Phylogenetic diversity of rhizobial strains nodulating *Robinia pseudoacacia* L.

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Lack of knowledge exists regarding the diversity of rhizobial strains nodulating black locust (*Robinia pseudoacacia* L.), which is a neophytic tree species widely distributed in Europe. Seventeen rhizobial strains isolated from nodules of black locust at a German location were examined by phenotypic characterization and 16S rDNA analysis. The isolates were classified in nine 16S rDNA genotypes using a set of seven endonucleases. Based on RFLP analysis and sequencing, the strains were shown to belong to the genera *Mesorhizobium* (76 %) and *Rhizobium* (24 %). Five genotypes were identical to the species *Mesorhizobium amorphae*, *Mesorhizobium loti*, *Mesorhizobium huakuii*, *Rhizobium leguminosarum* and *Rhizobium tropici*. A strong similarity between the 16S rDNA sequence of another two genotypes and *M. amorphae* (99.9 %) as well as the *Mesorhizobium* strain R88b (99.8 %) was found. The two remaining genotypes were classified in the genus *Rhizobium*, without a significant relationship at the species level. Comparing isolates nodulating *Rob. pseudoacacia* and *Amorpha fruticosa*, a parallel picture of phylogenetic diversity was detected with a range of phylogenetically different rhizobia and *M. amorphae* dominating. For this study, 18 rhizobial strains which had originally been isolated from a forest in Maryland where black locust is native were additionally analysed. Results revealed seven genotypes all belonging to the genus *Mesorhizobium*, with four genotypes identical to the isolates from the German sampling location. Whereas the genotype identical to *M. amorphae* dominated within the strains obtained from the German location, the dominance of a genotype identical to *M. huakuii* was found among the strains from the native location. Summarizing data from both locations, *Rob. pseudoacacia* was nodulated with various genomic species, most of which belonged to the genus *Mesorhizobium*. Concerning phenotypic features such as growth rate, pH tolerance or use of certain carbohydrates, most isolates corresponded to described species and genera. However, there were differences in salt tolerance between these isolates and the corresponding reference strains. Overall, the results demonstrated a high phenotypic and phylogenetic diversity of rhizobial strains nodulating *Rob. pseudoacacia*. This may be a characteristic of neophytic and other widely spread legumes and may contribute to the success of black locust as a pioneer tree species for the temperate zone.

Keywords: *Mesorhizobium*, *Robinia pseudoacacia*, black locust, 16S rDNA sequences, PCR-RFLP

INTRODUCTION

Black locust (*Robinia pseudoacacia* L.) originated from

The EMBL accession numbers for the sequences reported in this paper are AJ271898–AJ271902 for the *Mesorhizobium* strains Rob6, Rob8 and Rob23 and the *Rhizobium* strains Rob18 and Rob20, respectively.

North America but is gaining more and more importance in middle Europe as a fast-growing tree species suitable for the afforestation of arable land and devastated areas with former mining activity. The tree is characterized by good growth on poor and nitrogen-deficient soils, resistance to wood-destroying organisms and a high wood quality, which is of increasing interest

to the timber industry. Consequently, breeding strategies that included plant tissue culture methods were developed for the cultivation of selected phenotypes with straight-growing trunks (Hanover, 1992). Experiences with micropropagation of selected cultivars and clones for mass production were reported by several authors (Gruber & Hanover, 1992). However, the process of micropropagation of black locust is difficult compared to other fast-growing deciduous tree species. Besides other problems in cultivation, the lack of rhizosphere micro-organisms on *in vitro* plantlets led to a high mortality rate under greenhouse conditions. The promotion of growth and especially the rooting ability of plantlets as well as acclimation of young plants derived from tissue culture is possible by inoculation with effective rhizobial strains (Balla *et al.*, 1998; Herrera *et al.*, 1993). Previous investigations of rhizobial strains nodulating black locust demonstrated a high variability in their nitrogen-fixing efficiency, biochemical properties and in the promotion of increased plant biomass (Hoffmann, 1964; Batzli *et al.*, 1992; Han, 1996). On the other hand, the knowledge of phenotypic features and phylogenetic classification of rhizobial strains nodulating black locust under temperate conditions is sparse in comparison to what is known about rhizobia infecting herbaceous plants.

