nucleotide sequence analysis of *Frankia*, samples were chosen based on the different sites and hosts. It was not possible to carry out nucleotide sequencing of all the samples collected. Therefore, five nodule samples, one each representing the three hosts, coupled with the different collection sites, were incorporated. It must be noted that these sequences may not represent the entire range of sequences present in the soil.

Similarly, three different samples were chosen based on the differences in the PCR-RFLP patterns (Chauhan and Misra, 2002) and sequenced for comparative study of *Alnus nepalensis* genotypes. It must again be noted that no claims are being made that these three samples represented all the different genotypes actually present in the area of collection.

Isolation of Frankia *DNA from nodules*

After the epidermis of the nodule lobes had been peeled off in PVPP/PBS [polyvinyl pyrrollidone (PVPP) 3% (w/v), phosphate buffer saline (PBS) 100.00 mL; PBS = NaCl−0.08% (w/v), NaH_2PO_4−0.12% (w/v), KH_2PO_4−0.02% (w/v), in water], one lobe per nodule cluster, was placed in 1.5 mL micro centrifuge tube. The nodule was crushed in 300 μL of extraction buffer [(Rouvier et al., 1996), 100 m*M* Tris base, 20 m*M* EDTA, 1.4 *M* NaCl, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 1% (w/v) PVPP, pH-8.0]. The resulting homogenate was incubated at 65 °C for 1 h and centrifuged for 10 min at 7000×*g*. The supernatant was first extracted with equal volume of phenol:chloroform (1:1; v:v) and then with chloroform:isoamyl alcohol (24:1; v:v) at 13 000×*g* at 4 °C for 30 min each. DNA from the aqueous phase was precipitated by addition of 2 volumes of ice cold ethanol followed by centrifugation at 13 000×*g* for 30 minutes at 4 °C. The pelleted DNA was washed twice with 70% ice cold ethanol, vacuum dried, re-dissolved in 10 μL of Tris buffer (Tris base- 50 m*M*, EDTA- 20 m*M*, pH- 8.0) and stored at −20 °C.

Isolation of Frankia *DNA from cultures*

Frankia strain ACN1^{AG} (kindly provided by Dr Philippe Normand, University of Claude Bernard, Lyon, France) was used for comparison of the nodule DNAs, both at the time of amplification reaction as positive control and subsequently for nucleotide sequence analysis. To isolate the total genomic DNA from this culture, the following protocol based on Simonet et al. (1985) was followed.

The cells were first washed in TE buffer to remove traces of culture medium. They were incubated in 1 mL of Tris buffer, with a pinch (equivalent to approximately 500 units) of achromopeptidase, for 60 min at 37 °C. Then 10 μL of 10% (w/v) sodium dodecyl sulphate (SDS) were added and the cell suspension was further incubated at 70 °C for 15 min. An equal volume of buffer equilibrated phenol was added to the lysate and it was centrifuged at 7000×*g*. An equal volume of chloroform was added to the aqueous phase. It was centrifuged for 30 min at room temperature. Two volumes of isopropyl alcohol were added to the aqueous phase and kept over night at room temperature followed by centrifugation at 13 000×*g* for 30 min. The pellet was vacuum dried and dissolved in 10 μL of ultra pure water.

Isolation of genomic DNA from alder leaves

Young leaves were surface sterilised with 30% H_2O_2 and thoroughly rinsed with ultra pure water. Care was taken to avoid any leaves showing signs of fungal in-

Table 1. Nodule samples and sites of their collection for studies on *Frankia* sequences

