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Direct amplification of *nodD* from community DNA reveals the genetic diversity of *Rhizobium leguminosarum* in soil

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Summary

Sequences of *nodD*, a gene found only in rhizobia, were amplified from total community DNA isolated from a pasture soil. The polymerase chain reaction (PCR) primers used, Y5 and Y6, match *nodD* from *Rhizobium leguminosarum* biovar *trifolii*, *R. leguminosarum* biovar *viciae* and *Sinorhizobium meliloti*. The PCR product was cloned and yielded 68 clones that were identified by restriction pattern as derived from biovar *trifolii* [11 restriction fragment length polymorphism (RFLP) types] and 15 clones identified as *viciae* (seven RFLP types). These identifications were confirmed by sequencing. There were no clones related to *S. meliloti nodD*. For comparison, 122 strains were isolated from nodules of white clover (*Trifolium repens*) growing at the field site, and 134 from nodules on trap plants of *T. repens* inoculated with the soil. The nodule isolates were of four *nodD* RFLP types, with 77% being of a single type. All four of these patterns were also found among the clones from soil DNA, and the same type was the most abundant, although it made up only 34% of the *trifolii*-like clones. We conclude that clover selects specific genotypes from the available soil population, and that *R. leguminosarum* biovar *trifolii* was approximately five times more abundant than biovar *viciae* in this pasture soil, whereas *S. meliloti* was rare.

Introduction

Rhizobia are alpha-proteobacteria that form a nitrogen-fixing symbiosis with the roots of legumes. The bacteria can be readily isolated from the interior of the root

nodules, where they often form an almost pure culture. Because this is very convenient, nearly all studies of rhizobia have started from cultures derived from root nodules. This includes numerous studies of genetic diversity, using methods such as enzyme electrophoresis (Young, 1985; Young *et al.*, 1987; Harrison *et al.*, 1989; Leung *et al.*, 1994), restriction fragment length polymorphism (RFLP; Young and Wexler, 1988; Kajjalainen and Lindström, 1989; Demezas *et al.*, 1991) or polymerase chain reaction (PCR)-based techniques (Harrison *et al.*, 1992; Amarger *et al.*, 1994; Laguerre *et al.*, 1994; Leung *et al.*, 1994; Strain *et al.*, 1994; Eardly *et al.*, 1995; Haukka *et al.*, 1996; Turner *et al.*, 1996). We have learned a great deal from this work, but it must be recognized that the diversity sampled in this way has been 'filtered' by the plant hosts. The interaction between the bacteria and the plant is complex and specific, so that the representation of different bacterial genotypes among isolates from nodules may be quite different from their abundance in the soil population.

In order to describe the soil population of rhizobia from which the plant has made its selection, it is necessary to bypass the nodules and look directly in the soil. Unfortunately, free-living rhizobia have few distinctive properties, so it has not in general been possible to devise a really effective selective medium to separate them from other soil bacteria. Nevertheless, there has been considerable interest in this question, and many successful protocols have been devised, although most are restricted to particular bacterial species (Segovia *et al.*, 1991; Laguerre *et al.*, 1993; Bromfield *et al.*, 1995; Louvrier *et al.*, 1995; 1996; Sullivan *et al.*, 1996; Hartmann *et al.*, 1998).

One general conclusion of such studies is that there is often a considerable population of bacteria that are similar in genetic background to the nodulating rhizobia but lack the genes necessary for the symbiosis. Even among the bacteria that do possess the symbiosis genes, the host plant can still be very selective. This has been demonstrated in numerous competition studies, in which the relative occupation of nodules is determined after a host is provided with strains mixed in known proportions (e.g. Sessitsch *et al.*, 1998). Such studies are inevitably artificial, in that the mix of strains available to the plant can only be known if it is provided by the experimenter.