Comparison of 16S rDNA nucleotide sequences can be applied for classification of isolates at species and higher levels (Woese, 1987; Cilia *et al.*, 1996). Such phylogenetic analyses of representative rhizobial strains revealed that rhizobia are polyphyletic, and resulted in a reclassification of bacteria able to nodulate legumes into five genera (Young & Haukka, 1996; Chen *et al.*, 1988; de Lajudie *et al.*, 1994; Dreyfus *et al.*, 1988; Jarvis *et al.*, 1997; Jordan, 1982). The data also confirm that there is no branch of the evolutionary tree exclusively consisting of rhizobial species (Young, 1996). Variations in the 16S rRNA gene sequences have been used for differentiation and classification of numerous rhizobial strains. These studies demonstrated that some legume species were infected by more than one species of rhizobia (Amarger *et al.*, 1994, 1997; Laguerre *et al.*, 1993; Lafay & Burdon, 1998). However, no strains capable of nodulating black locust were included in these phylogenetic studies. Our objective was to analyse phenotypic features and variation in 16S rRNA genes of rhizobial strains promoting growth of black locust plantlets.

METHODS

Isolation of rhizobia and growth conditions. Most isolates were obtained from nodules of *in vitro* propagated young plants of *Rob. pseudoacacia* var. *rectissima* grown for 2–3 years in the nursery of Waldsiedersdorf, Germany. Three other isolates came from 80-year-old trees of this variety (Rob19, Rob25) or from a natural regenerated young plant (Rob16) grown in a stand located near the nursery. The strains Rob17, Rob18 and Rob20 were isolated from trees of *Rob. pseudoacacia* var. *rectissima* ROKO, *Rob. pseudoacacia* var. *monophylla* and *Rob. pseudoacacia* cv. 'Casque rouge' also grown in the nursery. The site belongs to the forest vegetation

type of the central Brandenburg pine (linden)–sessile oak area. The mean annual temperature is 8.2 °C; the mean annual precipitation amounts to 520 mm. The soils are moderately fertile sands (degraded brown earth) without connection to groundwater.

Nodules with pink- or red-stained tissue were taken from the root system at a soil depth of about 5 cm. The nodules were surface-sterilized with 0.05 % AgNO₃ for 1 min and washed several times with sterile deionized water (Fox, 1993). The nodule contents were streaked on a glucose medium enriched with pea extract and incubated at 28 °C. The medium contained (l⁻¹) 10 g glucose, 0.5 g K₂HPO₄, 0.5 g KH₂PO₄, 0.2 g MgSO₄, 0.2 g NaCl, 0.1 g CaSO₄, 1 mg (NH₄)₂MoO₄, 500 ml pea extract (boiled from 100 g peas) and 15 g agar adjusted to pH 6.8. Bacteria from single colonies were subcultured on a modified yeast extract-mannitol agar (YMA), which contained 0.5 g instead of 3 g yeast extract (Elkan & Bunn, 1992).

Reference strains. As a reference strain, LMG 4270 (ATCC 10320), isolated from black locust in central Iowa, was used (Thorne & Walker, 1936). Furthermore, the rhizobial strains USDA 4197, USDA 4207, USDA 4212, USDA 4214, USDA 4222, USDA 4224, USDA 4236, USDA 4242 and USDA 4255, and USDA 4262, USDA 4279, USDA 4283, USDA 4297, USDA 4313, USDA 4315, USDA 4318, USDA 4322 and USDA 4340, respectively, isolated by Batzli *et al.* (1992) from black locust trees at two sites in Green Ridge State Forest in Allegany County, MD, USA, were used to characterize the phylogenetic diversity of rhizobia from a native location.

Phenotypic characterization. The growth rates of the 17 isolates were estimated by the incubation time necessary to achieve a colony size of 2–3 mm on YMA at 28 °C (Jarvis *et al.*, 1997). Colony colour was examined macroscopically after growth on YMA for 6 d. Carbohydrate utilization was tested in yeast extract-mannitol medium in which mannitol was substituted by inositol or D-raffinose. NaCl tolerance was determined on YMA containing 5, 7.5, 10 and 12.5 g NaCl l⁻¹ with three replications. The growth at different pH ranges was tested in 5 ml yeast extract-mannitol broth adjusted at pH 5.0–9.0 in steps of 0.5 (Batzli *et al.*, 1992; modified). Each broth was inoculated with 200 µl of a fresh liquid culture and incubated for 24 h at 27 °C. The OD₆₆₀ was measured. For assaying antibiotic resistances, single colonies from 4-d-old cultures were spread on YMA containing either 10 µg rifampicin ml⁻¹, 25 µg nalidixic acid ml⁻¹, 50 µg kanamycin ml⁻¹, 25 µg ampicillin ml⁻¹, 15 µg chloramphenicol ml⁻¹, 30 µg streptomycin ml⁻¹ or 15 µg oxytetracycline ml⁻¹ (Sigma). The plates were read for growth after 7 d incubation.