Country	Province	Site	Host	Code assigned
India	Arunachal Pradesh	Hapoli	*Alnus nepalensis*	AnpHR
	Meghalaya	Upper Shillong	*Alnus nepalensis*	AnpUSR
		Nonkrem Hills	*Alnus nepalensis*	AnpNHR
	Tamilnadu	Ooty	*Alnus nepalensis*	AnpOR
	Himachal Pradesh	Kulu	*Alnus nitida*	AntKR
Germany		Tuebingen	*Alnus glutinosa*	AgTR

fection. Two leaves were crushed in 1 ml of warm extraction buffer [1 *M* Tris HCl, 1.4 *M* NaCl, 0.5 *M* EDTA, 2% (w/v) CTAB and 3% (w/v) PVPP, pH 8.0]. The macerate was filtered through cotton into a 1.5 mL micro-centrifuge tube and centrifuged for 5 min at 8000×*g* at room temperature. The supernatant was discarded and the pellet re-suspended in 300 μL of the extraction buffer. Ten μL of 20% (w/v) SDS were added, gently mixed and the homogenate incubated in a water bath at 65 °C for 1 h. It was then centrifuged at 12 000×*g* for 15 min. The supernatant was transferred to a fresh tube and extracted with equal volume of chloroform:isoamyl alcohol (24:1;v:v). After centrifugation at 12 000×*g* for 15 min, the aqueous phase was transferred to a fresh tube and 30 μL of 3 *M* sodium acetate (pH 5.2) and 1.2 mL of ice cold ethanol were added to precipitate the DNA. The DNA was pelleted by centrifugation at 13 000×*g* for 30 min at 4 °C. The pellet was washed 2–3 times, with ice cold 70% ethanol and vacuum dried. DNA was dissolved in 10 μL of ultra pure water and stored at −20 °C for further use. Two μL of the isolated DNA from each sample was tested for purity by agarose gel electrophoresis.

Amplification of Frankia *DNA*

Amplification reactions were carried out for two DNA regions, partial 16S rDNA and 16S–23S rDNA ITS. Amplification was performed using primers FGPS 989ac (Table 2, Bosco et al., 1992) and FGPS 1509' (Table 2, Navarro et al., 1992) for the partial 16S rDNA region, and primers FGPS 989ac and FGPL 2054′ (Table 2, Simonet et al., 1991) for the 16S–23S rDNA ITS region. Primer FGPS989ac is specific to *Frankia* compatible to genera *Alnus* or *Casuarina* (Bosco et al., 1992), therefore, the amplification of host plastid DNA was not likely. DNA isolated from the strain ACN1^{AG} was used as positive control.

Table 2. Oligonucleotide primers used for amplification and nucleotide sequencing of *Frankia* and *Alnus* DNAs.

Primers	Sequence
Frankia	
FGPS989ac	5′GGGGTCCGTAAGGGTC3′
FGPS1509′	5′AAGGAGGGGATCCAGCCGCA3′
FGPL2054′	5′CCGGGTTTCCCCATTCGG3′
M13F	5′GATGGATATCTTGGTTC3′
M13R	5′CAGGAAACAGCTATGAC3′
RFRITF3	5′CCGTCACGTCACGAAAGTCG3′
RFRITR2	5′GCTCGCGTCCACTGTGCAG3′
Alnus	
ITS1-PLANT	5′CGCGAGAAGTCCACTG3′
ITSC26A	5′GTTTCTTTTCCTCCGCT3′

Amplification was carried out in a total volume of 25 μL per tube. Each reaction mixture contained 3 μL of each primer (5 pM), 2.5 μL of 10×PCR buffer (Bangalore Genei, India), 2.5 μL of each dNTP (1.25 m*M*), 0.3 μL of *Taq* polymerase (5 units/μL, Bangalore Genei, India) and 1 μL of template DNA. Amplification was carried out for 35 cycles. Each cycle comprised of 1 minute of denaturation at 94 °C, 1 min of annealing at 53 °C for partial 16S rDNA and 49 °C for the ITS regions respectively, and 1 min of elongation at 72 °C. The PCR products were checked by electrophoresis using 0.8% agarose gel.