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When the inoculum is a natural soil population, it is not usually possible to determine whether the strains that form the majority of the nodules do so because they are particularly competitive or because they are abundant in the soil population. It would be of considerable interest to be able to estimate their frequency in the population available to the plant. Although rhizobia are certainly not 'unculturable', an approach that does not require culturing would be advantageous, because it is so hard to isolate them selectively from the general microbial population of the soil. In recent years, many studies have described the diversity of microbial communities by amplifying a particular class of genes from a DNA extract of the whole community. Early work (Giovannoni *et al.*, 1990; Ward *et al.*, 1990) concentrated on aqueous habitats, from which clean DNA can easily be extracted, but reliable methods have also been developed for extracting DNA from soil (Zhou *et al.*, 1996; Liu *et al.*, 1997). The main emphasis has been on ribosomal RNA genes, because they are universal and allow easy taxonomic identification. Other genes have also been targeted, including enzymes for nitrogen fixation (Kirshtein *et al.*, 1991) and methanotrophy (McDonald *et al.*, 1995) and a number of genes associated with mobile genetic elements (Smalla *et al.*, 2000).

We have developed a method to characterize the soil population of rhizobia that depends on the fact that the genes responsible for initiating the symbiosis, the *nod* genes, are found in all rhizobia, but the nearest homologues known in other bacteria are very distantly related. PCR primers that match *nod* gene sequences can be used, with soil bacterial DNA as a template, to amplify sequences that must come from rhizobia, and the specific origin of these sequences can be identified by comparing them with the extensive database of published *nod* gene sequences. We have compared the diversity of *nodD* sequences amplified from a soil sample with that of a set of isolates of *Rhizobium leguminosarum* biovar *trifolii* obtained from root nodules of white clover, *Trifolium repens*, inoculated with the same soil.

Results

RFLP of rhizobia isolated from clover nodules

Ten plants of white clover (*Trifolium repens*) were dug up from each of two patches in the permanent grassland of Walmgate Stray, York, and a single rhizobium isolate was purified from each root nodule. The number of isolates per plant varied from three to 12. PCR-RFLP of the *nodD* gene region revealed four distinct *AluI* digest patterns (Fig. 1). The frequency of the different patterns was similar on each plant, and there was no significant difference in the frequencies at the two sites (Table 1).

Table 1. Numbers of *R. leguminosarum* isolates, classified by *AluI* RFLP of the *nodD* region, obtained from clover plants growing at two grassland sites and from trap plants inoculated with soil from one of the sites.

Source of nodules	<i>nodD</i> RFLP types					Total
	A	B	C	D	NA ^a	
Plants at field site I	42	10	2		2	56
Plants at field site II	39	16	1	1	9	66
Plants from I with soil I	55	4	1	1		61
Plants from II with soil I	60	10	1	2		73

a. No amplification with NBA12/NBF12' primers, but positive with Y5/Y6 (see text).

Overall, type A was by far the most abundant, making up 66% of the sample, with 21% of type B and small numbers of types C and D and of strains that yielded no amplification product with the NBA12/NBF12' primers. The presence of *nodD* in these latter strains was confirmed by PCR using the Y5/Y6 primers, which are internal to the *nodD* gene. It is not known why amplification does not occur with NBA12/NBF12', but it is likely that there is a genetic rearrangement of the region around *nodD*. A similar phenomenon has been observed in *R. leguminosarum* biovar *viciae* (L. A. Mutch and J. P. W. Young, unpublished).

An analysis of plant diversity based on random amplified polymorphic DNA (RAPD, results not shown) indicated that all 10 clover plants taken from each site were almost identical genetically, but there were some differences between plants from the two sites. In order to test whether there were genetic differences in strain preference, sterile cuttings of clover plants originating from sites I and II were inoculated with soil taken from site I. Rhizobia were isolated from the resulting nodules and typed as before. There were no significant differences in RFLP frequency between the isolates trapped by the plants from the two sites, or between these isolates from trap plants and the isolates originally obtained from the field (Table 1). Again, type A predominated and type B made up the majority of the remainder.