Plant inoculation test. Rooted plantlets of five different genotypes of *Rob. pseudoacacia* var. *rectissima* were placed on sterile filter paper saturated with 2 ml sterile water and 1 ml Herridge's plant nutrient solution (Qian *et al.*, 1996) and inoculated with 250 µl bacterial suspension of approximately 10⁶ cells ml⁻¹. The root system of the plants was covered with moist filter paper, and the dishes were closed with Parafilm strips and cultivated for 30 d at 23 °C and 2000 lx (Philips TLD58W/93). During the first week, the dishes were covered with a thin layer of cellulose to protect green parts of plants against light. The test was performed in triplicate.

PCR amplification. For template preparation, DNA was isolated from bacteria grown on YMA for 2–4 d at 28 °C. Single colonies were washed with 0.3 % NaCl, resuspended in 20 µl 25 mM NaOH/0.25 % SDS and heated for 15 min at 95 °C. Aliquots (0.2 µl) of the resulting lysate were directly

Table 1. Phenotypic characteristics of isolates nodulating *Rob. pseudoacacia*

Isolate	Colony colour*	Incubation time (d)	Colony diam. (mm)	Growth			No. nodules†	AB‡
				pH range	Max. NaCl concn (%)	Inositol as sole C source		
Rob6	p	5	2–4	5.5–9.0	1.00	+	4.5 ^{ab}	—
Rob8	y	5	2	5.0–9.0	1.00	+	4.4 ^{ab}	Na
Rob9	p	5	1	5.5–8.5	1.00	+	3.9 ^{abc}	—
Rob10	p	5	1–2	5.5–8.5	1.00	+	4.3 ^{ab}	—
Rob11	p	5	1–2	6.0–8.5	0.10	—	5.4 ^a	Km
Rob12	p	5–6	1–2	5.5–8.0	1.00	+	4.9 ^{ab}	Na
Rob13	p	5	2–3	5.5–8.0	1.00	+	2.2 ^{bc}	Na
Rob16	p	5	2–3	5.5–8.5	1.00	+	3.2 ^{abc}	Na
Rob17	w	3	5	5.5–8.5	0.10	—	1.6 ^c	—
Rob18	p	3	2–4	5.5–8.5	1.00	+	3.3 ^{abc}	—
Rob19	p	3–4	3–4	5.5–8.0	1.00	+	3.1 ^{abc}	—
Rob20	p	4	2–3	6.5–8.0	0.75	—	4.1 ^{abc}	—
Rob21	p	4	5	5.0–9.0	1.00	+	2.8 ^{abc}	Na
Rob22	p	5	3	5.0–9.0	1.00	+	3.0 ^{abc}	—
Rob23	p	5	1–2	5.0–9.0	1.00	+	3.2 ^{abc}	Na
Rob24	p	5	2	5.5–8.5	1.00	+	3.7 ^{abc}	Na
Rob25	p	5	3	5.5–8.5	1.00	+	5.1 ^a	Na
<i>Rh. leguminosarum</i> DSM 30132 ^T	p	3–4	1–2	5.5–8.0	1.00	+	ND	—
<i>Rh. tropici</i> CIAT 899 ^T	p	3	2–3	5.5–8.5	2.50	+	ND	Ap, Na
<i>M. loti</i> DSM 2626 ^T	p	5	1–2	5.5–8.5	1.50	+	ND	Ap, Km, Na
<i>M. huakuii</i> DSM 6573 ^T	p	6	1–2	5.5–8.5	2.50	—	ND	Km, Na

* Colonies were opaque with a pale (p), white (w) or yellowish (y) colour.

† Mean number of nodules obtained after inoculation of 15 black locust plantlets. Different letters denote groups with significantly different numbers of nodules and were determined with the Student–Newman–Keuls test at probability level $\alpha = 0.05$.

‡ AB^R, resistant to the indicated antibiotic. The following antibiotics were screened: 10 µg rifampicin ml⁻¹, 25 µg nalidixic acid (Na) ml⁻¹, 50 µg kanamycin (Km) ml⁻¹, 25 µg ampicillin (Ap) ml⁻¹, 15 µg chloramphenicol ml⁻¹, 30 µg streptomycin ml⁻¹ and 15 µg oxytetracycline ml⁻¹.

used for PCR without further purification. The primers fD1 and rD1 (Weisburg *et al.*, 1991) used in this study are capable of amplifying almost the entire 16S rRNA gene of most eubacteria. For routine assays, a 50 µl reaction mixture containing 10 pmol of each primer in a standard buffer (Gibco-BRL) was used. The amplifications were performed in a GeneAmp PCR System 2400 (PE Biosystems) with the following protocol: initial denaturation at 95 °C for 2 min; 30 cycles of 40 s at 94 °C, 40 s at 52 °C, 1 min at 72 °C; followed by a single final extension at 72 °C for 3 min and a final soak at 4 °C (Ulrich & Müller, 1998; modified). After the reaction, aliquots (3 µl) of the PCR products were examined electrophoretically in a 1% agarose gel.