Amplification of Alnus *DNA*

The 18S–28S ITS region was amplified using universal primers ITS1-PLANT (Table 2, Normand, personal communication) and ITSC26A (Table 2, Wen and Zimmer, 1996). PCR amplifications were performed in

a 25 μL mix containing 2.5 μL of each of the primers from a 0.5 mM stock, 2.5 μL of 10×PCR buffer, 2.5 μL of the dNTP mix (1.25 m*M*) and 0.3 μL of *Taq* Polymerase (5 units/μL). One μL of the appropriate sample DNA was added to each tube just before starting the PCR run. The remaining volume was made up with ultra pure water. A negative control without the template DNA was also included in each PCR reaction. Each amplification reaction was run for 35 cycles and each cycle comprised of 30 s of denaturation at 94 °C, 30 s of annealing at 45 °C and 30 s of elongation at 72 °C. This was followed by an extension time of 3 min at 72 °C at the end of the run.

Sequencing of Frankia *amplicons*

Only five representative samples, representing different sites from India, were randomly selected for sequencing (see the section on sampling). One sample was sequenced as a representative of the microsymbiont found in *Alnus glutinosa*. Before sequencing, the PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Germany). An M13 cloning vector was used for sequencing the 16S–23S rRNA ITS and the sequencing was carried out by the dideoxy chain termination technique (Sanger et al., 1977) using T7 sequencing kit (Pharmacia). The primers used for sequencing studies are listed in Table 2.

Sequencing of Alnus *amplicons*

Three samples representing different PCR-RFLP profiles were selected for phylogenetic analysis (see the section on sampling). For this, after agarose gel electrophoresis, the band of interest was excised out and purified using the protocol of Byrnes et al. (1995). These samples were then sequenced commercially by M/s Bangalore Genei, India, using an automated DNA sequencer that used fluorescent label dye terminators or fluorescent label primers. The primers used for sequencing were the same used for amplification of DNA (Table 2). Each primer was used singly in a sequencing cycle.

Nucleotide sequence analysis of Frankia *amplicons*

The nucleotide sequence of each sample was entered into the computer program DNASIS (version 7.0). The sequences (Table 3) retrieved from the GenBank database using BLAST search were aligned with the studied sequences using multiple sequence alignment program CLUSTAL W (version 1.74; Thomson et al., 1994). The software PHYLIP (version 3.5c; Felsenstein, 1993) was used for inferring phylogenetic relationships. DNADIST was used to calculate evolutionary distance with Kimura 2 parameter ratio of 2. The NEIGHBOR JOINING method reconstructed phylogenetic trees from the evolutionary distance data. Parsimony trees were reconstructed with DNAPARS. The reliability of the clustering pattern obtained in the trees drawn was assessed by 100 bootstrap replicates. The trees were re-done using online (http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html) latest version of Phylip and were downloaded too from Phylip' s home page, http://evolution.genetics.washington.edu/phylip.html. They were rechecked with other online tools for phylogenetic analysis including GeneBee: Molecular biological server http://www.genebee.msu.su/index.html.

Nucleotide sequence analysis of Alnus *amplicons*

Alnus sequences were used for the Blast search of the GenBank. The sequences used for phylogenetic analysis are listed in Table 4. The sequences that were retrieved from the GenBank and our sequences were then aligned together using the multiple sequence alignment program CLUSTAL W (version 1.75, Thomson et al., 1994). Neighbour Joining trees were constructed using the program PHYLODENDRON (version 0.8d beta, Gilbert, 1999). 1000 bootstrap replicates were considered. The program DNADIST of the PHYLIP (version 3.5c; Felsenstein, 1993) inference package was used to calculate evolutionary distances with Kimura-2-parameter having a transition/transversion ratio of 2.0. Parsimony analysis was carried out using the program DNAPARS of the same inference package. To be confident of the parsimony trees constructed, 500 bootstrap replicates were considered.