RFLP of nodD clones from soil community DNA

Total DNA was extracted from a subsample of the same soil from site I that was used for the trap plant experiment described above. PCR using primers Y5 and Y6, which match sites internal to the *nodD* gene, gave a band of 850 bp, the expected size. There was also a minor band of around 300 bp, presumably reflecting amplification of some non-target sequence, as such a band is not seen when pure cultures are used. After random cloning of the PCR product, inserts varying in size from 300 to 1000 bp

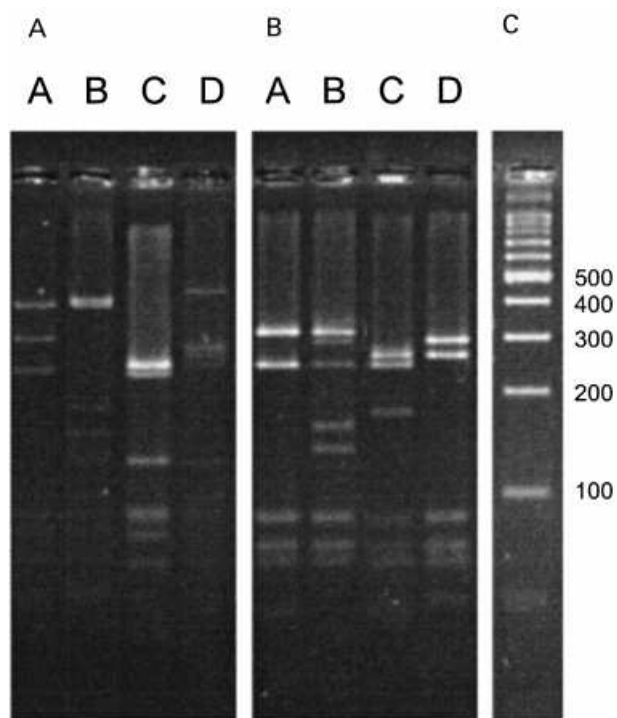


Fig. 1. *AluI* restriction digests of PCR products of the *nodD* region amplified using NBA12/NBF12' primers (A) and Y5/Y6 primers (B). The templates were DNA from nodule isolates representing RFLP types A, B, C and D as indicated. C. Molecular size standards, 100 bp ladder.

were obtained. In order to identify genuine *nodD* clones, inserts from 179 randomly chosen clones were amplified, electrophoresed, blotted and incubated with a *nodD* probe; 132 clones had inserts that hybridized with various signal intensities. Of these, 83 appeared to have a single copy of the expected size.

The 83 putative *nodD* clones were classified by restriction digestion with *AluI*. Eighteen RFLP patterns were found (Table 2). Four of the more abundant types (represented by 23, eight, five and seven clones respectively) had patterns that corresponded to the types A, B, C and D found among nodule isolates (Fig. 1; note that the Y5/Y6 fragment amplified in the soil DNA experiment is internal to the NBA12/NBF12' fragment amplified from the nodule isolates).

Phylogeny of *nodD* sequences

Examples of the four RFLP types identified among nodule isolates and the 18 RFLP types found in clones from soil were sequenced (EMBL/GenBank accession numbers AJ306460–AJ306480). Phylogenetic analysis (Fig. 2) places them in two clear clades that include published *nodD* sequences from *R. leguminosarum* biovar *trifolii* and

R. leguminosarum biovar *viciae* respectively. Soil clones 1, 2, 3 and 4 are similar in sequence to examples of RFLP types A, B, C and D, respectively, confirming the assignment based on restriction patterns (Table 2). Soil clones 5–11 also fell in the *trifolii* clade. On the other hand, soil clones 12–16, which were all closely related, grouped unambiguously with the two published sequences for biovar *viciae* (Fig. 2). Altogether, 68 clones had RFLP patterns represented in the *trifolii* clade, whereas 15 had patterns characteristic of *viciae*.

Discussion

The diversity of rhizobia from nodules

The work of Chanway *et al.* (1989) on the three-way interaction of *Lolium perenne*, *T. repens* and *R. leguminosarum* biovar *trifolii* suggests that rhizobium and clover genotypes might vary in a correlated fashion in a long-established pasture such as the one we sampled. In fact, however, the proportions of *nodD* genotypes were similar among the isolates from the two sites in the field, and also among the isolates from trap plants regardless of the plant genotype (Table 1). Admittedly, the level of polymorphism detected by the *AluI* digest is limited, but the good correlation between RFLP and sequence similarity (Fig. 2) indicates that this polymorphism is an accurate reflection of the underlying level of DNA sequence diversity at the *nodD* locus. This conclusion is consistent with the study by Laguerre *et al.* (1996), who used the

Table 2. Numbers of *nodD* clones from soil community DNA, classified by *AluI* RFLP.