RFLP analysis. PCR products (2–8 µl) were digested with each of the following enzymes: *Cfo*I, *Hae*III, *Alu*I, *Hin*FI, *Msp*I, *Sau*3A and *Scr*FI (Roche Molecular Biochemicals, New England Biolabs). To detect minor differences between bands, the DNA fragments were separated in 2–3.5% Metaphor agarose gels (FMC Bioproducts) depending on the fragment sizes to be distinguished. The DNA molecular mass markers V and VI were used as size standards (Roche Molecular Biochemicals). The gels were stained with ethidium bromide and documented with a video camera image system (Easy-Image plus, Herolab).

Cloning and sequencing. The isolates Rob20, Rob18, Rob6, Rob8 and Rob23 representing genotypes III, IV, V, VI and IX, respectively, were used in 16S rDNA sequence determinations. The PCR products obtained after amplification with *Pfu* DNA polymerase (Promega) were purified using the QIAquick PCR purification kit (Qiagen), digested at the *Hind*III and *Sall* restriction endonuclease sites within the linker sequence of the primers and subsequently cloned into the vector pSVB30 (Arnold & Pühler, 1988), which had been digested with the

same enzymes. A cycle-sequencing protocol was applied for sequencing both DNA strands with a Li-Cor Sequencer, model 4200 (MWG Biotech), using standard M13 primers. The resulting rDNA sequences of 1436 or 1440 nucleotides, corresponding to the genotypes III and IV, and V, VI and IX, respectively, were analysed for homologies to sequences deposited in the GenBank and EMBL databases. The similarity values were based on a pair-wise comparison of sequences. The determined DNA sequences and various 16S rDNA sequences from selected rhizobial species were aligned using the CLUSTAL W algorithm (program version 1.74; Thompson *et al.*, 1994). The phylogenetic analyses were performed with the PHYLIP computer program package, version 3.55c (Felsenstein, 1993). The neighbour-joining algorithm and the parsimony method were used to generate phylogenetic trees. The neighbour-joining algorithm (Saitou & Nei, 1987; NEIGHBOR using *Bradyrhizobium japonicum* as outgroup) was based on a matrix of pair-wise distances corrected for multiple base substitutions by the method of Kimura (1980) (DNADIST with transition/transversion ratio of 2.0). The parsimony method (DNAPARS) was applied with ten jumbles of the data set. Both trees were constructed using the original data set and 100 bootstrap data sets.

RESULTS

Phenotypic characteristics

17 rhizobial strains investigated in this study were isolated from black locust grown in a nursery or nearby woodland located in the north-eastern German pine-sessile oak area. All isolates were verified to be rhizobia by inoculation of sterile plantlets from five *Rob.*

Table 2. Restriction patterns of amplified 16S rDNA of rhizobial strains isolated from black locust and comparison of the resulting 16S rDNA genotypes with those of the reference strains

Isolate or reference strain	Restriction pattern of amplified 16S rDNA digested with:*							16S rDNA genotype†
	<i>AluI</i>	<i>CfoI</i>	<i>HaeIII</i>	<i>HinfI</i>	<i>MspI</i>	<i>Sau3A</i>	<i>ScrFI</i>	
Rob19, <i>Rh. leguminosarum</i> DSM 30132 ^T	a	a	a	a	a	a	a	I
Rob17, <i>Rh. tropici</i> CIAT 899 ^T	a	b	a	a	b	a	b	II
Rob 20	x	a	b	b	a	b	x	III
Rob 18	y	y	b	b	y	y	y	IV
Rob6, Rob9, Rob10, Rob13, Rob22, LMG 4270, USDA 4255, USDA 4207	c	d	a	e	x	e	c	V
Rob8, Rob12, Rob16	c	d	a	d	x	e	c	VI
Rob21, <i>M. loti</i> DSM 2626 ^T	c	d	a	e	e	e	c	VII
Rob11, Rob25, <i>M. loti</i> DSM 5235, <i>M. huakuii</i> DSM 6573 ^T , USDA 4212, USDA 4214, USDA 4224, USDA 4242, USDA 4262, USDA 4279, USDA 4283, USDA 4313, USDA 4340	c	g	a	g	j	e	c	VIII
Rob23, Rob24, USDA 4222, USDA 4315, USDA 4318	c	g	a	g	e	e	c	IX
USDA 4197	c	d	a	d	x	x	c	
USDA 4236	c	d	a	g	j	e	c	
<i>Rh. etli</i> CFN 42 ^T	a	a	b	b	a	b	a	
<i>M. loti</i> DSM 2627, USDA 4322	c	g	a	h	j	e	c	
<i>Mesorhizobium ciceri</i> DSM 11540 ^T	c	d	a	d	e	e	c	
<i>Mesorhizobium mediterraneum</i> DSM 11555 ^T	c	g	a	d	x	e	c	
<i>M. tianshanense</i> DSM 11417 ^T , USDA 4297	z	z	z	b	z	e	y	
<i>Sinorhizobium fredii</i> DSM 5851 ^T	a	a	a	d	d	d	b	
<i>Sinorhizobium teranga</i> DSM 11282 ^T	a	a	a	d	w	e	w	
<i>Sinorhizobium saheli</i> DSM 11273 ^T	a	a	a	d	w	w	w	