Results and Discussion

Sequence analyses

The numbers of samples presented here is small but have been collected from their natural habitat representing different geographic locations, and hence can be considered as representative of different sites. Although they may not represent the entire range of

Table 3. Sequences of bacterial strains and nodule microsymbionts used for the 16S rDNA phylogenetic analysis

Sequence/Host	Accession Number	Source/Reference
AnpHR/*Alnus nepalensis*	AJ404866	Present study
AnpNHR/*Alnus nepalensis*	AJ404867	Present study
AnpOR/*Alnus nepalensis*	AJ404868	Present study
AnpUSR/*Alnus nepalensis*	AJ404869	Present study
AntKR/*Alnus nitida*	AJ404870	Present study
AgTR/*Alnus glutinosa*	AJ404871	Present study
ACN1^{AG}/*Alnus* sp.	-	Present study
Rugosa/*Alnus rugosa*	L40956	Normand et al., 1996
Dryas/*Dryas drummondii*	L40616	Normand et al., 1996
Myrica/*Myrica nagi*	L40622	Normand et al., 1996
DARJN/*Alnus nepalensis*	U60284	Misra and Normand, 1997*
AVN17S/*Alnus* sp.	L40613	Normand et al., 1996
ArgP5/*Alnus rugosa*	L40612	Normand et al., 1996
ACN14a/*Alnus crispa*	M88466	Johnson, 1992*
ORS020606/*Casuarina* sp.	M58598	Normand et al., 1992
HR27-14/*Hippophae rhamnoides*	L40617	Normand et al., 1996
SCN10a/*Shepherdia canandensis*	L40619	Normand et al., 1996
PtI1/*Purshia tridentata*	L41048	Normand et al., 1996
M.ratis/*Mycobacterium ratisbonense*	AF055331	Reischl et al., 1998*

*Sequences are direct submissions to the GenBank and are available at the site http://www.ncbi.nlm.nih.gov.

Table 4. Sequences of 18S-28S ITS region used for phylogenetic analysis of Betulaceae

Genus & species	Accession Number	Source/Reference
Alnus nepalensis (S.1)	-	Present study
Alnus nepalensis (S.2)	-	Present study
Alnus nepalensis (S.3)	-	Present study
Alnus matsumurae	-	Normand (Personal Communication)
Alnus glutinosa	AF081529	Chen et al., 1999
Alnus incana	X68138	Savard et al., 1993
Alnus crispa	X68137	Savard et al., 1993
Alnus maritima	X68135	Savard et al., 1993
Betula glandulosa	X68134	Savard et al. ,1993
Betula pendula	AJ006445	Leskinen E, 1999*
Carpinus hupeana	AF081521	Chen et al., 1999
Carpinus turczaninowii	AF081518	Chen et al., 1999
Corylus chinensis	AF081520	Chen et al., 1999
Corylus heterophylla	AF081519	Chen et al., 1999

*Sequences are direct submissions to the GenBank and are available at the site http://www.ncbi.nlm.nih.gov.

Frankia strains present in the soil, they do take into consideration at least one sample each from the three different species of *Alnus* mentioned above. The nucleotide sequences for the six samples studied have been submitted to the EMBL Nucleotide Sequence Database. The accession numbers assigned are listed in Table 3. Sequence analysis of partial 16S and 23S rRNA genes (520 bases at 3′ end of 16S rRNA gene and 132 bases at 5′ end of 23S rRNA gene) from the analysed samples confirmed the conserved nature of the ribosomal RNA gene for these samples from *Frankia* nodulating the three *Alnus* species. However, when compared with the *Frankia* strain ORS020606 (retrieved from GenBank, Table 3), differences were found for 16S rRNA gene and 23S rRNA gene. The 3′ end of 16S rDNA is the most conserved part of the gene.

Sequence analyses of the 16S–23S rDNA ITS region revealed variations, with one exception (AnpUSR). The ITS sequence analyses gave only four genotypes for the seven samples analysed. All the samples, except AnpUSR, analysed from *Alnus nepalensis* (AnpOR, AnpNHR and AnpHR) grouped together with *Frankia* isolate ACN1^{AG}. All these samples had similar sequences and exhibited 102 nt differences compared to ACN1^{AG} strain used as reference. It may be pertinent to mention that the higher number of differences observed were due to the presence of more variable ITS region in the amplified fragment. In contrast to this, the 16S rDNA analysis showed differences that only differentiated AnpNHR (2 nt) and ACN1^{AG} (1 nt) samples.