RFLP type	Number of clones	Equivalent isolate type
<i>Biovar trifolii</i>		
1	23	A
2	8	B
3	5	C
4	7	D
5	12	
6	7	
7	2	
8	1	
9	1	
10	1	
11	1	
<i>Biovar viciae</i>		
12	7	
13	2	
14	1	
15	1	
16	1	
17	2	
18	1	

The putative *R. leguminosarum* biovar of origin and the equivalence to the RFLP classes identified among isolates from clover nodules are based on the sequence similarities illustrated in Fig. 2.

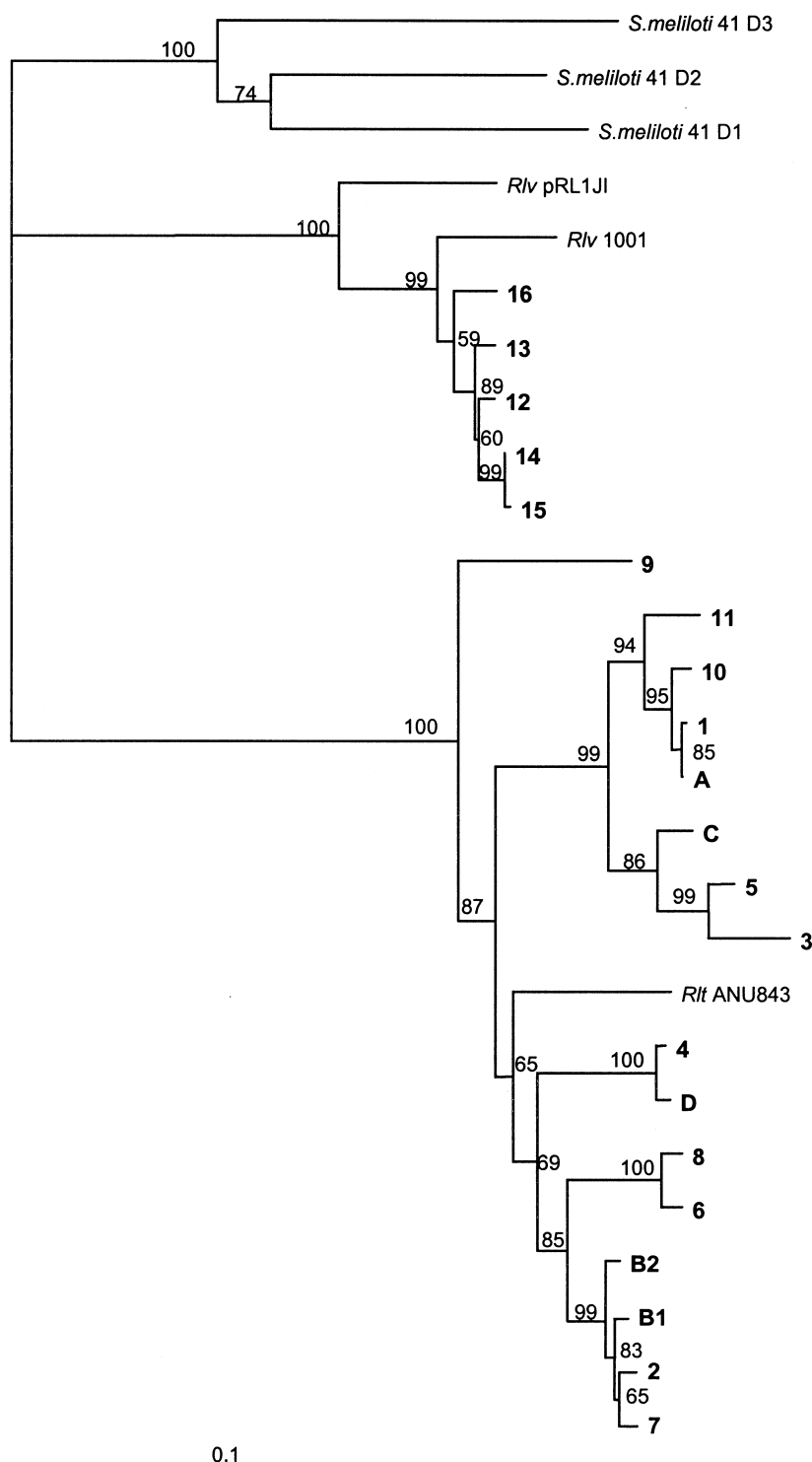


Fig. 2. Phylogenetic tree of *nodD* sequences representing RFLP types amplified from nodule isolates (A, two examples of B, C and D; see Table 1) or amplified and cloned from soil DNA (1–16, see Table 2). Accession numbers of published sequences used for comparison are X03721 (*R. leguminosarum* biovar *trifolii* ANU843 *nodD*), Y00548 (*R. leguminosarum* biovar *viciae* pRL1JI *nodD*), J03671 (*Rlv* 1001 *nodD*), X04473 (*S. melliloti* 41 *nodD*1), X04474 (*S. melliloti* 41 *nodD*2), X53820 (*S. melliloti* SU47 *nodD*3). The tree was constructed by the neighbour-joining method, and bootstrap values based on 1000 replicates are shown. The scale bar represents 0.1 substitutions per nucleotide.

same PCR primers and restriction enzyme and found that six out of 11 strains of *R. leguminosarum* biovar *trifolii* had a single RFLP pattern, and only minor differences were revealed by seven additional restriction enzymes. If we had sampled loci that were more polymorphic, it is possible that we would have picked

up differences in frequency reflecting site or host effects. There are some reports of differences in preference among *T. repens* cultivars (Russell and Jones, 1975; Roughley *et al.*, 1976; Jones and Hardarson, 1979), but in other cases there was little effect (Roughley *et al.*, 1976; Harrison *et al.*, 1987).

Comparison of soil and nodule populations