* Different restriction patterns were indicated with different letters for each endonuclease used. Designation of the restriction patterns is (if possible) in accordance with that used by Laguerre *et al.* (1994).

† The 16S rDNA genotypes resulted from the combination of the seven restriction patterns of each strain tested. Only genotypes found among the isolates are shown.

pseudoacacia clones. The rhizobial isolates did not show any differences in nodulation activity with respect to the various *Rob. pseudoacacia* clones in three independent tests. Most isolates formed three to five nodules per plantlet within the observation period. The highest numbers were detected for isolates Rob11, Rob12 and Rob25 (Table 1). Distinctly lower numbers were found on roots inoculated with the isolates Rob13 and Rob17. Significant differences of nodulation activity were revealed for Rob11 and Rob25, as compared to Rob13 and Rob17. The isolate with the lowest nodulation ability (Rob17) was obtained from the black locust clone ROKO, which originated from Hungary. On the one hand we found a low nodulation ability of Rob17 with all tested plant clones as well as a generally low ability of the ROKO clone to form nodules, but on the other hand there was no preference or increased nodulation ability of Rob17 with the ROKO clone. The growth rate, which is often referred to as a mean characteristic to distinguish rhizobial genera (Young, 1996), was estimated using incubation time. In our study, isolates formed colonies within 3–5 d and Rob17–Rob19 were distinguished by incubation times of less than 4 d (Table 1). Both the *Rhizobium legum-*

inosarum and the *Rhizobium tropici* reference strains could be differentiated from *Mesorhizobium* after 4 d incubation time. Concerning the colony characteristics, all isolates were opaque but they differed in colour. All isolates except Rob11, Rob17 and Rob20 grew well on medium enriched with NaCl up to a concentration of 1%. This is in contrast to the salt tolerance of the *Mesorhizobium* species investigated as well as that of *Rh. tropici*. Only *Rh. leguminosarum* showed a similar low salt tolerance. The pH growth optimum generally ranged from 5.5 to 8.5. However, two isolates (Rob11 and Rob20) were unable to grow at pH 5.5. All strains including the reference strains utilized D-raffinose as sole carbon source, whereas three isolates and the *Mesorhizobium huakuii* strain were unable to grow with inositol. In general, the isolates showed a low tolerance to antibiotics, but about 50% of isolates were resistant to nalidixic acid.

RFLP analysis of amplified 16S rDNA

The rDNA of all 17 isolates was amplified using the primers rd1 and fd1, resulting in a characteristic single band of about 1500 bp. Restriction analysis of the PCR

Table 3. Band sizes within the restriction patterns for rhizobial strains isolated from black locust