AnpUSR and AgTR showed 100% sequence similarity for the analysed region of 16S rDNA, although they were obtained from two different *Alnus* species. However, for these samples, differences were found in the ITS region (76 nt). Besides this, two differences were also seen in 23S rDNA. Further, AntKR sample, obtained from *Alnus nitida*, showed differences for bases both in the coding and the non-coding regions. The 16S–23S rRNA ITS of this sample showed a long stretch of inserted nucleotides (15 nt).

Sequencing of the *Alnus nepalensis* sample with primer ITS1-PLANT gave sequence information of about 130–140 bp including the distal region of the 18S rRNA gene and part of ITS I. While the size of the PCR product was approximately 750 bp, the sequence obtained was only about 130 bp. It was likely that the enzyme stuttered and failed to proceed further due to the presence of several GC residues around 130 bp. We, therefore, used the reverse primer

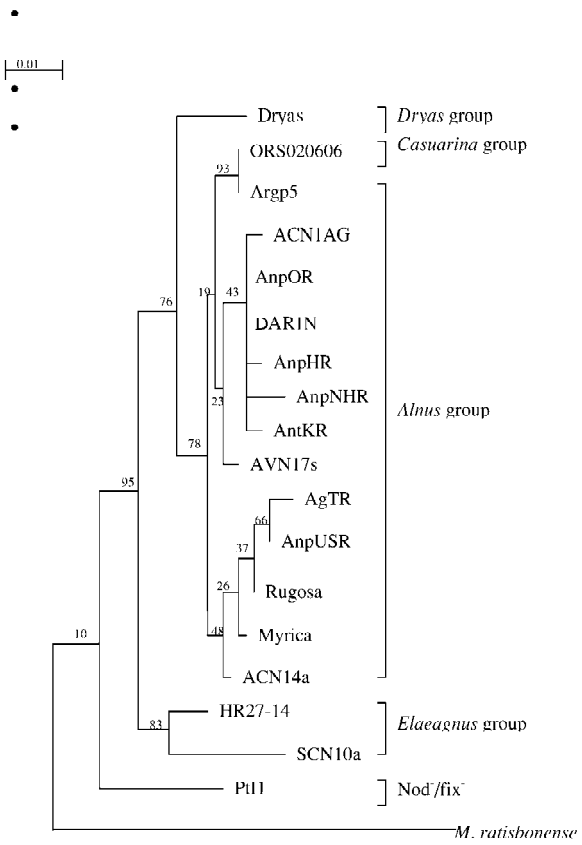

Figure 1. Neighbour-joining consensus tree for aligned sequences of the distal part of the 16S rRNA gene of *Frankia*. The numbers at the forks correspond to the bootstrap values out of 100.

(primer ITSC26A) along with DMSO to sequence the three samples. The primer ITSC26A is located in the initial part of the 28S rRNA gene and therefore, should give sequence information of the opposite strand i.e., the strand complementary to the one sequenced earlier. A computer simulated analysis of the sequence of the 18S–28S ITS region of *Alnus matsumurae* (kindly provided by Dr Normand) was done using Mac Vector® software. This analysis revealed that 413 bp downstream the start of the sequence of this region, an alternate annealing site for this primer was present on the positive strand. This gave us sequence information for about 467, 318 and 200 bp, respectively, for the three samples. The sequence obtained included complete ITS II and the initial part of the 28S rRNA gene.

Phylogenetic inferences for the microsymbiont

The phylogenetic analyses of the microsymbionts and related organisms based on the partial 16S-rDNA sequences were conducted using neighbour-joining and parsimony analyses. The trees obtained via both neighbour-joining (Figure 1) and DNA parsimony (tree not presented here) analyses, showed similar topology, clustering together of strain/microsymbionts from *Alnus* species (Betulaceae) with those from *Casuarina* sp. (Casuarinaceae) and *Myrica* sp. (Myricaceae). The topology of the tree obtained was similar to those deduced by Normand et al. (1996) and Clawson et al. (1998).