The contrast between the diversity of *nodD* types in the soil (Table 2) and in the nodule populations (Table 1) is all the more striking because of the relative uniformity of the various samples from nodules. All four of the RFLP types found in nodules were also recovered in clones recovered from the soil community DNA, which confirms that the cloning approach really is sampling the *R. leguminosarum* biovar *trifolii* population. However, the frequency of type A was only 34% (23/68) among the clones of the *trifolii* clade, significantly lower than the 77% (196/256) in nodules. Only types A, B, C and D were found in nodules but, even if the analysis is restricted to these four types, the frequency of type A was much higher in the nodule sample than among soil clones, where it was 53% (23/43). Thus, we must conclude that above-average competitiveness for nodulation is responsible in part for the high frequency of type A among nodule isolates. In fact, we can estimate that a cell of type A is 2.4 times more likely to form a nodule than a cell of types B, C or D $[(196/23)/(60/20) = 2.4]$.

The clones from soil also included a number of RFLP types (types 5–11) that were not found in nodule isolates, but clearly clustered with biovar *trifolii* on the basis of DNA sequence. The strains that these clones represent were clearly unable to compete successfully for nodulation of *T. repens* either in the field or in the trap experiment. It is quite possible, in fact, that they were totally unable to induce nodules because of some defect in their nodulation genes, although of course the presence of *nodD* demonstrates that they were not completely devoid of the symbiosis plasmid. Alternatively, they may have been competent symbionts, but of a host plant other than *T. repens*.