Restriction enzyme	Restriction pattern	Size of restriction fragments (bp)*								Corresponding 16S rDNA genotypes†
<i>AluI</i>	a	400	225	210	210	185	145	100		I, II
	x	240 + 160	.	.	.	.	.	.		III
	y	240 + 160	160	.	.	.	.	.		IV
	c	370	160	280	.	200	.	—		V, VI, VII, VIII, IX
<i>CfoI</i>	a	330	280	280	170	135	110	110	80	I, III
	b	.	.	.	.	—	.	.	.	II
	y	.	.	.	.	100	.	.	.	IV
	d	.	450	.	—	140	.	.	.	V, VI, VII
	g	.	.	.	.	140	.	.	.	VIII, IX
<i>HaeIII</i>	a	490	280	240	180	180	85			I, II, V, VI, VII, VIII, IX
	b	.	.	210	.	.	.			III, IV
<i>HinfI</i>	a	1170	—	220	120					I, II
	b	1290	—	.	—					III, IV
	d	690	490	.	.					VI
	e	370 + 320	490	.	.					V, VII
	g	370 + 320	290 + 200	.	.					VIII, IX
<i>MspI</i>	a	500	420	225	180	125	—	—		I, III
	b	660	.	.	.	—	—	—		II
	e	.	310	.	.	.	140	—		VII, IX
	j	250	310	.	.	.	140	110 + 85		VIII
	x	370	310	.	.	.	140	110		V, VI
	y	660	310	.	.	—	140	—		IV
<i>Sau3A</i>	a	710	230	175	175	85				I, II
	b	.	175	.	.	.				III
	e	950	.	.	—	.				V, VI, VII, VIII, IX
	y	950	175	.	—	.				IV
<i>ScrFI</i>	a	440	350	180	180	120	110	85	—	I
	b	.	500	.	.	—	.	.	—	II
	c	385	310	.	.	.	.	.	120	V, VI, VII, VIII, IX
	x	.	500	.	—	—	300	.	—	III
	y	310	500	.	—	—	300	.	120	IV

* Band sizes of restriction fragments as determined by electrophoresis. Dots represent fragment sizes identical to those of pattern a. Dashes indicate the absence of restriction fragments.

† The 16S rDNA genotypes are as given in Table 2.

products using seven endonucleases revealed two to six restriction patterns per enzyme (Table 2). DNA fragments smaller than 80 bp were not properly resolved by electrophoresis and were therefore not used for the comparison of patterns. On average, 41 restriction fragments per 16S rDNA genotype were used for the classification of isolates, and nine distinct 16S rDNA genotypes, each comprising one to five isolates, were found. To differentiate the nine genotypes, at least two enzymes (*MspI*, *HinfI*) were required. Band sizes of restriction patterns are shown in Table 3 to provide an overview of the differences between the various restriction patterns. The reference strains used in this study were also classified by restriction analysis of 16S rDNA (Table 2). Designation of the corresponding restriction patterns followed that used by Laguerre *et al.* (1994). Four of the 16S rDNA genotypes obtained from the

isolates were found among the type strains of the rhizobial species tested. Thus genotypes I, II, VII and VIII were identical to *Rh. leguminosarum*, *Rh. tropici*, *Mesorhizobium loti* and *M. huakuii*, respectively. However, genotype VIII was identical to both the *M. huakuii* type strain as well as to a *M. loti* strain. Moreover, the 16S rDNA genotypes of the other isolates were not identical to those of rhizobial species or strains analysed by Laguerre *et al.* (1994).

The reference strain LMG 4270 isolated from black locust in central Iowa showed the 16S rDNA genotype V, which was also found in 5 of the 17 isolates. To assess phylogenetic diversity of rhizobia isolated within an area where black locust is native, 18 strains isolated by Batzli *et al.* (1992) were investigated. In total, seven 16S rDNA genotypes were found. Only two strains belonged to genotype V, whereas nine strains were identical to

Fig. 1. Phylogenetic tree showing the relationships of the identified 16S rDNA genotypes of black locust-nodulating isolates within rhizobia. The tree is based on a 1448 bp alignment and was constructed using the neighbour-joining method (Saitou & Nei, 1987). The values are numbers of times that a branch appeared in 100 bootstrap replications. Genotypes identified in this study are in bold characters. The bar indicates the relative sequence divergence. Representative sequences used for phylogenetic analysis: *B. japonicum* (accession no. U69638); *M. amorphae* (AF041445); *M. ciceri* (U07934); *M. huakuii* (D13431); *M. loti* DSM 2626^T (67229); *Mesorhizobium* sp. R88b (U50165); *M. mediterraneum* (L38825); *M. tianshanense* (AF041447); *Sinorhizobium fredii* (X67231); *Rhizobium* sp. RCR 36189 (U29387); *Rh. etli* (U29386); *Rh. gallicum* (U86343); *Rh. leguminosarum* bv. *viciae* (U29386); *Rh. mongolense* (U89822); *Rh. tropici* (U89832); *Sinorhizobium meliloti* (D14509); *Sinorhizobium saheli* (X68390); *Sinorhizobium terangae* (X68387).

genotype VIII and three strains to genotype IX. The other four genotypes were not found at the German location. Two genotypes were identical to *Mesorhizobium tianshanense* and a *M. loti* strain, whereas the remaining two genotypes were not found among the other strains investigated. In general, there were only a few differences between the seven genotypes identified from the strains from Maryland.