The distant relationship of the AnpUSR with other nodule samples studied from India was clearly reflected in its rDNA sequence. In the trees drawn, it clustered together with AgTR as close neighbour followed by *Myrica*, *Rugosa* and ACN14a. The SCN10a and HR27-14 strains from the *Elaeagnus* group formed a separate cluster of their own. This agrees with the inference made by Nazaret et al. (1991) who showed the close relationship of *Casuarina* strains with the *Alnus* infectivity group. They also found that the *Alnus* infectivity and the *Casuarina* infective groups had greater distance from *Elaeagnus* infectivity group. Further, *nod*$^{-}$/*fix*$^{-}$ PtI1 strain was placed distantly in the tree (Figure 1).

The sequence information of the ITS gave a general view of relationship between the *Frankia* nodule microsymbionts studied. The obtained sequences were aligned with the corresponding sequence of ORS020606, a *Casuarina* infective strain. The most variable region of 450 bases incorporating the 420 bases of the ITS, was taken for the comparative sequence analysis. The samples studied joined the *Alnus* infective group. Both neighbour-joining and parsimony (trees not presented here) analyses showed that the nearest neighbour for the nodule microsymbiont AnpUSR was AgTR.

Thus, the microsymbiont from nodules collected from Upper Shillong, clustered away from the other nodule samples analysed from the same host (*Alnus nepalensis*). The evolutionary separation of AnpUSR from the other samples was reflected in the PCR-RFLP pattern (data not presented here) that was confirmed by rDNA sequence analysis. This is in accordance with the observation made by Ganesh et al. (1994) who suggested the possibility of existence of genetically diverse *Frankia* strains in this region. It is surprising that the microsymbiont AnpUSR showed close relationship with AgTR sample and clustered far away from the other Indian samples. It suggests that AnpUSR could be a distinct *Alnus* infective *Frankia* strain with a distinct genetic history. It may be mentioned that we have studied only one sample from Germany. Additional information may become available if more samples are studied. We are only emphasising here the similarity detected by us.

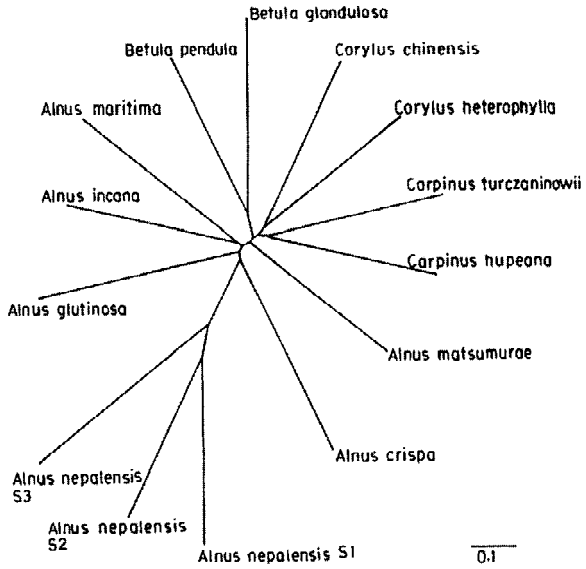

Figure 2. Neighbour joining Phylogenetic tree constructed using aligned sequences of the initial part of the 28S rRNA gene and part of ITSII of alder. The tree has been selected after considering 1000 bootstrap replicates. 0.1 represents the substitution rate per 100bp.

Phylogenetic analysis of alder using Neighbour joining method

For this analysis, sequences of the distal part of 18S rRNA gene and a part of ITS I and the ITS II and proximal part of the 28S rRNA gene were used separately. Bootstrap analysis was used to assess the robustness of the tree.