Besides the 68 clones that grouped unambiguously with *nodD* sequences from *R. leguminosarum* biovar *trifolii*, there was another group of 15 clones that clustered even more convincingly with *R. leguminosarum* biovar *viciae*. This biovar is the normal symbiont of *Vicia*, *Lathyrus*, *Pisum* and *Lens* species, and several wild *Vicia* and *Lathyrus* species occur in hedges and grassland near the sample sites, although none has been recorded on the sites themselves. Thus, it is not surprising that this biovar is present. If the amplification efficiency is comparable for all the sequences, we can conclude that there were roughly four or five times as many *R. leguminosarum* biovar *trifolii* as biovar *viciae* in the soil sample, which is consistent with the observation that *Trifolium* is present on the site but hosts of biovar *viciae* are not. This is the first time that it has been possible to assess directly the relative abundance of two rhizobium populations with different host specificity. The fact that only these two biovars were represented among the soil clones may

result in part from the specificity of the PCR primers. Although the primer Y5 matches the published *nodD* sequences from *R. leguminosarum* biovar *phaseoli*, *Mesorhizobium loti*, *Bradyrhizobium japonicum* and various other rhizobia, the reverse primer Y6 has two or more mismatches to these sequences, so it is unlikely that such sequences would be recovered. On the other hand, the published *nodD1* and *nodD2* sequences of *Sinorhizobium meliloti* do match the primers Y5 and Y6 exactly, and such sequences should have been amplified if they had been present. The apparent absence (or, at least, low abundance) of this species is consistent with previous findings that it does not persist at high densities in UK conditions in the absence of its host (Hirsch, 1996).

Amplification of *nodD* sequences directly from soil community DNA has allowed us to draw several conclusions about the soil population of rhizobia. In the future, this method could be applied to describe in more detail the dynamics of competition for nodulation in natural populations. The approach could also be extended to other microbial populations for which a distinctive genetic marker exists, and could allow an exploration of the population genetics even of unculturable bacteria.

Experimental procedures

Field site and first sampling of nodule isolates

In June 1998, 10 white clover plants (*T. repens*) were sampled from each of two discrete patches of clover (sites I and II) 3 m apart on Walmgate Stray, a long-established pasture field adjacent to the University of York. All the nodules were collected from each plant, and a rhizobium culture was established from each nodule by standard methods (Palmer and Young, 2000). To propagate multiple clones of each plant, 5–10 shoots were sterilized as described by Lieven-Antoniou and Whittam (1997), planted in pots filled with an autoclaved mixture of moist peat and vermiculite (1:3) and grown in a greenhouse, where they remained free of nodules.

Second sampling of nodule isolates using trap plants

In March 1999, soil was collected from the upper 10 cm at site I and mixed well. A portion was reserved for DNA extraction, and the remainder was transferred to sterile plant pots. Propagated plants, descendants of three original plants from each of the two sites, were transplanted into these pots and grown in a greenhouse for 3 months. Rhizobia were isolated from the resulting nodules as before.

DNA extraction from bacterial cultures

After 2 days growth in 2–5 ml of TY liquid medium, 500 μ l of cells was centrifuged for 5 min at 13 000 r.p.m. The pellet was resuspended in 100 μ l of Tris-HCl (10 mM, pH 8). After vortexing, 8 μ l of proteinase K (1 mg ml⁻¹) was added. The

cells were incubated at 55°C for at least 2 h followed by boiling for 10 min.

PCR amplification and RFLP of *nodD* region

Bacterial DNA extract (1 µl) was used in a 50 µl PCR reaction to amplify the *nodD* gene region using primers NBA12 and NBF12' (Laguerre *et al.*, 1996), which target the Nod box regulatory elements upstream of *nodA* and *nodF* respectively. The amplified region includes the whole of *nodD* and is ≈ 1450 bp in length. When required, a fragment internal to *nodD* was amplified using the primers Y5 (ATG CGK TTY ARR GGM CTN GAT CT) and Y6 (CGC AWC CAN ATR TTY CCN GGR TC). These primers are 128-fold and 256-fold degenerate, respectively, with mixed bases at six positions in each (Y = T or C, R = A or G, K = T or G, M = A or C, N = any base). They amplify a fragment of about 850 bp beginning at the first codon of *nodD* and match published sequences for *R. leguminosarum* biovars *trifolii* (accession no. X03721) and *viciae* (Y00548, J03671) and *S. meliloti* (M11268, M29367, X04473, X04474). The PCR used 1× reaction buffer, 3 mM MgCl₂ and 1 U of *Taq* polymerase (all Gibco), 0.2 mM each dNTP and 16–20 pmol of each primer. The temperature regime was an initial 120 s at 94°C, 25 cycles of 93°C for 60 s, 55°C for 60 s, 72°C for 120 s and, finally, 72°C for 300 s. Amplified products (5–10 µl) were digested with *AluI*, and fragments were separated on 4% 3:1 Nusieve–agarose or 8% acrylamide gels.