DNA sequence analysis of isolates with unknown RFLP patterns

16S rDNA sequencing was performed on a representative isolate of the genotypes that could not be classified by restriction analysis. All sequences showed a high homology to sequences from rhizobial species. The sequence of Rob6 (genotype V) was identical to that of *Mesorhizobium amorphae*. Best conformities of Rob8 (VI) and Rob23 (IX) were found with rDNA sequences of *M. amorphae* (99.9%) and the *Mesorhizobium* strain R88b (99.8%), respectively. The 16S rDNA sequences of Rob6 and Rob8 differed only in two nucleotides, but both genotypes were differentiated by RFLP analysis, illustrating the high resolution of this technique.

With respect to their restriction patterns, genotypes III and IV were more distinct in comparison to genotypes

V–IX (Tables 2 and 3). These differences were also demonstrated by the similarity of 16S rDNA sequences; thus genotypes III and IV (Rob20 and Rob18) showed a similarity of 91.1% and 91.9%, respectively, to genotype V (Rob6). The search for homologous sequences for Rob20 and for Rob18 resulted in a similarity index of 98.1% to a *Rhizobium* strain nodulating *Phaseolus vulgaris* (RCR 3618D) and of 96.4% to *Rhizobium gallicum*, respectively.

An unrooted phylogenetic tree including further rhizobial species was constructed to illustrate the phylogenetic position of the identified genotypes (Fig. 1). As a result, isolates of groups V, VI, VII, VIII and IX were assigned to the genus *Mesorhizobium* (bootstrap values of 100%). These genotypes represent 76% of the isolates. The isolates Rob6 and Rob8 were unequivocally clustered with *M. amorphae*. Furthermore, the phylogenetic tree showed the strong similarity of isolate Rob23 and *Mesorhizobium* strain R88b. Like the *M. loti* type strain, this strain was isolated from *Lotus corniculatus*. The isolate Rob18 branched with the *Rh. gallicum*/*Rhizobium mongolense* grouping with a relatively low similarity and weak bootstrap support (58%). Therefore, phylogenetic assignment of this isolate remains open. There is a good support (97%) for the branching of Rob20 with the *Rhizobium* sp. strain

RCR 3618D. Both isolates form a branch together with *Rh. leguminosarum/Rhizobium etli* (72% bootstrap support).

DISCUSSION

It seems to be common that rhizobial isolates are capable of nodulating more than one legume species and several legume species can be nodulated by more than one rhizobial species (Young, 1996). Thus until recently, rhizobia able to nodulate *P. vulgaris* were assigned to *Rh. leguminosarum* bv. *phaseoli*. However, at present, at least five species (*Rh. leguminosarum* bv. *phaseoli*, *Rh. tropici*, *Rh. etli*, *Rh. gallicum* and *Rhizobium giardinii*) can be distinguished based on both the organization of the symbiotic plasmid and sequence divergence of the 16S rRNA gene (Amarger *et al.*, 1997; Segovia *et al.*, 1993; Martinez-Romero *et al.*, 1991). Likewise, various other legumes were able to form nodules with different rhizobial species (Lafay & Burdon, 1998; Perret *et al.*, 2000). In our study, 17 rhizobial isolates were differentiated into nine 16S rDNA genotypes. Most of the isolates (76%) were classified in the genus *Mesorhizobium* (genotypes V–IX). The other genotypes (I–IV) assigned to the genus *Rhizobium* each consisted of only one isolate and showed a low relatedness to each other. Both genera belong to different evolutionary branches (Fig. 1; Jarvis *et al.*, 1997), which is also shown by several phenotypic and genetic traits such as growth rate, fatty acid composition or similarity of *nod* genes (Jarvis *et al.*, 1996; Haukka *et al.*, 1998). Hence, isolates nodulating black locust represent a phylogenetically wide range of rhizobial species. Previous studies indicated that *Rob. pseudoacacia* can be nodulated by rhizobial strains obtained from nodules of *Vicia*, *Trifolium*, *Lathyrus*, *Melilotus* and *Medicago* species (Lyr, 1992; Hoffmann, 1964). With respect to the 160 legume species tested by Wilson (1939), *Rob. pseudoacacia*, along with another tree, had the broadest capacity to nodulate. Only some strains originally isolated from black locust were further investigated and the strain which was most effective in terms of nodulation and nitrogen fixation was classified as *M. loti* (Röhm & Werner, 1992; Schäfer & Werner, 1993). However, phylogenetic studies of indigenous rhizobia nodulating black locust have not been done previously. Comparing the phylogenetic diversity of our isolates with that of the strains obtained by Batzli *et al.* (1992) from an area where *Rob. pseudoacacia* is native revealed some similarities. Among the 18 strains tested, seven genotypes could be identified with four genotypes identical to the isolates from the German location. However, as shown by the similarity of the restriction patterns, all strains which originated from the native area, including the reference strain LMG 4270, can be assigned to the genus *Mesorhizobium*. Therefore, genetic diversity of the strains nodulating black locust seems to be higher at the German location. Whereas the genotype identical to *M. amorphae* dominated within the strains obtained from the German location, the dominance of a genotype identical to *M. huakuii* was