The tree constructed using the aligned sequences of the distal part of 18S rRNA gene and a part of ITS I (tree not presented here) showed that all the genera studied had a common ancestor. These observations suggest a monophyletic origin of the family Betulaceae, supportive of the reports of Chen et al. (1999). From the point of origin, trifurcations were apparent. One of the branches further bifurcated into two giving rise to *Corylus* and *Carpinus*. These two genera formed a single clade. The second branch gave rise to *Betula* lying in close proximity to the alders,

which originated from the third branch. It is likely that *Betula* and *Alnus* belonged to a single clade. Among the alders a clear differentiation of two subclades was apparent. One of these included all the alders of the eastern region while the other included those of the western region. Hence, the tree showed a distinct demarcation of the alders based on their geographic distribution. Apparently this region of the 18S–28S *rrn* operon diversified significantly after the geographic isolation of the alders.

The topology of the tree constructed using the aligned sequences of the ITS II and the initial part of the 28S rRNA gene (Figure 2) did not show distinct demarcation of the alders based on geographic distribution. Hence, it is likely that this region of the 18S–28S *rrn* operon did not diversify substantially after the separation of the land masses. This tree also supported the hypothesis of common ancestry. *Corylus* and *Carpinus* grouped together as a single group, more appropriately termed a clade. The two species of *Betula* and those of *Alnus* clustered together as one clade. Apparently, during the course of evolution after the origin of *Betula*, the branch divided at regular intervals diversifying into the different species of *Alnus*. *Alnus matsumurae* appeared closest to the non-actinorhizal genus *Betula*. However, all the three samples of *Alnus nepalensis* clustered together. It was also visible that *Alnus nepalensis* had evolved more compared to *Alnus matsumurae* because it was more distant from the point of origin.

Phylogenetic analysis of alder using Parsimony method

Two most parsimonious trees were separately derived from the sequences of the distal region of the 18S rRNA gene (including a part of ITS I) and the sequences of ITS II (including the proximal part of the 28S rRNA gene). Both trees were more or less similar except for a few minor differences (trees not presented here). *Alnus matsumurae* again appeared closest to the non-nitrogen fixing genus *Betula*. The three samples of *Alnus nepalensis* clustered together and the European alder *Alnus glutinosa* appeared closest to *Alnus nepalensis*. Since we report above the similarity between *Frankia* DNA sequences from *Alnus nepalensis* and *Alnus glutinosa* root nodules, a common origin of the frankiae found associated with both these species of *Alnus* is likely.

From the observations made in the present study it is evident that all the studied members of the family Betulaceae originated from a single progenitor. This trifurcated into *Alnus*, *Betula* and *Carpinus-Corylus* branches. It is difficult to say whether this common progenitor had the ability to host *Frankia* or not. It is more likely that this symbiotic association developed after the trifurcation. Swensen (1996) had suggested multiple origins of the nitrogen fixing symbioses.

Parsimony analysis suggested a close relationship between *Alnus nepalensis* and *Alnus glutinosa*. Apparently our sample 3 of *Alnus nepalensis* represented earlier genotypes closer to the alder progenitor which first entered into the actinorhizal association. Not only the *Frankia* genotypes have diversified since, the *Alnus nepalensis* genotypes also have diversified considerably.

In concluding, we found close similarity between an Indian strain and a German strain of *Frankia* for the DNA region studied. Parsimony analysis showed similar results for the alder trees belonging to *nepalensis* and *glutinosa* species. This finding is in line with our initial hypothesis. It is possible that the proximity between the two microsymbionts found by us could be due to co-evolution of the host and the microsymbiont. We are aware that co-evolution would imply a gene-to-gene relationship between the host and the microsymbiont. In such a case, the alder *Frankia* should not infect other plants. We have not tested this. The evidence presented here is only preliminary in nature and thus this contention needs confirmation by further experiments with improved sampling strategy.

At this stage it would be speculative to say whether the common ancestor of AnpUSR and AgTR belonged to India or elsewhere (say Far East). The differences we observed are based on a limited number of sequences studied. It is quite likely that many more variations are detected in a more exhaustive study. But the fact that diversity was detected in such a small area of India does indicate that the alder compatible frankiae have been evolving in India for several million years. There is a possibility of alder compatible frankiae reaching Europe from India or through India.

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