DNA sequencing and analysis

Representative NBA12/NBF12'-amplified products were reamplified using primers Y5/Y6, and PCR products were purified using the Qiaquick PCR purification kit (Qiagen). DNA sequencing was performed using primers Y5 and Y6, the ABI Prism dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS and an ABI automatic sequencer model 377. CLUSTAL X (Thompson *et al.*, 1997) was used to align sequences and reconstruct their phylogeny using the neighbour-joining method (Saitou and Nei, 1987). Trees were displayed using TREEVIEW (Page, 1996).

Soil DNA extraction

Soil from the second sampling (March 1999) was mixed thoroughly, and 5 g was used for DNA extraction by a modification of the method of Zhou *et al.* (1996). The soil was suspended in 13.5 ml of DNA extraction buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB, pH 8.0) with 100 µl of proteinase K (10 mg ml⁻¹) in a 50 ml tube (Falcon 2098). It was then shaken horizontally (225 r.p.m.) at 37°C overnight. The following steps were performed according to the method of Zhou *et al.* (1996), except that SDS extraction was carried out six times instead of three to allow better DNA recovery. All supernatants were combined and extracted twice in an equal volume of phenol–chloroform–isoamyl alcohol (4:4:1) and once in an equal volume of chloroform. Nucleic acids were then precipitated overnight at –20°C by the addition of

1/5 volume of sodium acetate (3 M) and two volumes of ethanol. After centrifugation at 12 000 *g* for 30 min at 4°C, the nucleic acid pellet was washed with cold 70% ethanol, air dried and dissolved in 500 µl of sterile water. The crude DNA extract was then purified twice using the Wizard DNA clean-up system (Promega) and checked on a 1% agarose gel.

PCR amplification and cloning of *nodD* sequences from soil community DNA

The DNA extract from soil was diluted 10-fold for PCR amplification. DNA (5–50 ng) was amplified in 50 µl with primers Y5/Y6 and the TaqPlus precision PCR system (Stratagene) under the temperature regime described above. The PCR products were cleaned using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T (Promega). The cloned products were transformed into *Escherichia coli* JM109 competent cells (Promega). Transformed cells (100 µl) were plated on LB containing 100 µg ml⁻¹ ampicillin, 0.5 mM IPTG and 40 µg ml⁻¹ Xgal. Cells from white colonies, potentially carrying inserts, were transferred using toothpicks to 100 µl of sterile water. After boiling for 5 min, 5 µl was used for PCR amplification in a volume of 50 µl using primers Y5/Y6 as described above.

Screening clones for the *nodD* gene

Amplified products from putative positive transformants were run on 1% agarose gels. After electrophoresis, the gels were rinsed briefly in distilled water, incubated in denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 30 min, neutralized in 1.0 M Tris-HCl (pH 8)–1.5 M NaCl for 30 min and rinsed in sterile water. The DNA was transferred to positively charged nylon membrane (Boehringer Mannheim) by standard Southern blotting (Sambrook *et al.*, 1989). After transfer, membranes were rinsed in 5× SSC, and DNA was fixed by UV light (312 nm) for 4 min. Membranes were prehybridized for 1 h in DigEasy buffer (Boehringer Mannheim) at 42°C, then hybridized with a probe prepared from a *nodD* fragment amplified by primers Y5 and Y6 from a nodule isolate of RFLP type A. The probe was labelled by random priming using the DIG-DNA labelling kit (Boehringer Mannheim), and the chemiluminescent hybridization signals were developed and detected as instructed by the manufacturer. Inserts yielding positive hybridization signals were typed by PCR–RFLP using Y5/Y6 and *AluI*, and representatives were sequenced as described above.

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