found among the strains from the native location. Summarizing data from both locations, *Rob. pseudoacacia* was nodulated with various genomic species, most of which belong to the genus *Mesorhizobium*. Furthermore, 16S rDNA sequences indicate some classification at the species level; however, additional studies involving DNA–DNA hybridization as well as phenotypic characterization would be necessary to confirm species assignment (Sullivan *et al.*, 1996; Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987).

Rob. pseudoacacia was introduced into middle Europe approximately 300 years ago. Although native only to the eastern USA, the tree species is now naturalized and widely planted throughout the world from temperate to subtropical areas. The rhizobia nodulating *Rob. pseudoacacia* are usually effective for nitrogen fixation as indicated by the red colour of the nodules or shown by their symbiotic host response (Batzli *et al.*, 1992). It may be possible that some rhizobia were seed-borne and introduced together with the neophytic plant, as has been suggested for the transmission of *Rh. etli* on seeds of *P. vulgaris* (Pérez-Ramírez *et al.*, 1998). However, both the high phylogenetic diversity of isolates effective with *Rob. pseudoacacia* and its broad nodulation capacity support the view that indigenous rhizobia may be associated with this tree species. On the other hand, about half of the 16S rDNA genotypes were identical at both locations and, as shown for *L. corniculatus*, symbiotic gene transfer could also cause diversity of nodulating strains (Sullivan *et al.*, 1996). The highest proportion of the isolates obtained from the German location (47%, genotypes V and VI) showed identical or closely related (99.9%) 16S rDNA sequences to *M. amorphae*. In general, we found a parallel picture of phylogenetic diversity of isolates nodulating *Rob. pseudoacacia* to those nodulating *Amorpha fruticosa* (Wang *et al.*, 1999). Wang *et al.* (1999) analysed 55 isolates and identified five 16S rDNA genotypes. The highest proportion of the isolates were *M. amorphae*, whilst two other genotypes belonging to the genus *Mesorhizobium* as well as some isolates related to *Rh. leguminosarum* and *Bradyrhizobium elkanii* were also identified. Similar to *Rob. pseudoacacia*, *A. fruticosa* is a neophytic plant native to south-eastern and mid-Western USA and was introduced into Asia more than 50 years ago. We suggest that neophytic and other legumes, especially when widely distributed, are less specific and form nodules with several phylogenetically different rhizobia, whereas archaeophytic plants could preferably host a singular or few specialist micro-symbionts. A narrow host range of rhizobial strains is considered a specialization which developed for certain plants in restricted niches (Perret *et al.*, 2000). However, further studies are necessary to evaluate this hypothesis.

The phylogenetic classification was mostly supported by phenotypic characteristics. Thus isolates classified in the genus *Mesorhizobium* generally showed a longer incubation time than the *Rhizobium* isolates (Rob17–Rob20). The growth rates of the reference strains used in the study are in the same range, although there were

some differences from the incubation time given by Jarvis *et al.* (1997). These authors showed an incubation time of 7 d for *M. loti* and of 5–6 d for *M. huakuii*. The markedly low salt tolerance of the isolates differed from the salt tolerance of the reference strains. However, our isolates were similar to all rhizobial groups isolated from *A. fruticosa* in that they did not grow at salt concentrations above 1 % NaCl (Wang *et al.*, 1999). The phenotypic characters tested indicate a diversity of black locust-nodulating rhizobia as already described by Batzli *et al.* (1992) for the rhizobial strains used as reference strains in this study. These strains demonstrated a high heterogeneity of several phenotypic characters, including carbohydrate utilization, nitrogen fixation, generation times between 3 and 9 h and different protein profiles. As shown for the phylogenetic diversity, there was a similar phenotypic heterogeneity of strains to those reported for rhizobia nodulating the leguminous shrub *A. fruticosa*.

Our results revealed a high phylogenetic diversity with seven genotypes among 17 isolates analysed, which suggests the existence of further taxa nodulating *Rob. pseudoacacia*. The high phenotypic and phylogenetic diversity of rhizobial strains obtained from black locust seems to be characteristic for widely distributed legumes. This could enhance their ability to spread into new areas. Thus rhizobial diversity is considered to be a reason for the success of black locust as a pioneer tree species outside its native range and the suitability of the tree for natural succession on former arable land and recultivation areas under temperate conditions.

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